

# Mycotoxins: Are They Perceived as a Serious Threat for the Human Health?

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Mycotoxins contamination and the associated risks are well known from the scientific community as it is possible to desume from the huge amount of the related scientific publications. Although the threat for human and animal health is really high and the worldwide diffusion of the contamination is for the most part uncontrollable, the public opinion is not really conscious of the problem and sometimes ignores it.

In the present paper, a brief description of different aspects related to mycotoxins contamination is reported as regard toxicology and risks for the health, recent European and global regulations, and laboratory methods for mycotoxins determination.

## 1. Introduction

The term mycotoxin was used for the first time in 1961 in the aftermath of a veterinary crisis in England, during which thousands of animals died. The disease was linked to a peanut meal, incorporated in the diet, contaminated with a toxin produced by the filamentous fungus *Aspergillus flavus* (Richard, 2007). In general, mycotoxins are low-molecular-weight compounds that are synthesized during secondary metabolism by filamentous fungi; their chemical structures vary from simple heterocyclic rings with molecular weights of up to 50 Da, to groups with 6 to 8 heterocyclic rings with a molecular weight of >500 Da. Mycotoxins are natural contaminants in raw materials, food and feed. Mould species that produce mycotoxins are extremely common, and they can grow on a wide range of substrates under a wide range of environmental conditions; they occur in agricultural products all around the world (Bosco and Mollea, 2012).

Most mycotoxins are relatively heat-stable within the conventional food processing temperature range (80–121°C), therefore so little or no destruction occurs under normal cooking conditions, such as boiling and frying, or even following pasteurization processes (Miličević et al., 2010).

It has been estimated that 25 % of the world's crops are affected by fungal growth, and commodities may be, both pre- and post-harvest, or during processing, storage and feeding, contaminated with mycotoxins. The mycotoxins that can be expected in food differ from country to country in relation to the different crops, agronomic practices and climatic conditions (Bryden, 2007). Currently, more than 400 mycotoxins are known. Scientific attention has mainly focused on those that have proven to be carcinogenic and/or toxic in humans and animals. Six classes of mycotoxins are considered the most significant in agriculture and in the food industry: aflatoxins (AFs), ochratoxins (OTs), fumonisins (FBs), zearalenone (ZEN), deoxynivalenol (DON) and other trichothecenes, and Patulin (Figure 1). They are the most widespread mycotoxins in animal feed and human food.

In general, mycotoxin exposure is more expected to occur in countries where poor methods of food handling and storage are common and few regulations exist to protect exposed populations. Nevertheless, even in developed countries, specific subgroups may be susceptible to mycotoxin exposure in relation to an higher consumption of particular contaminated products or in relation to high concentration of toxigenic moulds in buildings.

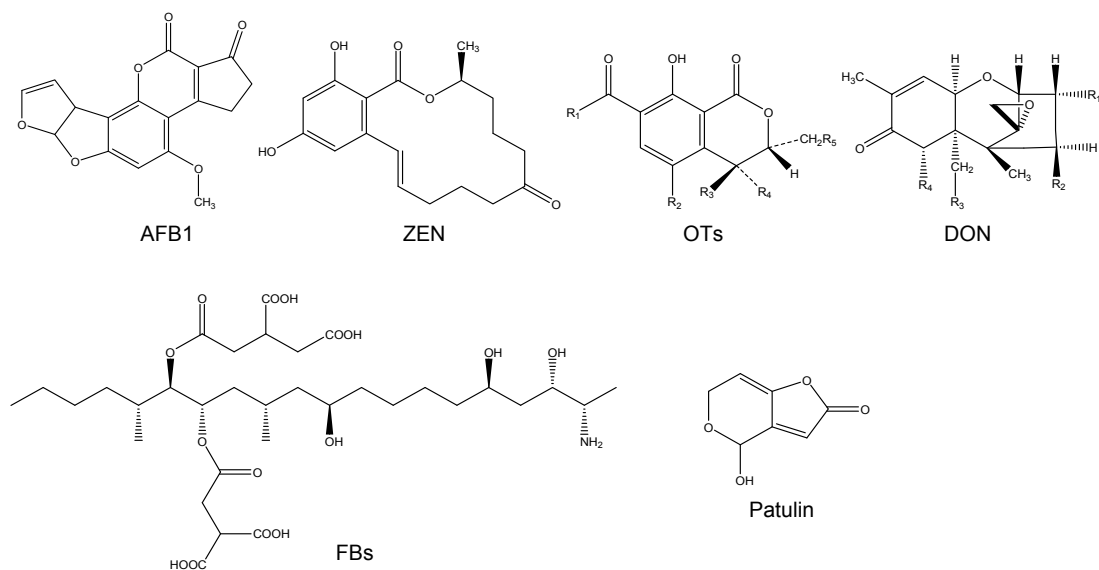


Figure 1: Chemical structures of some of the major mycotoxins.

At present, the perceived risk related to the presence of mycotoxins in food and feed is lower than the real one; insomuch as in the last EUROBAROMETER survey report, edited in 2010, on the concerns of consumers across Europe about the possible risks associated with food, mycotoxins were not clearly reported among the possible toxic substances associated to food consumption (European Food Safety Authority, EFSA, 2010).

## 2. Toxicology and health

Mycotoxins have adverse effects on human and animal health. Many of them may be toxic to vertebrates and other animal groups and, in low concentrations, some of them can cause autoimmune illnesses, exhibit hormonal activity, and have allergenic properties, while others are teratogenic, carcinogenic, and mutagenic (Bezerra da Rocha et al., 2014). Their main effects on DNA, RNA, protein synthesis and their pro-apoptotic action may cause changes in physiological functions including reproduction, growth and development. Development defects, including birth ones, are possible adverse effect following exposure to mycotoxins. In addition to these different actions, mycotoxins may affect the gastrointestinal system, cause skin irradiation, have hematological effects and reduce growth.

Exposure to mycotoxins may occur through ingestion, inhalation, and dermal contact, and it is almost always accidental. Most cases of mycotoxicoses (animals and humans) result from eating contaminated food. Human exposure can be direct via cereals or indirect via animal products (e.g. meat, milk and eggs) (Council for Agricultural Science and Technology, CAST, 2003). Mycotoxicoses in humans or animals have been characterized as food or feed related, non-contagious, non-transferable, and non-infectious. Depending on the species, mycotoxins have various acute and chronic effects; within a given species, the impact of mycotoxins on health is influenced by age, sex, weight, diet, exposure to infectious agents, and by the presence of other mycotoxins (synergistic effect) and/or pharmacologically active substances (Zain, 2011). Moreover, differences in response amongst different races of humans that bear on genetic basis and even amongst different individuals of the same race have been reported (Bezerra da Rocha et al., 2014). The majority of mycotoxins currently known are grouped, according to their toxic activity, under chronic conditions as mutagenic, carcinogenic or teratogenic. Grouping according to their site of action results in hemo-, hepato-, nephron-, dermato-, neuro- or immunotoxins (Niessen, 2007).

Nowadays, carcinogenic properties have been recognized with regard to AFs and FBs (Mazzoni et al., 2011). Due to their capability to bind cell DNA, aflatoxins affect protein synthesis and their immunosuppressive properties induce infections in contaminated people; many studies in poultry, pigs and rodents showed that exposure to aflatoxins results in suppression of various aspects of the cell-mediated immune response. Aflatoxins are lipophilic and able to cross the placental barrier. Aflatoxin B1 (AFB1) has been linked to human primary liver cancer, in which it acts synergistically with HBV infection and it has been classified as a carcinogen in humans (Group 1 carcinogen).

Fumonisin B1 (FB1), the most abundant of the numerous fumonisin analogues, was classified as a Group 2B carcinogen (possibly carcinogenic to humans) (Wild and Gong, 2010). The carcinogenic property of fumonisins does not seem to involve interaction with DNA (Coulombe, 1993). On the other hand, its similarity with sphingosine suggests the probable intervention in the sphingolipids biosynthesis (Shier, 1992). The inhibition of sphingolipid biosynthesis leads to serious problems related to cell activity, since these substances are essential for membrane composition. Animal studies have demonstrated that FB1 exposure can cause neural tube defects (NTDs) giving rise to further concerns that this mycotoxin could cause similar effects in humans (Sherif et al., 2009).

ZEN can adopt a conformation resembling 17-beta-estradiol that allows to bind the estrogen receptor in target cells. ZEN and its derivatives have estrogenic and anabolic activity: zearalanol can cause precocious puberty in children; F-2 toxin can cause oestrogenisation (Sherif et al., 2009).

OTA can have different effects on animals: nephrotoxic, hepatotoxic, neurotoxic, teratogenic and immunotoxic. This mycotoxin was associated with human nephropathy and it is suspected to be the cause of the human Balkan Endemic Nephropathy (BEN) and the Tunisian Nephropathy (TCIN). As regard DON (vomitoxin) the main cellular mechanism is the inhibition of protein synthesis. Human exposure to DON-contaminated grains has been reported to cause acute temporary vomiting, diarrhea, abdominal pain, headache, and fever. Patulin has an immunosuppressive effect and inhibits DNA synthesis. Exposure to Patulin is associated with immunological, neurological and gastrointestinal symptoms. Patulin-induced nephropathy and gastrointestinal tract malfunction have been demonstrated in animal models (Bosco and Mollea, 2012).

A mixture of mycotoxins may occur simultaneously, depending on the environmental conditions and substrate availability. Therefore it can be expected that humans and animals are exposed to a mixture rather than to individual compounds (Milićević et al., 2010).

### 3. Legislation

In several countries worldwide, mycotoxins, like the other food contaminants, are regulated to protect human health; regulations concern specific maximum limits for food, feed, and commodities, but also references to sampling methods and analysis to be used.

*Table 1: Comparison of limits for the major mycotoxins in EU, S.A., and USA (Source: European Mycotoxins Awareness Network, 2014).*

Mycotoxins	Country	Groups of foodstuffs	Limit (µg/kg)
AFB1, AFs	EU	Peanuts, nuts with exception, dried fruits, all cereals and derivatives	4
	S.A.	Peanuts	15
	USA	Brazil nuts, peanuts and derivatives, pistachio products	20
AFM1	EU	Raw or heat treated milk, milk destined to derivatives production	0.05
	S.A.	Milk	0.05
	USA	Milk	0.5
OTA	EU	Wine, grapes juices and related products	2
	S.A.	No specific limits for OTA in foodstuffs are set in S.A.	-----
	USA	No specific limits for OTA in foodstuffs are set in USA	-----
DON	EU	Bread, sweets, biscuits, cereals snacks and breakfast cereals	500
	S.A.	No specific limits for DON in foodstuffs are set in S.A.	-----
	USA	Finished wheat products	1,000
ZEN	EU	Bread, sweets, biscuits, cereals snacks and breakfast cereals with exceptions	50
	S.A.	S.A. do not establish specific maximum limits for ZEN	-----
	USA	USA do not establish specific maximum limits for ZEN	-----
FBs	EU	Bread, sweets, biscuits, cereals snacks and breakfast cereals	800
	S.A.	No specific limits for FBs in foodstuffs are set in S.A.	-----
	USA	Whole or partially degermed dry milled corn products	4,000
Patulin	EU	Apples and derivatives	25
	S.A.	Apple juice, apple juice ingredients in other juice	50
	USA	Apple juice, concentrates and products	50

In the Europe Community, apart from the overall Regulation (EC) No 178/2002, adopted by the European Parliament and the Council to define general principles and requirements of food law and obligation of food trade, the presence of mycotoxins is controlled by specific Regulations; these last are mainly focused on

establishing maximum levels in different kinds of raw materials, food or animal feed. First of all the Commission Regulation (EC) No 1881/2006 sets maximum levels for the main mycotoxins (AFs, OTA, Patulin, DON, ZEN, FBs, T-2 and HT-2 toxins) according to different foodstuffs and sets that the foodstuffs listed in the Annex cannot be placed on the market if they contain a contaminant at a level exceeding the maximum one set out. Later this Regulation has been amended as regard specific mycotoxins levels, namely: by the Commission Regulation (EC) No 1126/2007, for *Fusarium* toxins in corn and corn products, by the Commission Regulation (EC) No 105/2010, related to maximum levels of OTA in different foodstuffs, and by the Commission Regulations (EU) No 165/2010, concerning maximum levels of AFs in groundnut, other oilseeds, tree nuts, dried fruit, rice, corn and cereals. Evaluating the maximum levels settled in the EU for the different mycotoxins, in all the cases the lowest limits are indicated for infant formula. Apart from this specific category of food, the highest limitations (e.g. concentration values) are typical for each mycotoxin depending on the substrate (food, feed or raw material) where it can be found more frequently. As an example, the lowest limits for OTA (2.0 µg/kg) are referred to wine, grapes juices and related products, while for Patulin they concern apples and derivatives (25 µg/kg) (European Commission, 2014). Like the EU, also different agencies and organizations like the Food and Agriculture Organization (FAO), the World Health Organization (WHO) or country specific agencies have settled limits to regulate mycotoxins content in different substrates, but these limits are not easily comparable from country to country. In Table 1, for example, is reported a comparison for the major mycotoxins among limits imposed by EU, South Africa (S.A.), and USA; for the EU the groups of foodstuffs indicated are those with the highest restrictions. As a matter of fact, for the same group of foodstuffs limits can be quite different also in the order of magnitude, groups of foodstuffs can include different foods or fruits or can be more detailed or generals. Finally, in the USA and in South Africa, limits for some mycotoxins have not been settled yet. It has to be mentioned that, evaluating limits in different countries worldwide, those imposed by the EU Regulations are the lowest.

The differences in mycotoxins limits depend on the fact that, as reported by Marroquin-Cardona et al. (2014), mycotoxins to be regulated and limits to be imposed are decided by each country even though most of these limits are based on worldwide set standards. Therefore, taking into account the free trade of foods which cover almost all the world, a global regulation is indispensable primarily to protect consumers health but also to avoid refusal of huge food lots with negative outcomes on the global economy. A first attempt in this direction has been done thanks to the report "Comparison of Regulatory Management of Authorized Ingredients, Approval Processes, and Risk-Assessment Procedures for Feed Ingredients" edited in 2013, on behalf of the International Feed Industry Federation, with the objective: "... To address similarities and differences among 7 regulatory jurisdictions (Brazil, Canada, China, European Union, Japan, South Africa, and United States) on the regulatory management of authorized (existing) feed ingredients, the approval process, and risk management assessment for feed ingredients. The tool can assist in global marketing as well as supporting in the harmonization/convergence efforts in identifying areas of dissimilarity" (Smedley, 2013).

#### 4. Mycotoxins analysis

Mycotoxins contamination of agricultural products is unavoidable both in the developed world, where modern agricultural practices and regulated food production exist, and in developing countries, for the most part lacking of a legislative regulation for food and agriculture (Miličević et al., 2010). Therefore mycotoxin control at any production stage is worldwide mandatory; this can be achieved through proper agricultural practices and food processing and storage but also by developing fast and reliable analytical methods for mycotoxins identification and surveillance. The quantification of mycotoxins is of utmost importance because it can fill up the lack of information about exposure; indeed, currently, also for most of the best known mycotoxins, the tolerable daily intakes (TDI) established by the FAO/WHO Joint Expert Committee on Food Additives are still temporary, provisional or at provisional maximum levels (Alkadri et al., 2014). Analytical methods must allow to obtain comparable and traceable results; since the 1960s various techniques have been developed but only some of them have been accepted and validated by official authorities like the AOAC International (Association of Official Agricultural Chemists) and the ISO (International Organization for Standardization) on the basis of international guidelines such as the ISO standard 5725-5:1998. Mycotoxins analysis consists of various steps, some of which represent key points to obtain consistent analytical results: a sampling, a mycotoxins/multi-mycotoxins extraction, an extract clean-up, and a final determination (Köppen, 2010). The sampling step, in particular, is a critical one and can contribute to the largest variability in mycotoxins determination because food matrices are complex, mycotoxins are present at low concentrations (ppm, ppb or ppt) and are heterogeneously distributed in many agricultural commodities and concentrated in "hot-spots" (Krska et al., 2008). This heterogeneous distribution is clearly explained by the example reported by Blanc (2006): a single peanut kernel, bringing a high AFs contamination, is sufficient to cause, in a 10 kg lot, an AFs contamination which is outside the limits allowed by the European regulation. Sampling step must consent to

obtain a sample which is representative of the overall suspected matrix: sampling is regulated by the Commission Regulation (EC) 401/2006 and the following Commission Regulation (EC) 178/2010. In this regulations different sampling methods are described in detail for grains and derivatives, dried fruits, nuts, spices, milk and derivatives, coffee and derivatives, fruit juices, apple derivatives, and baby food (European Commission, 2014).

As regard mycotoxins extraction, analysis and detection, it is impossible to use only one technique because their variety is wide due to different chemical structures, concentrations, and contaminated substrates. These differences influence, first of all, the extraction which has to be carried out with a combination of different solvents depending on the analite/analites. The following clean-up step can also be realized with traditional solid-phase extraction (SPE) protocols or with mycotoxins molecular imprinted polymers (MIPs), but currently there is a strong trend towards the use of immunoaffinity columns (IACs). IACs technique consents to produce clean extracts, reducing the interference of matrix components, but it is limited by the availability of immune-antibodies and by the fact that some mycotoxins are underestimated (Krska et al., 2008). On the other hand SPE cartridges are less expensive and selective than IACs, but they are difficult to be used for the analysis of multiple mycotoxins. To overcome these limitations, the so called QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) methodology has been widely used in the last years. It is simple and allows an effective cleaning-up of complex samples; it involves an extraction step and a dispersive SPE step that includes further clean-up using combinations of MgSO<sub>4</sub> and different sorbents, to remove interfering substances (Arroyo-Manzanares et al., 2014).

*Table 2: AOAC approved methods for mycotoxins analysis (Source: AOAC International, 2014).*

Analites (Method number)	Analytical technique	Analytical range	Matrices
AFB1, AFB2, AFG1 (990.34)	ELISA	>= 30 ng/g	Corn and raw peanuts
AFs (994.08)	HPLC	5-30 ng/g	Almonds, Peanuts, Pistachio nuts
FB1,FB2, FB3 (995.15)	HPLC	>= 1 µg/g	Vegetables/Corn
FBs (2001.06)	Competitive direct ELISA	>1.0 mg/kg	Vegetables/Corn
DON (986.17)	TLC	>= 300 ng/g	Grains/Wheat
DON (986.18)	GC	>= 350 ng/g	Grains/Wheat
OTA (2000.03)	IAC-HPLC	> 1 ng/g	Grains/Barley
OTA (2000.09)	IAC-HPLC	> 1.2 ng/g	Coffee/Roasted coffee
ZEN (994.01)	ELISA	>= 800 ng/g	Grains/Wheat, Animal feed

As regard analytical methods for mycotoxins determination they can be divided in two main classes, namely classical ones and immunochemical or rapid techniques. Classical methods can in turn be based mainly on thin-layer chromatography (TLC), gas chromatography (GC) or high-performance liquid chromatography (HPLC) coupled to FLD, DAD or MS (Köppen et al., 2010). In the last decade, in particular, HPLC tandem mass spectrometry (HPLC-MS/MS) have allowed to reach the best advance in mycotoxins analysis; thanks to this technique the simultaneous determination of different mycotoxins, also less known ones coupled with other metabolites, in different food matrices, and detection of low mycotoxins concentrations in biological fluids and tissues are possible. Apart from highly reliable methods, also rapid tests for mycotoxins determination, characterized by fast sample preparation and analysis, exist; they are employed to determine approximate levels of contamination, also for many samples even in a non-laboratory environment. For this purpose different immunochemical tests exist: the widely used enzyme-linked immunosorbent assay (ELISA), the immunochemical method based on fluorescence polarization, which allows to obtain results within a few minutes, the surface plasmon resonance biosensor for which portable equipment may be used, the really fast non-instrumental antibody based applications (lateral flow tests, dipstick or flow-through tests), and the semi-quantitative rapid tests used to determine if the sample is contaminated or not (Köppen et al., 2010). Clearly, these fast techniques can be an useful tool for the food or agricultural industry to obtain rapid results as regard, for example, the received raw materials, but later they have to be confirmed by chromatographic methods for an accurate determination of the analite concentration. Some example of the AOAC approved methods, chromatographic and rapid ones, for the analysis of different mycotoxins are reported in Table 2 together with the analytical range and the pertaining matrices.

## 5. Conclusions

As it is possible to desume from the present paper, mycotoxins contamination of food and feed represents a serious risk for human and animal health, which is well known among the scientific community but almost

unknown among the consumers one. This lack of information regards not only people in developing countries but also the well-informed consumers of the developed world. For this reason, it is necessary to disseminate the knowledge about the probable presence of mycotoxins in food and the consequent risk for human health. To this purpose, Frutic Symposium and EXPO 2015 certainly represent a precious opportunity.

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