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Chapter 16

Aflatoxins: Risk, Exposure and Remediation

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Additional information is available at the end of the chapter

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1. Introduction

Aflatoxins family includes a great number of lipophilic molecules produced by aerobic microscopic fungi belonging to the genus *Aspergillus*. The chapter describes their chemical structure, chemical and physical properties, and aspects related to their presence in food and commodities. Aflatoxins presence in food is considered a real and severe risk to consumers for their toxicity. Aflatoxins levels and frequency of foods natural contamination as reported in the scientific literature are briefly analyzed. Focus is given to the different foodstuffs that may be at risk of contamination by *Aspergillus* and the subsequent accumulation of aflatoxins in the food chain. Bioavailability and bioaccessibility of aflatoxins will be discussed considering that these unwanted molecules can be assumed by the humans with the diet. Bioaccessibility, that deals with the fraction of micro-nutrients released from the food matrix during digestion and gastro-intestinal available for absorption, will be discussed with reference to aflatoxins bioaccessibility of during the digestion process, considering the relationships between the food matrix and its influences on aflatoxins fate. Bioavailability of the aflatoxins assumed from the diet depends on their stability during digestion, since they are released from the food matrix (bioaccessibility) and on the efficiency of their passage through the gastro-intestinal mucosa. The term bioavailability includes the concepts of availability to the absorption, metabolism, distribution of nutrients to tissues and bioactivity and indicates the fraction of micro-nutrients absorbed by the body and the speed with which these molecules are absorbed and made available at their site of action. Despite of the practical difficulties in measuring the distribution and bioactivity of aflatoxins on a specific human body organ, the bioavailability is the fraction of an oral dose of a compound or precursor of an active metabolite that reaches the bloodstream. Bioaccessibility includes the entire sequence of events that take place during the digestion of food material that can be assimilated by the body through the epithelial cells of the gastro-intestinal mucosa. Aflatoxins are often present in very small amounts or in traces and, for this reason, a part of the

chapter addresses the advanced new chromatographic and spectrometric methods described in the literature and applied to research, that can reveal, even in trace amounts, aflatoxins in biological fluids as free form or as by-products, e.g. non-covalent adducts.

2. Structure and chemistry of aflatoxins

Aflatoxins were isolated and characterized after the Turkey X desease, that caused the death of more than 100.000 turkey poultries due to the intake of a contaminated peanut meal produced in South America starting from contaminated raw material (Blout, 1961; Goldblatt, 1969).

The most important aflatoxins, among the about 13 compounds so far identified, are the aflatoxin B_1 and B_2, the aflatoxin G_1 and G_2 and the aflatoxin metabolic byproducts M_1 and M_2. The four major aflatoxins are called B_1, B_2, G_1, and G_2 based on their fluorescence under UV light (blue or green) and relative chromatographic mobility during thin-layer chromatography. Figure 1 shows the chemical structures of the main aflatoxins. Their chemical structure incorporates dihydrofuran and tetrahydrofuran moieties coupled to a substituted coumarin. They are produced by a polyketide pathway by many strains of *Aspergillus flavus* and *Aspergillus parasiticus*; in particular, *Aspergillus flavus* is a common contaminant in agriculture. *Aspergillus bombycis*, *Aspergillus ochraceoroseus*, *Aspergillus nomius*, and *Aspergillus pseudotamari* are also aflatoxin-producing species, but they are encountered less frequently (Goto, Wicklow, Ito, 1996; Klich, Mullaney, Daly, Cary, 2000; Peterson, Ito, Horn, Goto, 2001). Table 1 gives some relevant chemical properties of these compounds. Aflatoxin B_1 is considered the most toxic and is produced, together with aflatoxin B_2 by both *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin G_1 and G_2 are produced exclusively by *Aspergillus parasiticus*. While the presence of *Aspergillus* spp. in food products does not always indicate harmful levels of aflatoxins are also present, it does imply a significant risk in consumption. Aflatoxins M_1 and M_2 were originally discovered in the milk of cows which fed on moldy grain. Aflatoxin M_1 has been observed also in the fermentation broth of *Aspergillus parasiticus*. These compounds are products of a conversion process in the animal's liver that try to make these molecules more hydrophilic to be easily excreted from body via the kidney. Aflatoxin M_1 is a metabolite of aflatoxin B_1 in humans and animals where exposure at ng levels can come from mother's milk. Similarly, aflatoxin M_2 is a metabolite of aflatoxin B_2 in milk of cattle fed on contaminated food (Tara, 2005). Other metabolites can derive from these main ones, like Aflatoxicol, that forms by biological reduction of aflatoxin B_1 (Pawlowski, Schoenhard, Lee, Libbey, Loveland, Sinnhuber, 1977). The levels considered safe for these compounds are reported in Table 2. Aflatoxin B_1 is the most potent natural carcinogen known, and is probably also the most studied aflatoxin being often the major aflatoxin produced by toxigenic strains (Squire, R. A. 1981). For this reason, it is also the best studied: in a large percentage of the papers published the term aflatoxin can be assumed to refer to aflatoxin B_1. However, many other aflatoxins (e.g., P_1. Q_1, B_{2a}, and G_{2a}) have been described, especially as mammalian biotransformation products of the major metabolites (Heathcote, Hibbert, 1978).

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Aflatoxin	MW (g/ mol)	Formula	Melting point (°C)	IUPAC name
B_1	312.28	$C_{17}H_{12}O_6$	268–269	2,3,6a,9a-tetrahydro-4-methoxycyclopenta(c)furo(3',2':4,5)furo(2,3-h)(1)benzo-pyran-1,11-dione
B_2	314.29	$C_{17}H_{14}O_6$	286–289	2,3,6aa,8,9,9aa-Hexahydro-4-methoxycyclopenta(c)furo(2',3':4,5)furo(2,3-h)chromene-1,11-dione
G_1	328.28	$C_{17}H_{12}O_7$	244-246	7AR,cis)3,4,7a,10a-tetrahydro-5-methoxy-1H,12H-furo(3',2':4,5)furo(2,3-h)pyrano(3,4-c)chromene-1,12-dione
G_2	330.29	$C_{17}H_{14}O_7$	237–240	1H,12H-furo(3',2':4,5)furo(2,3-h)pyrano(3,4-c)(1)benzopyran-1,12-dione
M_1	328.28	$C_{17}H_{12}O_7$	299	(6AR-cis)-2,3,6a,9a-tetrahydro-9a-hydroxy-4-methoxycyclopenta(c)furo(3',2':4,5)furo(2,3-h)(1)benzopyran-1,11-dione
M_2	330.29	$C_{17}H_{14}O_7$	293	2,3,6a,8,9,9a-Hexahydro-9a-hydroxy-4-methoxycyclopenta(c)furo(3',2':4,5)furo(2,3-h)(1)benzopyran-1,11-dione

Table 1. Chemical relevant data for main aflatoxins (O'Neil, Smith, Heckelman, 2001).

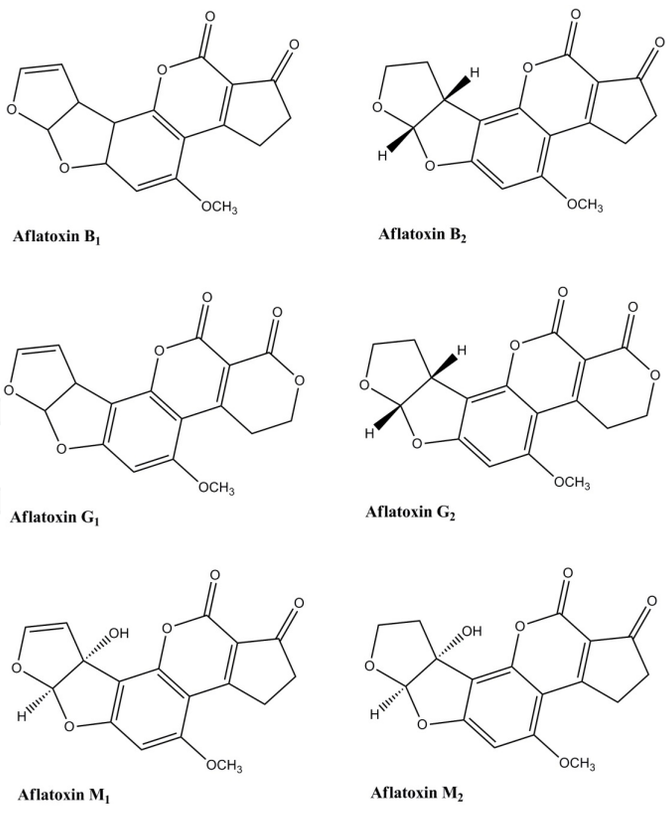

Figure 1. Chemical structures of the main aflatoxins.

µg/kg	Food
20	Food addressed to humans consumption. Corn and grains for animal feeds.
100	Corn and grains for breeding beef cattle, breeding swine, or poultry.
200	Corn and grains intended for swine.
300	Corn and grains for finishing beef cattle, swine, poultry.

Table 2. Aflatoxins levels limits generally considered as safe.

3. Biosynthesis of aflatoxins

Many relevant aspects of aflatoxins biosynthesis and molecular biology have been studied and extensively described. The first step in the biosynthetic pathway is considered the production of norsolorinic acid, an anthraquinone precursor, by a type II polyketide synthase. A series of about 15 post-polyketide synthase steps follows, yielding increasingly toxigenic metabolites (Bennett, Chang, Bhatnagar, 1997; Cleveland, Bhatnagar, 1992; Hicks, Shimizu, Keller, 2002; Payne, Brown, 1998; Townsend, 1997; Trail, Mahanti, Linz, 1995). Sterigmatocystin, a related dihydrofuran toxin, mutagenic and tumorigenic but less potent than aflatoxin (Berry, 1988), is a late metabolite in the aflatoxin pathway, and is also produced as a final biosynthetic product by a number of species like *Aspergillus versicolor* and *Aspergillus nidulans*. Analysis of the molecular genetics of sterigmatocystin biosynthesis in the genetically tractable species *Aspergillus nidulans* has provided a useful model system. The genes for the sterigmatocystin gene cluster from *Aspergillus nidulans* have been cloned and sequenced (Brown, Yu, Kelkar, Fernandes, Nesbitt, Keller, Adams, Leonard, 1996). Cognate genes for aflatoxins pathway enzymes from *Aspergillus flavus* and *Aspergillus parasiticus* show high sequence similarity to the sterigmatocystin pathway genes (Payne, Brown, 1998; Yu, Chang, Bhatnagar, Cleveland, 2000; Yu, Woloshuk, Bhatnagar, Cleveland, 2000). Genes organization for *Aspergillus flavus, Aspergillus nidulans*, and *Aspergillus parasiticus* sterigmatocystin-aflatoxin pathway has been studied as reported by Cary et al. (Cary, Chang, Bhatnagar, 2001) and Hicks et al. (Hicks, Shimizu, Keller, 2002).

Aspergillus oryzae and *Aspergillus sojae*, species that are widely used in Asian food fermentations such as soy sauce, miso, and sake, are closely related to the aflatoxigenic species *Aspergillus flavus* and *Aspergillus parasiticus*. Although these food fungi have never been shown to produce aflatoxin (Wei, Jong. 1986), they contain homologues of several aflatoxin biosynthesis pathway genes (Klich, Yu, Chang, Mullaney, Bhatnagar, Cleveland, 1995). Deletions and other genetic defects have led to silencing of the aflatoxin pathway in both *Aspergillus oryzae* and *Aspergillus sojae* (Takahashi, Chang, Matsushima, Abe, Bhatnagar, Cleveland, Koyama, 2002; Watson, Fuller, Jeens, Archer, 1999; Bennett, Klich, 2003).

4. Frequency and levels of contamination in food

Aflatoxins have received greater attention than any other mycotoxins because of their demonstrated potent carcinogenic effect in susceptible laboratory animals and their acute toxicological effects in humans. Many countries have attempted to limit exposure to aflatoxins by imposing regulatory limits on commodities to be used as food and feed. The two species of *Aspergillus* fungi, aflatoxin producing, are especially found in areas with hot and humid climate. Since aflatoxins are known to be genotoxic and carcinogenic, exposure through food should be kept as low as possible. Aflatoxins have been also associated with various diseases, such as aflatoxicosis. Aflatoxin B_1 is the most common in food, and has the most potent genotoxic and carcinogenic effects. Aflatoxin M_1 is a major metabolite of aflatoxin B_1 in humans and animals, which may be present in milk from animals fed with aflatoxin B_1 contaminated feed. Aflatoxins can occur in foods, such as groundnuts, treenuts, maize, rice, figs, grapes, raisins, and other dried foods, spices and crude vegetable oils, and cocoa beans, as a result of fungal contamination before and after harvest. The biosynthesis and the occurrence of aflatoxins is influenced by environmental factors; consequently the extent of contamination varies with geographic location, agricultural and agronomic practices. The susceptibility of commodities to fungal invasion during preharvest, storage, and/or processing periods is also important to assess the possible contamination.

5. Aflatoxins in food and commodities

From the mycological perspective, there are great qualitative and quantitative differences in the toxigenic abilities displayed by different strains within each aflatoxigenic species. For example, only about half of *Aspergillus flavus* strains produce aflatoxins (Klich, Pitt, 1988), while those that do may produce more than 10^6 μg/kg (Cotty, Bayman, Egel, Elias, 1994). Many substrates support growth and aflatoxin production by aflatoxigenic molds. Natural contamination of cereals, figs, oilseeds, nuts, tobacco, and a long list of other commodities is a common occurrence (Detroy, Lillehoj, Ciegler, 1971; Diener, Cole, Sanders, Payne, Lee, Klich, 1987).

Crops can be contaminated with aflatoxins in the field before harvest (Diener, Cole, Sanders, Payne, Lee, Klich, 1987; Klich, 1987). Even more problematic is the fate of crops stored under conditions that favor mold growth. The most relevant variables to keep under control during the storage are considered the moisture content of the substrate and the relative humidity of the surroundings (Detroy, Lillehoj, Ciegler, 1971; Wilson, Payne, 1994). There are many side implications of aflatoxins contamination. Aflatoxin contamination has been linked to increased mortality in farm animals and thus significantly lowers the value of grains as an animal feed and as an export commodity (Smith, Moss, 1985). Milk products can also be an indirect source of information on aflatoxins presence in the diet, and considering the broad diffusion of these products mainly addressed to infants, children, and people affected by health conditions, the risk associated to aflatoxins M_1 and M_2 is relevant. When cows assume

aflatoxin-contaminated feed, they metabolically biotransform aflatoxin B_1 into a hydroxylated form, namely aflatoxin M_1, as a detoxification way for animal exposed to aflatoxins B_1 or B_2 (Van Egmond, 1989).

6. Occurence

Aflatoxins often occur in crops in the field before harvest so frequently that they are considered mycotoxins originating from the field compared to other mycotoxins that are commonly found in post-harvesting of field crops. Postharvest contamination can occur if crop drying is delayed and during crop storage if water is present in the amount required for the mold growth. Insect or rodent presence can facilitate mold onset on stored commodities. Aflatoxins have been also detected in milk, cheese, corn, peanuts, cottonseed, nuts, almonds, figs, grape berries, spices, and a variety of other foods and feeds. Milk, eggs and meat products are contaminated sometimes due to the consumption by the animal of aflatoxin contaminated feed, and are a clear example of carry-over. A few years after the discovery of mycotoxins, scientific understanding of the carry-over phenomenon raised immediately the interest of scientists and put focus on the risk related to food contaminated by molds. The commodities with the highest risk of aflatoxin contamination are corn, peanuts, and cottonseed. Corn is probably the commodity of greatest worldwide concern, because it is grown in climates that are likely to have perennial contamination with aflatoxins. Corn is the staple food of many countries, and, also for some population corn represents the main ingredient of the diet. It is usually named as single-food with all nutritional and unwanted contaminants related to its consumption. Corn can be used to produce flour and starch products and this links back to the problem statement such as aflatoxins is a likely toxin to be found in foodstuff. However, procedures used in the processing of corn help to reduce contamination of the resulting food product. This is because although aflatoxins are stable to moderately stable in most food processes, they are unstable in processes such as those used in making tortillas that employ alkaline conditions or oxidizing steps. Aflatoxin-contaminated corn and cottonseed meal in dairy rations have resulted in aflatoxin M_1 contaminated milk and milk products, including non-fat dry milk, cheese, ice creams and yogurts. Even in the case of the butter, during its production due to its chemical lipid rich compostion, the accumulation and concentration of any aflatoxin M_1 present in milk is usually involved.

7. Aflatoxins toxicity

Aflatoxins, and especially aflatoxin B_1, are associated with both toxicity and carcinogenicity in human and animal populations. The International Agency for Research on Cancer has classified aflatoxin B_1 as a group I carcinogen (International Agency for Research on Cancer, 1982).

In particular, aflatoxin B_1 is considered by medicine doctors and toxicologists as the most hepatocarcinogenic compound not produced by human activites but produced by a life or-

ganism (Newberne, Butler, 1969; Shank, Bhamarapravati, Gordon, Wogan, 1972; Peers, Linsell, 1973; Eaton, Groopman, 1994). The diseases caused by aflatoxin consumption are loosely called aflatoxicoses. Acute aflatoxicosis results in death; chronic aflatoxicosis results in cancer, immune suppression, and other "slow" pathological conditions (Hsieh, 1988). The liver is the primary target organ, with liver damage occurring when poultry, fish, rodents, and non human primates are fed aflatoxin B_1 contaminated foodstuff. This data is not unexpected because the liver is a lipophilic organ and all compounds carried by blood stream, i.e. drugs, contaminants, mycotoxins etc., are stored and concentrated in the hepatocytes that, with a long exposure time, may transform themselves in a cancer cell line. There are substantial differences in species susceptibility. Moreover, within a given species, the magnitude of the response is influenced by age, sex, weight, diet, exposure to infectious agents, and the presence of other mycotoxins and pharmacologically active substances. Thousands of studies on aflatoxin toxicity have been conducted, mostly on laboratory models or agricultural important species (Cullen, Newberne, 1994; Eaton, Groopman, 1994; Newberne, Butler, 1969).

Cytochrome P450 enzymes convert aflatoxins to the reactive 8,9-epoxide form (also known as aflatoxin-2,3 epoxide), which is capable of binding to both DNA and proteins (Eaton, Groopman, 1994). The reactive aflatoxin epoxide binds to the N^7 position of guanines. Moreover, aflatoxin B_1-DNA adducts can result in GC to TA transversions. A reactive glutathione *S*-transferase system found in the cytosol and microsomes catalyzes the conjugation of activated aflatoxins with reduced glutathione, leading to the excretion of aflatoxins (Raj, Prasanna, Mage, Lotlikar, 1986). Variation in the level of the glutathione transferase system as well as variations in the cytochrome P450 system are considered contributor to the differences observed in interspecies aflatoxin susceptibility (Eaton, Ramsdel, 1992; Eaton, Groopman, 1994).

Considering the differences exhisting in aflatoxin susceptibility in test animals, it has been proven not easy to extrapolate the possible effects of aflatoxins to humans. Acute toxicity of aflatoxins in humans however represent a serious threat.

In 1974 it has been reported in India an outbreak of hepatitis and 100 cases of death attributed to the consumption is heavily aflatoxins contaminated maize, causing an aflatoxins intake of 2 to 6 mg per day (Krishnamachari, Bhat, Nagarajan, Tilnak, 1975). Based on these data, it has been estimated that the acute lethal dose (LD) for adults is approximately 10 to 20 mg of aflatoxins (Pitt, 2000). Aflatoxins have been in years associated to various health conditions and are considered a poison. For example it has been associated kwashiorkor, a severe malnutrition disease, to a form of pediatric aflatoxicosis (Hendrickse, 1997). Aflatoxins, according to reported studies non completely assessed, could be involved in Reye's syndrome, an encephalopathy, and to fatty degeneration of some target organs in children and adolescents (Hayes, 1980).

Exposure to aflatoxins in the diet is considered an important risk factor for the development of primary hepatocellular carcinoma, particularly in individuals already exposed to hepatitis B. There are also observed nonhepatic effects of aflatoxin B_1 as reported by Coulombe (Coulombe, 1994). Several epidemiological studies have linked liver cancer incidence to estimated

aflatoxin consumption in the diet (Peers, Linsell, 1973; Van Rensburg, Cook-Mazaffari, van Schalkwyk, van der Watt, Vincent, Purchase, 1985; Li, Yoshizawa, Kawamura, Luo, Li, 2001) even if the long term quantification of individual exposure to aflatoxins is difficult. The incidence of liver cancer varies widely from country to country, but it is one of the most common occurring in China, the Philippines, Thailand, and many African countries. The presence of hepatitis B virus infection, an important risk factor for primary liver cancer, complicates many of the epidemiological studies. In one case-control study involving more than 18.000 urine samples collected over 3.5 years in Shanghai, China, aflatoxin exposure alone yielded a relative risk of about 2; hepatitis B virus antigen alone yielded a relative risk of about 5; combined exposure to aflatoxin and hepatitis B yielded a relative risk of about 60 (Ross, Yuan, Yu, Wogan, Qian, Tu, Groopman, Gao, Henderson, 1992).

Using molecular epidemiology, it is possible to asses a link exhisting between putative carcinogens and specific cancers. Biomonitoring of aflatoxins can be done by analyzing for the presence of aflatoxin metabolites in blood, milk, and urine. In addition, excreted DNA adducts and blood protein adducts can also be monitored (Sabbioni, Sepai, 1994). The aflatoxin B_1-N^7-guanine adduct is considered a reliable urinary biomarker for aflatoxin exposure but reflects only recent exposure. Many studies have shown that carcinogenic potency is highly correlated with the extent of total DNA adducts formed *in vivo* (Eaton, Gallagher, 1994; Eaton, Groopman, 1994).

Inactivation of the p53 tumor suppressor gene may be important in the development of primary hepatocellular carcinoma. Studies of liver cancer patients in Africa and China have shown that a mutation in the p53 tumor suppressor gene at codon 249 is associated with a G-to-T transversion (Bressac, Kew, Wands, Ozturk, 1991; Hsu, Metcalf, Sun, Welsh, Wang, Harris, 1991). It is known that the reactive aflatoxin epoxide binds to the N^7 position of guanines. Moreover, aflatoxin B_1-DNA adducts can result in GC to TA inversion. The specific mutation in codon 249 of the p53 gene has been called the first example of a "carcinogen-specific" biomarker that remains fixed in the tumor tissue (Eaton, Gallagher, 1994).

There is also considerable evidence associating aflatoxin with neoplasms in extrahepatic tissues, particularly the lungs. For example, one early epidemiological study of Dutch peanut processing workers exposed to dust contaminated with aflatoxin B_1 showed a correlation between both respiratory cancer and total cancer in the exposed group compared with unexposed cohorts (Hayes, van Nienwenhuise, Raatgever, Ten Kate, 1984). Exposition even indirect to aflatoxins can result in a severe health issue: Deger (Deger, 1976) reported for example that dust from scrapings of chromatographic plates from aflatoxin analyses contributed to causing cancer in two young adults.

In developed countries, sufficient amounts of food combined with regulations that monitor aflatoxin levels protect human populations from significant aflatoxins ingestion. However, in countries where populations are facing starvation or where regulations are either not enforced or nonexistent, routine ingestion of aflatoxin may occur (Cotty, Bayman, Egel, Elias, 1994). Worldwide, liver cancer incidence rates are 2 to 10 times higher in developing countries than in developed countries (Henry, Bosch, Troxell, Bolger, 1999). A joint Food and Agriculture Or-

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ganization/World Health Organization/United Nations Environment Programme Conference report stated that "in developing countries, where food supplies are already limited, drastic legal measure may lead to lack of food and to excessive prices. It must be remembered that people living in these countries cannot exercise the option of starving to death today in order to live a better life tomorrow" (Henry, Bosch,. Troxell, Bolger, 1999).

8. Monitoring techniques for assessing human exposure to aflatoxins

In the last few years, new technologies have been developed that more accurately monitor individual exposures to aflatoxins. Particular attention has been paid to the analysis of aflatoxin DNA adducts and albumin adducts as surrogates for genotoxicity in people. Autrup et al. (Autrup, Bradly, Shamsuddin, Wakhisi, Wasunna, 1983) proposed for the first time the use of synchronous fluorescence spectroscopy for the measurement of aflatoxin DNA adducts in urine. Urine samples collected after exposure to alfatoxins were found to contain 2,3-dihydroxy-2-(N7-guanyl)-3-hydroxyaflatoxin B_1, trivially known as aflatoxin B-Gual. Wild et al. used highly sensitive immunoassays to quantitate aflatoxins in human body fluids (Wild, Umbenhauer, Chapot, Montesano, 1986).

An enzyme linked immunosorbent assay (ELISA) was used to quantitate aflatoxin B_1 in a range from 0.01 ng/mL to 10 ng/mL, and was validated in human urine samples. Using this method, aflatoxin-DNA adduct excretion into urine was found to be positively correlated with dietary intake, and the major aflatoxin B_1-DNA adduct excreted in urine was shown to be an appropriate dosimeter for monitoring aflatoxin dietary exposure. Several epidemiological studies have found positive association between aflatoxin B_1 dietary exposure and an increased risk of human liver cancer (Sudakin, 2003; Zhu, Zhang, Hu, Xiao, Chen, Xu, Fremy, Chu, 1987; Groopman, Donahue,1988; Bean, Yourtee, 1989). Cytochrome P-450 enzymes further convert aflatoxins to different metabolites (Eaton, Ramsdell, Neal, 1994), e.g. aflatoxin B_1 is converted to metabolites like aflatoxin B_1-epoxide and the hydroxylated aflatoxins M_1, P_1 and Q_1. The hydroxylated metabolites form glucuronide and sulfate conjugates that can be enzymatically hydrolysed by b-glucuronidase and sulfatase (Wei, Marshall, Hsieh, 1985).

The European Union (EU) introduced measures to minimise the presence of aflatoxins in different foodstuffs. Maximum levels of aflatoxins are laid down in Commission Regulation (EC) No. 1881/2006. In an opinion adopted in January 2007, the European Food Safety Authority (EFSA) scientific Panel on contaminants in the food chain (CONTAM), concluded that increasing the current EU maximum levels of 4 μg/kg total aflatoxins in nuts to 8 or 10 μg/kg total afatoxins would have had minor effects on the estimated dietary exposure, cancer risk and calculated margin of exposure. The Panel also concluded that exposure to aflatoxins from all food sources should be kept as low as reasonably achievable because aflatoxins are genotoxic and carcinogenic. In June 2009 the European Commission asked EFSA to assess the effect on public health of an increase of the maximum level for total aflatoxins from 4 μg/kg to 10 μg/kg allowed for tree nuts other than almonds, hazelnuts and pistachios (e.g. Brazil nuts and cashews). This would facilitate the enforcement of the maxi-

mum levels, in particular regarding commercially available mixtures of nuts. The Panel concluded that public health would not be adversely affected by increasing the levels for total aflatoxins from 4 μg/kg to 8 or 10 μg/kg. However, the Panel reiterated its previous conclusions regarding the importance of reducing the number of highly contaminated foods reaching the market.

9. Bioavailability

In human health risk assessment, ingestion of contaminated food is considered a major route of exposure to many contaminants either caused by industrial or environmental contamination or as result of production processes. The total amount of an ingested contaminant (intake) does not always reflect the amount that is available to the body. Only a certain amount of the contaminant will be bioavailable (Versantvoort, Oomen, Van de Kamp, Rompelberg, Sips, 2005). Bioavailability is a term used to describe the proportion of the ingested contaminant in food that reaches the systemic circulation and then the organ or the apparatus. Studies in animals and humans show that oral bioavailability of compounds from food can be significantly different depending on the food source (food product), food processing or food preparation (Wienk, Marx, Beynen, 1999; van het Hof, West, Weststrate, Hautvast, 2000). As a consequence, the intake of a contaminant in food matrix A can lead to toxicity whereas the intake of the same amount of contaminant in food matrix B will not exert a toxic effects. Thus, a better insight in the effect of the matrix on the oral bioavailability of a contaminant will lead to a more accurate health risk assessment (Versantvoort, Oomen, Van de Kamp, Rompelberg, Sips, 2005).

Oral bioavailability of a compound can be seen as the resultant of three processes, namely the release of the compound from its matrix into digestive juice in the gastrointestinal tract (bioaccessibility); the transport across the intestinal epithelium into the vena Portae (intestinal transport); and the degradation of the compound in the liver and intestine (metabolism).

Release of the contaminant from the ingested product in the gastrointestinal tract is a prerequisite for uptake and bioavailability of a contaminant in the body. The oral bioavailability of the contaminant can be reduced subsequently by partial transport of the contaminant across the intestinal epithelium, or by degradation of the contaminant. Thus, determination of the bioaccessibility of a contaminant from its matrix can be seen as an indicator for the maximal oral bioavailability of the contaminant. Quantification of bioavailability and bioaccessibility of a compound from a certain matrix is difficult and often hampered by complex processes comprising digestion. The last decade there is an increasing interest in the use of *in vitro* methodologies to study the human oral bioavailability of compounds from the food chain (Minekus, Marteau, Havenaar, Huis, 1995; Glahn, Wien, Van Campen, Miller, 1996; Garrett, Failla, Sarama, 1999; Ruby, Schoof, Brattin, Goldade, Post, Harnois, Mosby, Casteel, Berti, Carpenter, Edwards, Cragin, Chappell, 1999; Oomen, Hack, Minekus, Zeijdner, Cornelis, Schoeters, Verstraete, Wiele, Wragg, Rompelberg, Sips, Wijnen, 2002).

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Most of the *in vitro* digestion models simulate in a simplified manner the digestion processes in mouth, stomach and small intestine, in order to enable investigation of the bioaccessibility of compounds from their matrix during transit in the gastrointestinal tract.

Extensive studies involving animal models have indicated that the primary site for absorption of aflatoxin is the small intestine, in particular the duodenum (Wogan, Edwards, Shank, 1967; Ramos, Hernandez, 1996). *Lactobacillus spp.* has previously proven to be capable to survive at the gastrointestinal tract after oral intake (Taranto, Medici, Perdigon, Ruiz-Holgado, Valdez, 2000; Valeur, Engel, Carbajal, Connolly, Ladefoged, 2004); therefore, it is probable that mycotoxins were in contact with bacteria in the intestinal lumen, which then favored aflatoxin B_1 binding by bacteria prior to its natu ral process of absorption.

It has been reported that the binding process might be dependent on the environmental pH (Bolognani, Rumney, Rowland, 1997) and that the presence of bile salts could produce significant effects in the aflatoxin B_1 binding ability of the bacteria (Hernandez-Mendoza, Garcia, Steele, 2009). These two factors are closely related during the normal digestive process and its relationship varies along the small intestine (Low, 1990). Hence, the difference on aflatoxin binding ability of *Lactobacillus spp.* observed at the different portions of the intestine could be influenced by conditions prevailing in each region of the gastrointestinal tract.

Once the aflatoxin B_1 has been absorbed at intestinal level, it proceeds to the bloodstream and binds with plasma proteins especially albumin to form aflatoxin B_1-albumin adduct (Verma, 2004). The average half-life of albumin (approximately 20 days in humans) allows accumulation of adducts after chronic exposure to the toxin (Chapot, Wild, 1991). According to this, the amount of adducts present in blood samples of rats treated only with aflatoxin B_1 represent the cumulative dose of aflatoxin intake over the experimental period, which indicates that the reduction of aflatoxin B_1-Lys adduct observed in animals treated with aflatoxin plus bacteria was originated by the ability of *Lactobacillus spp.*to bind aflatoxin B_1 inside the intestinal lumen, thus avoiding its passage into the bloodstream. In a related work (Gratz, Täubel, Juvonen, Viluksela, Turner, 2006) no significant differences were found in the amounts of aflatoxin B_1-Lys adduct present in animals receiving *Lactobacillus rhamnosus* GG daily for 3 d before and 3 d after a single oral dose of aflatoxin B_1 compared with those receiving only the mycotoxin. Other reports suggested that probiotics are less capable of binding aflatoxin B_1 in the presence of mucus and are more susceptible to interfere factors in the intestinal tract, which may explain the behavior observed in the levels of adduct (Gratz, Mykkänen, Ouwehand, Juvonen, Salminen, 2004; Gratz, Täubel, Juvonen, Viluksela, Turner, 2006). This effect could have been surmounted by the numbers of bacteria implanted before oral dose of aflatoxin B_1, and the constant administration of probiotic bacteria during the experimental period (Gratz, Mykkänen, Ouwehand, Juvonen, Salminen, 2004).

In agreement with earlier reports (Ward, Sontag, Weisburger, Brown, 1975; Maurice, Bodine, Rehrer, 1983), body weight gain was not adversely affected. However, there was a reduction in feed intake in rats receiving only aflatoxin B_1. This effect could be induced by the dose of aflatoxin received, since it has been reported that aflatoxin B_1 induces reduction of food intake in some animal species, including rats and birds, in a dose-dependent manner (Maurice, Bodine, Rehrer, 1983). In addition, toxicological studies in rats have shown that aflatoxin

B_1 consumption may produce a significant decrease of serum leptin levels (Abdel-Wahhab, Ahmed, Hagazi, 2006). Leptin concentration is usually associated with the high levels of cortisol and interleukin-6, which act together to influence the feeding response (Barber, McMillan, Wallace, Ross, Preston, 2004). *Lactobacillus reuteri* might have contributed to reduce the aflatoxin B_1 absorption in bacteria-treated rats and thus diminish its effect on leptin levels in blood serum.

The volume of the stomach is considered an important parameter for oral dosing in experimental animals. For rats, maximum oral dosage volume recommended is 10 mL kg^{-1} of body weight; for a 200 g rat this would mean a dosing volume of 2 mL (McConnell, Basit, Murdan, 2008). Therefore, it is possible that the volume supplied (every third day) by oral gavage of aflatoxin and/or bacteria over the experiment, had partially met the basic water needs of the rats, which may explain the observed reduction in water consumption at the end of the experimental period (21 days).

A world-wide-accepted method for protecting animals against mycotoxicosis is the use of adsorbent materials. An effective adsorbent is one that tightly binds the mycotoxin in contaminated feed without dissociating in the gastrointestinal tract of the animal. The toxin–adsorbent complex passes then through the gastrointestinal tract without absorption and is eliminated via the faeces. In other words, the bioavailability of the mycotoxin is reduced as less mycotoxin is absorbed because it is bound to the adsorbent, i.e. lower bioaccessibility. Therefore, these adsorbents can be used to evaluate the use of the in vitro digestion model as indicator for the in vivo bioavailability. The following materials, representative for different classes of adsorbents, have been selected: an aluminosilicate (HSCAS), which is a common anticaking additive in animal feeds to reduce mycotoxicosis in animals; activated charcoal, which is used in humans and animals as an antidote against poisoning; cholestyramine is an anion exchange resin and binds bile acids in the gastrointestinal tract and it has been used for over 20 years in the clinic for reduction of lowdensit y lipoproteins and cholesterol.

The effect of chlorophyllin on intestinal transport of aflatoxin B_1 was studied by measurement of the transport of aflatoxin B_1 with the intestinal Caco-2 cells. The rate at which compounds are transported across the Caco-2 cells, which is expressed as a permeability coefficient, is correlated with absorption in humans (Artursson, Karlsson, 1991).

Transport of 5ng/mL aflatoxin B_1 across Caco-2 cells revealed that after 4h, 25±6% aflatoxin B_1 was transported across Caco-2 cells into the basolateral compartment. Addition of chlorophyllin (1 mg/mL) greatly reduced (>20-fold) the transport of aflatoxin B_1 to only 1±1%. From this transport, a permeability coefficient can be calculated for aflatoxin B_1 of $9x10^{-6}$ cm/s in absence, and $0.4x10^{-6}$ cm/s in presence of chlorophyllin. When we compare these transport rates with the S-shaped correlation found for absorption of compounds in humans, the permeability coefficient of aflatoxin B_1 alone ($9x10^{-6}$ cm/s) corresponds with high absorption in humans whereas the permeability coefficient of aflatoxin B_1 in presence of chlorophyllin ($0.4x10^{-6}$ cm/s) indicates an intermediate absorption. Thus, these data are in accordance with the human intervention study on chlorophyllin and aflatoxin B_1, where a 50%

reduction in excretion of aflatoxin metabolites in urine was found in presence of chlorophyllin (Versantvoort, Oomen, Van de Kamp, Rompelberg, Sips, 2005).

Furthermore, these results provide evidence for the hypothesis that chlorophyllin reduces the absorption of aflatoxin B_1 in humans.

In risk assessment, a dose proportional relationship between contamination level and bioavailability is taken as basic assumption. This assumption simplifies risk assessment, since it can be assumed that regardless the level of contamination, a constant percentage of the contaminant will be bioavailable. The extreme sensitivity of turkeys to the toxic effects of aflatoxin B_1, a condition associated with a combination of efficient CYP-mediated activation and deficient GST-mediated detoxification of aflatoxin B_1 (Klein, Buckner, Kelly, Coulombe, 2000), makes turkeys an excellent model in which to study various chemopreventives. We have recently shown that the observed chemopreventive properties of BHT in turkeys is due, at least in part, to its ability to inhibit hepatic conversion of aflatoxin B_1 to the exo-aflatoxin B_1-8,9-epoxide (AFBO) in vivo and in vitro (Guarisco, Hall, Coulombe, 2008).

Determining the outcome of inhibition of hepatic aflatoxin B_1 bioactivation in whole animals is relevant to veterinary medicine and to food safety. Dietary butylated hydroxytoluene (BHT) can reduce aflatoxin B_1 bioavailability, as demonstrated by serum concentrations of radiolabel which were reduced at every time interval after aflatoxin B_1 administration. Among the possible explanations for reduced bioavailability is high first pass elimination prior to absorption into the blood, and/or an attenuation of mucosal aflatoxin B_1 absorption. However, since no quantitative difference in the biliary elimination of aflatoxin B_1 or its metabolites was identified, any change due to increased first pass effect would have to result in increased elimination by non-biliary pathways.

The observed reduction in hepatic aflatoxin B_1–DNA adducts in BHT fed animals is consistent with the fact that this antioxidant is a competitive inhibitor of hepatic in vitro CYP1A5-mediated aflatoxin B_1 epoxidation to AFBO. Because of the critical role of AFBO and subsequent adduct formation in aflatoxicosis (as well as longer-term consequences such as tumor formation), a reduction in hepatic aflatoxin B_1–DNA adducts would be expected to have a positive effect on the overall health of aflatoxin B_1-exposed turkeys.

10. Bioaccessibility

Bioaccessibility has been defined as the fraction of a compound that is released from its matrix in the gastrointestinal tract and thus becomes available for intestinal absorption i.e. enters the blood stream (Benito, Miller, 1998). Bioaccessibility includes the entire sequence of events that take place during the digestive transformation of food into material that can be assimilated by the body, the absorption/assimilation into the cells of the intestinal epithelium, and lastly, the presystemic metabolism (both intestinal and hepatic). Bioaccessibility analyses can be approached using general experimental techniques (there are systematic techniques common to all types of foods) that can be adapted to all types of claims regarding nutritional content.

In vivo, as soon as a compound is released from its matrix in the chyme, the compound can be transported across the intestinal epithelium into the body thereby keeping the compound concentration low in the chyme.

Different analytical approaches can be applied to measure bioaccessibility of nutrients and bioactive compounds: *in vivo* and *in vitro* studies both present strengths and drawbacks. Within *in vivo* studies, balance studies and tissue concentration are two strategies that allow determination of the absorbed amount of nutrients, bioactive compounds, or their metabolites. Balance studies determine the absorbed amount by measuring the difference between the fed and excreted amounts of the nutrient or bioactive compound. Tissue concentration consists of monitoring the increase in plasma/serum concentration of the nutrient or bioactive compound. These approaches have been applied these approaches have been used with both animals and humans to determine absorption of carbohydrates, minerals, vitamins, phytochemicals, and different compounds (Benito, Miller, 1998; Hallberg, 1991). *In vivo* human studies are the criterion standard approach to determine bioaccessibility of food nutrients or bioactive compounds, although some experimental approaches are ethically and technically unaffordable.

Digestion and absorption involve several different steps, and each one could cause an effect on the nutrient or bioactive compound so that a detailed picture is not obtained with the balance and bioassay studies. *In vitro* studies have been developed to simulate the physiologic conditions and the sequence of events that occur during digestion in the human gastrointestinal tract. In a first step, an in vitro gastrointestinal method is applied to the food, mirroring the physiochemical conditions that take place during human digestion, considering the three areas of the human digestive system (mouth, stomach, and intestine).

The main features of the in vitro gastrointestinal methods are temperature, shaking or agitation, and the chemical and enzymatic composition of saliva, gastric juice, duodenal juice, and bile juice (Wittsiepe, Schrey, Hack, Selenka, Wilhelm, 2001). When physical processes that occur in vivo are not reproduced (shear, mixing, hydration, changes in conditions over time, peristalsis), the in vitro gastrointestinal model is defined as a static or biochemical model. The dynamic models mimic the *in vivo* physical processes so that they take into account new variables, such as changes on viscosity of the digesta, particle size reduction, diffusion, and partitioning of nutrients. Several examples of *in vitro* gastrointestinal static and dynamic models have been described (Rotard, Christmann, Knoth, Mailahn, 1995; Arcand, Mainville, Farnworth, 2007). During the application of the in vitro gastrointestinal method, food nutrients or bioactive compounds can be monitored to determine whether they are affected by digestion conditions (pH, enzymes) or if interactions with other food components (fiber, sucrose polyester, fat replacers) take place, which could affect efficiency of digestion. The final processed material of the experimental procedure is a digesta or intestinal preparation.

To analyze the lipophilic content that has been effectively incorporated to mixed micelles, the micellar fraction can be isolated from that processed material by the application of an ultracentrifugation protocol (Hernell, Staggers, Carey, 1999). In the digestion model, the compounds are not removed from the chyme during digestion and therefore, bioaccesibility

may be underestimated when saturation of the compound occurs in the chyme. Thus, one factor potentially affecting bioaccessibility is the level of contamination.

The bioaccessibility of aflatoxin B_1 in chime has been determined from nine peanut slurries ranging from 0.6 to 14 μg/kg aflatoxin B_1 (contamination level in peanuts 1.5-36 μg/kg). Aflatoxin B_1 was almost completely mobilised from the peanut slurries during digestion evidencing a mean bioaccessibility of 94%. The concentration of aflatoxin B_1 in chyme of the two highest contaminated peanut slurries was higher than those in the calibration curve and a smaller volume of chyme was used for analysis (Versantvoort, Oomen, Van de Kamp, Rompelberg, Sips, 2005).

The amount of aflatoxin B_1 released from the peanut slurries into the chyme is dose proportional to the contamination level in the peanut slurries. These results showed no saturation of the chyme with aflatoxin B_1. The amount of food in the digestion model was varied in order to study whether release of the contaminant from its food matrix was linearly, i.e. whether bioaccessibility was independent from the amount of food in the model. Application of 0.5 g and 4.5 g peanut slurry in the in vitro digestion model corresponds to the consumption of approximately 10 and 100 g peanuts, respectively. The bioaccessibility of some bioactive compounds can be influenced by the food composition as observed by Versantvoort et al. (Versantvoort, Oomen, Van de Kamp, Rompelberg, Sips, 2005) that studied the effects of different food components on the bioaccessibility of aflatoxin B_1 from peanut slurry considering an average meal. Bioaccessibilities of aflatoxin B_1 (108±11%) from 6 g food-mix (4.5g standard meal + 0.5g peanut slurry + 1g buckwheat) were compared to the bioaccessibility of aflatoxin B_1 from 0.5g corresponding peanut slurry (83±18%) showing that the bioaccessibility of aflatoxin B_1 did not vary significantly.

11. Advanced analysis of aflatoxins in biological fluids

11.1. Sampling and sample preparation

Sampling and sample preparation remain a considerable source of error in the analytical identification of aflatoxins. Thus, systematic approaches to sampling, sample preparation, and analysis are absolutely necessary to determine aflatoxins 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 modeled 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. Methods of sampling and analysis for the official control of mycotoxins, including aflatoxins, are laid down in Commission Regulation No 401/2006. This ensures that the same sampling criteria intended for the control of mycotoxin content in food are applied to the same products by the competent authorities throughout the EU and that certain performance criteria, such as recovery and precision, are fulfilled. In 2008, the Codex Alimentarius set a maximum

level of 10 μg/kg total aflatoxins in ready-to-eat almonds, hazelnuts, and pistachios at a level higher than that currently in force in the EU (4 μg/kg total aflatoxins).

11.2. 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. Extracts are 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.

11.3. 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 analysis. Since 1990, it has been considered the AOAC official method and the method of choice to identify and quantitate aflatoxins at levels as low as 1 ng/g. The TLC method is also used to screen and corroborate findings by newer, more rapid techniques.

11.4. Liquid chromatograph

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 aflatoxins 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.

11.5. Immunochemical methods

Thin layer chromatography and LC methods for determining aflatoxins in food are laborious and time consuming. Often, these techniques require knowledge and experience of chromatographic techniques to solve separation and and interference problems. Through advances in biotechnology, highly specific antibody-based tests are now commercially available that can identify and measure aflatoxins in food in less than 10 minutes. These tests are based on the affinities of the monoclonal or polyclonal antibodies for aflatoxins. The three types of immunochemical methods are radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and immunoaffinity column assay (ICA). These are mostly chemical methods of detection but still provide an insight into the immunochemical methods such as ELISA and RIA which can used to detect aflatoxins in foods, such as flour and starch products.

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12. Aflatoxins identity assessment

Although analytical methods might consist of different extraction, clean-up, and quantitation steps, the results of the analyses by such methods should be similar when the methods are applied properly. Since the reliability of the quantitative data is not in question, the problem still to be solved is the confirmation of identity of the aflatoxins. The confirmation techniques used involve either chemical derivatization or mass spectrometry (MS).

Different analytical methods have been reported in the literature in order to facilitate the investigation of the role of ingested aflatoxins in small volumes of human sera (Grio, Jose, Frenich, Martinez Vidal, Luis, Romero-Gonzalez, 2010; Yuanjing, Yi, Huiming, Bingnan, Haicheng, Fanli, Miaomiao, Wei, Wendong, 2010). Aflatoxin B_1 has been extracted from 1 mL or less of human sera spiked with a known concentration of aflatoxin B_1 and analyzed using high-performance liquid chromatography (HPLC) as the detection system. Several methods have been used to analyze feed, foods and bodyfluids, human and animal plasma, serum, milk, etc. (Santini, Ferracane, Meca, Ritieni, 2009; Rampone, Piccinelli, Aliberti, Rastrelli, 2009; Monbaliu, Van Poucke, Detavernier, Dumoulin, Van De Velde, Schoeters, Van Dyck, Averkieva, Van Peteghem, De Saeger, 2010). The ELISA (Zhu, Zhang, Hu, Xiao, Chen, Xu, Fremy, Chu, 1987) or radioimmunoassay (RIA) methods (Groopman, Donahue, 1988; Tang, Pang, 2009; Li, Zhang, Zang, 2009) allow the quantification of the total amount of aflatoxins, and results are expressed in term of aflatoxin B_1 equivalents. Both methods however involve the use of specific antibodies not commercially available. Recently immunoensors (Sun, Yan, Tang, Zhang, 2012) and biosensor have been proposed for the analyses of mycotoxins in different matrices (Campàs, Garibo, Prieto-Simón, 2012).

The International Agency for Research on Cancer has classified aflatoxin B_1 as a human carcinogen and aflatoxins B_2, G_1 and G_2 as possible nephrotoxic natural compounds and carcinogenic to humans (IARC, 1993; Commission Regulation (EC) No. 1525/98, 1998). Due to carryover in food and feed they are considered nowadays to have the most severe impact of all mycotoxins on human health. Maximum residue levels have been set down to the ▪ g/mL range in a wide variety of agricultural commodities, food, feed and milk, e.g. 0.01 mg/kg of aflatoxin M_1 in milk for infants (Groopman, Donahue, Zhu, Chen, Wogan,1985).

Methods like liquid chromatography-mass spectroscopy (LC/MS) have been repeatedly used for structural elucidation in metabolism on aflatoxin containing analytes and specific matrices but only a limited number of quantitative methods have been published to determine the more common aflatoxins present in food (Papp, Otta, Zaray, Mincsovics, 2002; Biselli, Hartig, Wegener, Hummert, 2004; Biselli, Hartig, Wegener, Hummert, 2005; Sorensen, Elbaek, 2005; Kokkonen, Jestoi, Rizzo, 2005) milk, (Sorensen, Elbaek, 2005) cheese, (Cavaliere, Foglia, Pastorini, Samperi, Lagana, 2006) herbs, (Ventura, Gomez, Anaya, Diaz, Broto, Agut, Comellas, 2004) urine, (Scholl, Musser, Groopman, 1997; Walton, Egner, Scholl, Walker, Kensler, Groopman, 2001; Egner, Yu, Johnson, Nathasingh, Groopman, Kensler, Roebuck, 2003; Wang-Buhler, Lee, Chung, Stevens, Tseng, Hseu, Hu, Westerfield, Yang, Miranda, Buhler, 2005) airborne dust (Kussak, Nilsson, Andersso, Langridge, 1995) and cigarette smoke (Edinboro, Karnes, 2005).

LC/MS has been used as a confirmation technique for the already well established, reliable and robust LC-FL methodology (Kussak, Nilsson, Andersson, Langridge, 1995; Abbas, Williams, Windham, Pringle, Xie, Shier, 2002; Blesa, Soriano, Molto, Marin, Manes, 2003; Abbas, Cartwright, Xie, Shier, 2006) and has also been used to confirm positive results of TLC and ELISA based screening analyses. All the aflatoxins exhibit good ESI ionisation efficiency in the positive ion mode with abundant protonated molecules $[MH]^+$ and sodium adduct ions (Blesa, Soriano, Molto, Marin, Manes, 2003; Ventura, Gomez, Anaya, Diaz, Broto, Agut, Comellas, 2004; Kussak, Nilsson, Andersson, Langridge, 1995) and typically, for aflatoxins B_1, B_2, G_1 and G_2, the formation of sodium adduct ions can easily be suppressed by the addition of ammonium ions to the mobile phase leading to a better mass spectroscopy (MS) sensitivity (Cavaliere, Foglia, Pastorini, Samperi, Lagana, 2006). Reports about the utility of atmospheric pressure chemical ionization (APCI) interfaces and ionization efficiencies in this mode seem to be highly dependent on the aflatoxin studied and the APCI interface geometry (Abbas, Williams, Windham, Pringle, Xie, Shier, 2002; Abbas, Cartwright, Xie, Shier, 2006).

This method has been proved to be more sensitive for the simultaneous determination of aflatoxins B_1, B_2, G_1, G_2, M_1, M_2, and moreover smaller sample volumes of serum can be used for the analysis. Aflatoxins are in free equilibrium with the albumin combined form and it is reported in the literature the effect of pH and/or serum concentration of fatty acids on the formation of the adducts. Moreover, a recent study showed that green tea polyphenols might modulate the formation of the adducts between aflatoxin B_1 and albumin (Tang, Tang, Xu, Luo, Huang, Yu, Zhang, Gao, Cox, Wang, 2008).

Advanced spectrometric methods, such as LC-MS/MS, permit quantification and recognition of the free aflatoxins in the sera with fewer problems on recovery, sensitivity and chemical identification (Santini, Ferracane, Meca, Ritieni, 2009; Huang, Zheng, Zengxuan, Yongjiang, Yiping, 2010) evaluating the aflatoxin exposure directly from their free forms.

13. Decontamination, detoxification, exposition

Aflatoxin produced by *Aspergillus flavus, A. parasiticus* and *A. nomius* crops contamination, is a worldwide food safety concern. Several strategies, including chemical, physical and biological control methods have been investigated to manage these potent toxic secondary metabolites in foods. Among them, biological control seems nowadays to be the most promising approach for the aflatoxins control. From the food safety point of view, fermentation with microorganisms, a technique quite commonly used in food production (e.g. fermentation with lactic acid bacteria, alcoholic fermentation, conventional fermentation of the protein from vegetables as common in South Asia, etc.) should be preferred. In optimal conditions, this procedure can result in a mycotoxin-free food or feed.

The reaction of aflatoxins to various physical conditions and reagents have been studied extensively because of the possible application of such reactions to the detoxification of aflatoxins contaminated material. Aflatoxins in dry state are stable to heat up to the melting

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point. However, in the presence of moisture and at elevated temperatures there is destruction of aflatoxin and this can occur with aflatoxin in oilseed meals, roasted peanuts or in aqueous solution at pH 7. Although the reaction products have not been examined in detail it seems likely that such treatment leads to opening of the lactone ring with the possibility of decarboxylation at elevated temperatures. At a temperature of about 100°C, ring opening followed by decarboxylation occurs, and reaction may proceed further, leading to the loss of the methoxy group from the aromatic ring.

In alkali solution reversible hydrolysis of the lactone moiety occurs. Recyclization has been observed after acidification of a basic aflatoxin containing solution.

In the presence of acids, aflatoxin B_1 and G_1 are converted in to aflatoxin B_{2A} and G_{2A} due to acid-catalyzed addition of water to the double bond in the furan ring. In the presence of acetic anhydride and hydrochloric acid the reation proceeds further to give the acetoxy derivative. Similar adducts of aflatoxin B_1 and G_1 are formed with formic acid-thionyl chloride, acetic acid-thionyl chloride and trifluoroacetic acid.

Many oxidizing agents, e.g. sodium hypochlorite, potassium permanganate, chlorine, hydrogen peroxide, ozone and sodium perborate react with aflatoxin and change the aflatoxin molecule in some way as indicated by the loss of fluorescence. The mechanisms of these reactions are uncertain and the reaction products remain unidentified in most cases. Reduction of aflatoxin B_1 and B_2 with sodium borohydride yielded aflatoxin RB_1 and RB_2, respectively. These arise as a result of opening of the lactone ring followed by reduction of the acid group and reduction of the keto group in the cyclopentene ring. Hydrogenation of aflatoxin B_1 and G_1 yields aflatoxin B_2 and G_2 respectively. Further reduction of aflatoxin B_1 using 3 moles of hydrogen yields tetrahydroxyaflatoxin.

Food and feed contaminated with mycotoxins pose a severe health risk to animals and they may cause big economical losses due to the lower efficacy of animal husbandry and crop performances.

In addition, directly or indirectly (carry on through animal products) contaminated foods may also pose a health risk to humans. For this reason it is understandable that many research has been addressed in an attempt to salvage mycotoxin contaminated commodities and to avert health risks associated with the toxins.

Relevant basic criteria to be followed when a decontamination strategy is assessed have been suggested (Scott, 1990; Pomeranz, Bechtel, Sauer, Seitz, 1990):

- the mycotoxin must be inactivated (destroyed) by transformation to non-toxic compounds;
- fungal spores and mycelia should be destroyed, so that new toxins are not produced;
- the food or feed material should retain its nutritive value and remain palatable for consumers;
- the physical properties of raw material should not change significantly;

- it must be economically feasible (the cost of decontamination should be less than the value of contaminated commodity).

The main three possibilities to avoid any possible harmful effects of contamination of food and feed caused by mycotoxins habe been described by Halàsz et al. (Halasz, Lasztity, Abonyi, Bata, 2009):

- prevention of contamination;
- decontamination of mycotoxin-containing food and feed;
- inhibition of absorption of mycotoxin in consumed food in the digestive tract.

Although the different methods used at present are to some extent successful, they have big disadvantages with limited efficacy and possible losses of important nutrients and normally with high costs. It is a common opinion that the best solution for decontamination should be detoxification by biodegradation, giving a possibility for removal of mycotoxins under mild conditions without using harmful chemicals without significant losses in nutritive value and palatability of decontaminated food and feed. One of the most frequently used strategies for biodegradation of mycotoxins includes isolation of microorganisms able to degrade the given mycotoxin and treatment of food or feed in an appropriate fermentation process.

Thousand of microorganisms habe been screened for their ability to degrade aflatoxins from solutions (Lillehoj, Ciegler, Hall, 1967; Ciegler, Lillehoj, Peterson, Hall, 1996). As a result it was found that only one bacterium, the *Flavobacterium aurantiacum* B-184, was able to eliminate aflatoxin from solutions and uptake of the mycotoxin by the cells was influenced by pH and temperature.

Another interesting result was that an high concentration populations of the cells, more than 10^{11} per mL, is more useful to remove the aflatoxin from solutions than lower cell concentrations. Large populations of heat inactivated cells were also shown to bind some aflatoxin from solution, which was easily recovered by washing with water (Line, Brackett, 1967). The ability of *Flavobacterium aurantiacum* B-184 to remove aflatoxins from foods was demonstrated in milk, vegetable oil, corn, peanut, peanut butter and peanut milk (Hao, Brackett, 1988; Hao, Brackett, 1989; Line, Brackett, 1995). To assess the exact fate of the aflatoxin B_1 treated with *Flavobacterium aurantiacum*, Line et al. used radio-labeled carbon (C_{14}) aflatoxin B_1 and detected the formed radioactive carbon dioxide confirming this way the biodegradation pathway of aflatoxin (Line, Brackett, Wilkinson, 1994).

It should be noted that the interest of the biological approach to degrade aflatoxin is increasing since the consumers prefer this tool to chemical treatments used on food and feed to eliminate aflatoxins.

Nevertheless, one of the big obstacle to the developing of biological approaches is the bright pigmentation associated with the bacterium treatment, that hampers the applicability for food and feed. Microorganisms that are able to degrade aflatoxin B_1 include *Corynebacterium rubrum*, *Aspergillus niger*, *Trichoderma viride*, *Mucor ambiguus*, *Dactylium dendroides*, *Mucor griseocyanus*, *Absidia repens*, *Helminthosporium sativum*, *Mucor alternans*, *Rhizopus archisus*, *Rhizo-*

pus oryzae, Rhizopus solonifer and a protozoan *Tetrahymena pyriformis* (Doyle, Applebaum, Brackett, Marth, 1982; Karlovsky, 1999).

Recently, a growing interest can be observed concerning the use of *Rhodococci* for aflatoxins degradation: these microorganisms have a wide-range ability to degrade compounds like aflatoxins (Alberts, Engelbrecht, Steyn, Holzapfel, van Zyl, 2006; Teniola, Addo, Brost, Farber, Jany, Alberts, Van Zyl, Steyn, Holzapfel, 2005). Teniola et al. (Teniola, Addo, Brost, Farber, Jany, Alberts, Van Zyl, Steyn, Holzapfel, 2005) reported the degradation of aflatoxin B_1 using liquid cultures of *Rhodococcus erythropolis* and the analysis of the intracellular extracts separated from *Rhodococcus erythropolis* liquid cultures suggested that a cascade of enzymatic reactions with loss of fluorescence (the intact aflatoxin is a fluorescent compound and degradation results in loss of fluorescence in time) occurred. Aflatoxin B_1 is probably degraded by the same enzymes (biphenyl-dioxygenases, dihydro-diol-dehydrogenases, and hydrolases) that are involved in catabolic pathways of polychlorinated biphenyls. Knowledge of gene coding for these enzymes may be helpful in development and production of new effective enzyme preparations for degradation of aflatoxins. The role of trace metal ions in microbial aflatoxin B_1 degradation has been studied studied by Souza et al. (Souza, Brackett, 1998) who found that copper and zinc ions may inhibit the degradation of aflatoxin B_1 by *Flavobacterium aurantiacum*. This effect is probably connected with an influence on the enzyme system involved in the degradation process. Peltonen et al. (Peltonen, El-Nezami, Salminen, Ahokas, 2000) and El Nezami et al. (El-Nezami, Kankaanpaa, Salminen, Ahokas, 1998) studied the ability of dairy strains of lactic acid bacteria to bind Aflatoxin B_1. It has been observed that *Lactobacillus rhamnosus* can significantly remove Aflatoxin B_1 compared with other strains. Removal was observed as very rapid, with 80% of toxin removed within the first 60 min of treatment.

Several bacterial species, such as *Bacillus*, *Lactobacilli*, *Pseudomonas*, *Ralstonia* and *Burkholderia* spp., have shown ability to inhibit fungal growth and production of aflatoxins by *Aspergillus* spp. in laboratory tests. Palumbo et al. (Palumbo, Baker, Mahoney, 2006) reported that a number of *Bacillus*, *Pseudomonas*, *Ralstonia* and *Burkholderia* strains could completely inhibit *A. flavus* growth. *B.subtilis* and *P. solanacearum* strains isolated from maize soil were also able to inhibit aflatoxin accumulation. In most cases, although these strains were highly effective against fungal growth and against the produced toxins in laboratory conditions, they did not give good efficacies on field. This could be attributed to the difficulty to bring the bacterial cells to the *Aspergillus* infection sites on commodities under field conditions. Saprophytic yeast species, such as *Candida krusei* and *Pichia anomala*, have revealed promising efficacy as biocontrol agents for aflatoxins decontamination (Yin, Yan, Jiang, Ma, 2008). In a similar way to bacterial agents, these yeast strains were able to significantly inhibit *Aspergillus* growth and resultant toxins in laboratory conditions. Shetty et al. (Shetty, Hald, Jespersen, 2007) observed that the ability of *S. cerevisiae* to bind aflatoxin B_1 was strain specific with 7 strains binding 10-20%, 8 strains binding 20-40% and 3 strains binding more than 40% of the added aflatoxin B_1. Though the yeasts are considered to be potential biocontrol agents for the aflatoxins management, further experiments conducted on field are necessary to test their efficacies in reducing aflatoxin contamination in real on field situations.

Many reports exists on the use of physically separated yeast cell walls obtained from brewery as feed additive in poultry diet resulting in amelioration of aflatoxins toxic effects (Shetty, Jespersen, 2003; (Santin, Paulillo, Maiorka, Okada Nakaghi, Macari, Fischer da Silva, Alessi, 2003). When dried, yeast and yeast cell walls have been added to rat-ration along with aflatoxin B_1, and a significant reduction in the toxicity has been observed (Baptista et al., 2004). In an *in vitro* study with the cell wall material, there was a dose dependent binding of as much as 77% (w/w) and modified mannan-oligosaccharides derived from the *S. cerevisiae* cell resulted in as much as 95% (w/w) binding (Girish and Devegowda, 2006).

14. Conclusions

Total quality of food is the main goal to reach and a mission both for food industry and for the world Government Institutions. Quality means also safety, and this aspect is the most relevant goal to pursue and achieve. Consumers have often prejudicials ideas about risks associated to food and feed; nowadays based on the information available, they tend to have a quite large knowledge about genetically modified microorganisms, phytopharmaceutical origin active principles, heavy metals contamination or unbalanced dietary habit. These are the main issues for the majority of the people. However, the perceived risk related to mycotoxin occurrence in food is very neglected and underestimated.

Aflatoxins are a serious problem for human health, and it is not possible to evaluate this threat without paying great attention to the exposure to these compounds. The frequency and level of mycotoxin presence in the food chain are grown up in the last decades, probably due to the changed global weather conditions, to the market globalisation, and to the worldwide deployment of mold. The development of new analytical methods, more sensitive and more specific to evaluate aflatoxins presence, ensures the managment of the risk and, consequently, could allow to guarantee the safety of food from aflatoxin contamination. It is not possible however to completely avoid aflatoxins contamination in the food chain since the colonisation by molds and their mycotoxins biosynthesis are not under the full humans control due to many different biological, genetic and biochemical reasons.

Consumers, together with safety food agencies and with the worldwide research, must maximize efforts to reduce the global aflatoxin exposure. Focus should not be given only to Aflatoxin B_1 for which the neologism ALARA where this acronym say "As Low As Reasonably Achievable" has been considered applicable.

It can be noted as final remark that, in general, consumers do not appreciate any chemical procedure, e.g. the use of alkali or acid solutions. The same results to control unwanted aflatoxins presence are achievable using ammonium or different physical or chemical approaches. Another aspect regards the develop of a biological protocols that use microrganism generally recognised as safe for food (GRAS), a procedure that is considered much more acceptable by the consumers. Nevertheless, aflatoxins fate should be determined considering also the toxicological bioactivities of aflatoxins byproducts, like their many metabolites. It is important to determine these aspects before to propose new microrganisms

able to detoxify aflatoxins without causeing any unwanted side effect, e.g. changes in sensorial and technological properties of foods. In addition, any new method should be economically convenient if compared with any actually used procedure, especially for food industry that may hamper for these economic reasons.

Safe food is a non-negotiable topic both for ethic reasons and for economic aspects. The social costs linked to an increase of health conditions like liver diseases, or the problems connected to crop destruction, withdrawal of food from the shelves, etc., can be more expensive than a preventive actions to reduce aflatoxin presence in the food chain.

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