

# PRINCIPLES OF RISK ASSESSMENT OF MYCOTOXINS IN FOOD AND FEED BY EUROPEAN FOOD SAFETY AUTHORITY (EFSA)

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## MYCOTOXINS: A REVIEW OF REGULATORY ASPECTS

There are several international organizations with scientific boards dealing with the problem of food and feed contaminants. Apart the International Agency for Research on Cancer (IARC, Lyon, France) which is focused on carcinogenicity of natural and manmade compounds, there is also a more recently founded (1980) International Programme on Chemical Safety (IPCS), a joint venture of the United Nations Environmental Programme (UNEP), the International Labour Organization (ILO) and WHO. IPCS organizes scientific meetings of experts called Joint FAO/WHO Expert Committees on Food Additives (JECFA) which is particularly concerned about the safety of food additives, residues of veterinary drugs, naturally occurring toxicants and contaminants of food. JECFA serves as scientific advisory body to FAO, WHO, their Member States and the Codex Alimentarius Commission.

In order to protect European consumers from the exposure of all kinds of possible lesions and diseases of food origin the European Commission (EC) founded the independent scientific advisory board called European Food Safety Authority (EFSA) [1]. In 2003, EFSA organized Panel on Contaminants in the Food Chain (CONTAM Panel) with the mandate to deliver scientific opinions on “contaminants in food and feed, associated areas and undesirable substances such as natural toxicants, mycotoxins and residues of non-authorized substances not covered by another Panel”. CONTAM Panel receives the requests from the EC (95 %), Member States (1 %) and the European Parliament (1 %). Since 2003 CONTAM Panel produced 107 opinions (55 on contaminants in food, 43 in feed and 9 combined assessments in food and feed), 15 out of them on mycotoxins. Evaluation of risk assessment of mycotoxins is specific because of their natural origin and it relies on public scientific information.

Mycotoxins are secondary metabolites produced by many species of filamentous fungi. Around 300 different mycotoxins have been described that are produced by about 200 different fungal species. However, there are only 20 mycotoxins that are regularly found in food and feedstuffs at concentrations likely to pose a health hazard for animals and people consuming these materials—so-called “primary exposure”. The commonly known and health relevant mycotoxins can be categorized into *Aspergillus* mycotoxins (e.g. aflatoxins, ochratoxins), *Fusarium* mycotoxins (e.g. fumonisins, trichothecenes, zearalenone, nivalenol, deoxynivalenol, T-2 toxin, enniatins and beauvericin) and *Penicillium* mycotoxins (e.g. ochratoxin A, citrinin). These mycotoxins often co-occurred naturally in cereals since one kind of crop can be infected by different toxigenic molds and also each mold can produce several kinds of mycotoxins simultaneously. The occurrence of these compounds depends on factors like strain of fungus, species, plant species, and environmental and ecological conditions such as humidity, temperature and presence of pests. This toxicity can range from the production of several hormonal disorders or immunosuppression to the induction of carcinogenic, teratogenic or mutagenic activities. Therefore, the actions of these co-occurring mycotoxins on human or animal can be antagonistic, additive or synergistic.

Risk assessment is the procedure of evaluation whether or not a hazardous chemical contaminant or undesirable substance in food is likely to be associated with adverse health effects in the population. Risk assessment is based on two components: the potential of substance to cause adverse effects (hazard) and knowledge about the human exposure to the substance.

In the procedure of hazard identification in case of mycotoxins all available data regarding the evaluated compound in the scientific literature are taken into account. These data include reports on the human and animal intoxication, studies on experimental animals as well as on the cell cultures. For the evaluation of human exposure CONTAM Panel relies on data on occurrence of contaminants submitted by Member States upon the calls for data.

### **Hazard identification**

In hazard identification procedure of mycotoxins all data on toxicity of the compound published in the available scientific literature are collected. The task of CONTAM Panel is to scrutinize thoroughly the quality of the presented results. Data on toxicity of mycotoxins in humans are rather rare and very often unreliable. Namely, in general, in reports on human lesions suspected to be caused by exposure to mycotoxins usually there is neither data on mycotoxin concentration in biological material (blood, urine, feces) of intoxicated persons nor the firm proves of human exposure to particular mycotoxin. The only exception is in the case of aflatoxin B1 (AFB1) with a number of excellent studies. More reliable are reports on toxicity deriving from experimental animals.

Hazard identification requires data on toxicokinetics, toxicity after acute and chronic exposure, mutagenicity, genotoxicity and carcinogenicity studies in with life-span exposure of experimental animals (rats and mice). Research on mycotoxins intensified in 1960-ies after the well known intoxication of turkeys with aflatoxins. When the carcinogenic features of AFB1 in experimental animals became evident, the researchers focused their studies on carcinogenic properties of other mycotoxins. This resulted with a large number of studies on mycotoxins other than AFB1 with continuous exposure of laboratory animals to high mycotoxin concentrations. Very often toxicokinetics and chronic toxicity studies are missing as well as toxicity testing. The lack of toxicity studies with low doses of mycotoxins results with difficulties in establishing HBGV which should be established based preferably on dose-response relationship.

### **Occurrence data**

Manmade products, (i.g. pesticides), require a large amount of research for marketing authorization procedure. Since mycotoxins are natural products nobody is responsible for their production occurrence data are collected from scientific literature (scientific journals), official national reports in Member States and risk assessment of other international organizations (JECFA, IARC, WHO). Due to the high occurrence of these compounds in food and feed and their implication in pathologies, mycotoxins are a global concern and they are included in monitoring food program to minimize the levels in these products. The occurrence data collected from Member States are usually part of the official food and feed control and are not intended for the purpose of risk assessment. Such data very often contain a large number of samples with contamination lower than limit of detection (LOD) or the limit of quantification (LOQ) (called also left censored data). The other problem is that LOD and LOQ are adjusted to the legal limits of the Member State and therefore there is no precise measurement at contamination levels below these limits. For the purpose of risk assessment CONTAM Panel launches calls for data on occurrence of contaminants in food and feed inviting all Member States to present data which should be given in special format requested from EFSA.

## Consumption data

In the evaluation of human exposure to contaminants, it is necessary to combine occurrence levels with food consumption data. Food consumption is certainly not equal in all European countries because of different nutritional habits in Member States. In order to obtain an European average of the consumption of various food, data from 30 national dietary surveys from 22 countries are included to calculate mean values. In these surveys data on food consumption of children was also recorded and they are used in the risk assessment for children.

Similar studies on feed consumption do not exist in Europe because of large variety of feed formula not only for animal species but also for age of animals. In the process of the risk assessment animal exposure is based on occurrence data collected from literature and submitted occurrence data.

With respect to “secondary exposure” (i.e. exposure of consumers to mycotoxins and their metabolites in primary animal products—meat, milk and eggs), the principal metabolite of aflatoxin B<sub>1</sub>, aflatoxin M<sub>1</sub>, is secreted in milk following consumption of aflatoxin B<sub>1</sub> by lactating cows. However, provided that the EU limits for aflatoxin B<sub>1</sub> (and other mycotoxins) are observed there should be no problems with harmful residues in edible tissues or milk. With regard to ochratoxin A, it has been estimated that pork and poultry products contribute to around 5% of the total human exposure. The little published data on the carry over from feed to animal products of some of the other toxins (fumonisins, zearalenone and deoxynivalenol) do not suggest that residues of these substances or their metabolites pose a threat to the consumer.

Traditional evaluation of human and animal exposure to mycotoxins is based on direct analysis of food and feed or more generally based on occurrence data combined with consumption data [2,3]. However, this approach has some unavoidable shortcomings. Firstly, there are some other routes for mycotoxins exposure such as dermal contact and inhalation. Secondly, during disease outbreak or toxicosis implicating mycotoxins, the feed or food is already destroyed before it can be analyzed [4]. Last but not the least, the traditional evaluation method is only suitable to assess the exposure of populations to some toxins or to identify the risk group; it cannot reflect accurate information of individual intake of mycotoxins. To circumvent all these shortcomings, biomarkers have been proposed as suitable targets to assess mycotoxin exposure. Different from the analysis of food and feed, the measurement of biomarkers of exposure can account for variations in food contamination levels. All the factors, such as food consumption, exposure routes, diet composition and food preparation techniques, metabolism and excretion of the toxin can be integrated into the formation of one indicator (biomarker), which will, out of question, greatly compromise all sources not being taken into account and simplify the analytical procedure [5,6].

Human exposure to aflatoxins (AFs) is a concern worldwide because AFs are potent cancer-promoting agents, especially liver cancer (International Agency for Research on Cancer (IARC), 1993) [7]. Animal studies have shown that under normal conditions, 50% of the orally administered dose of AFB<sub>1</sub> is quickly absorbed from the duodenal region of the small intestine and enters the liver through the hepatic portal blood supply, where it is metabolized in several derivatives [8]. The metabolites of AFB<sub>1</sub> detected in human urine include aflatoxin P<sub>1</sub> (AFP<sub>1</sub>), aflatoxin Q<sub>1</sub> (AFQ<sub>1</sub>), aflatoxin M<sub>1</sub> (AFM<sub>1</sub>) and DNA-adduct (AFB<sub>1</sub>-N<sub>7</sub>Guanine). The excretion rate of the different aflatoxin metabolites in human urine is not clearly defined. However, in vitro studies using primate and human liver microsomes have demonstrated that AFQ<sub>1</sub> is a major AFB<sub>1</sub> metabolite, with AFM<sub>1</sub>, the hydroxylated metabolite constituting less than 10% of the total metabolized AFB<sub>1</sub> [9,10]. Accordingly, in a study carried out in China, the levels of urinary AFQ<sub>1</sub> were 60 fold higher than

those of AFM1. The levels of AFQ1 strongly correlated ( $r = 0.673$ ) with detected levels of AFB1-N7Guanine adduct (AFB1-N7Gua), a strong biomarker for cancer effect. Consequently, AFQ1 was suggested as a predictive marker for AFB1 exposure [11]. However, the use of this metabolite is strongly compromised due to the lack of commercial standards.

Biomarkers of exposure to OTA have been thoroughly investigated and documented in many scientific publications. Gilbert et al. [12], found a positive correlation between the urinary concentration of OTA and the consumption of OTA, while Munoz et al. [13] reported the presence of OTA and its major metabolite ochratoxin alpha (OT $\alpha$ ) in 100% of the samples analyzed. OT $\alpha$  which is produced from the hydrolysis of OTA by the gut microflora in the intestine was the major metabolite detected in the urine of rats [14]. The hydroxylated form of OTA (4-OH OTA) was detected in urine of 96% of children under 5 years old in Sierra Leone at concentrations of 0.04–21 ng mL<sup>-1</sup> [15]. Data on the formation of genotoxic products of OTA still remain inconclusive as in vivo experiments conducted with male Fischer-344 rats treated orally with high levels (1–2 mg kg<sup>-1</sup> bodyweight) of 3<sup>[H]</sup>OTA did not reveal significant detectable levels of OTA-DNA adduct [16,17].

Zearalenone (ZEN), a mycoestrogen which is a frequent contaminant of cereals and especially breakfast cereals, was suspected to be a triggering factor for central precocious puberty observed in adolescent females in the United States [18]. Review on the toxicity, occurrence, metabolism and detoxification of ZEN suggested two major biotransformation pathways in animals: (1) hydroxylation resulting in the formation of alpha zearalenol ( $\alpha$ -ZOL) and beta zearalenol ( $\beta$ -ZOL) assumed to be catalyzed by 3 $\alpha$  and 3 $\beta$  hydroxyl steroid dehydrogenase respectively and (2) conjugation of ZEN and its reduced metabolites with glucuronic acid [19]. Till date, no paper has reported the presence of  $\alpha$ -ZOL-glucuronide and  $\beta$ -ZOLglucuronide in human urine samples.

Deoxynivalenol (DON) and its detoxification metabolite DON-3-glucuronide (DON-3Glu) has often been reported in the urine of exposed humans. In a study carried out in the United Kingdom by Turner et al. [20] a strong correlation was found between these urinary metabolites (the sum of the free DON and DON-3Glu) and cereal intake of the study population. Still in a related study performed by the same group of authors, the mean transfer of DON to urine was estimated to be 72% [21]. For these reasons, detecting and or quantifying the sum of these metabolites (DON and DON-3-glucuronide) in human urine has been recommended for biomonitoring of this toxin.

The increase in the sphinganine (Sa) to sphingosine (So) (Sa/So) ratio in urine and serum was proposed as a functional biomarker to evaluate exposure to FB1 in exposed animals [22]. When this biomarker was investigated in serum and urine in several human studies, it resulted in inconclusive outcomes [23-26]. FB1 does not appear to undergo any major metabolism as incubation with primary rat hepatocyte cultures and subcellular enzyme fractions failed to produce detectable metabolites [27]. Furthermore, FB1 was recovered unaltered in the urine, feces and bile of dosed animals. For these reasons, recent studies have recommended the use of the parent analyte (FB1) as an alternative biomarker [28]. Hydrolysis of the two tricarballic acid ester groups of FB1 has been reported to occur in the gut of vervet monkeys [29,30] and could possibly serve as an alternative biomarker. However, hydrolysis of FB1 has not yet been reported with human cell culture studies.

Several acute and chronic toxic effects were observed in humans after consumption of food contaminated with T-2 toxin. T-2 toxin is rapidly metabolized by esterases, resulting in several metabolites being detected in vivo and in vitro after ingestion. The spectrum and the ratios of T-2 metabolites in animals strongly depend on the investigated species [31]. The main biotransformation pathway is deacetylation of the C-4 acetyl group which leads to HT-2 toxin. In cell culture studies

with human fibroblast cells and isolated microsomes from liver, kidney and spleen of various animals, HT-2 toxin was detected as the sole metabolite of T-2 toxin [32-35]. Other metabolites detected after incubation of T2 toxin with the Chinese hamster ovary cells and the African green monkey kidney cells included traces of T-2 triol and T-2 tetraol [33].

Citrinin (CIT), a nephrotoxin, has been implicated in several disease outbreaks in animals and humans (IARC, 1986) [36]. Literature on the toxicokinetics and metabolism of CIT in humans is very scarce. However, Dunn et al. [37] isolated and successfully identified dihydrocitrone as the main urinary metabolite of CIT in rats. Low levels (2–5 ng mL<sup>-1</sup>) of the un-metabolized toxin were also detected in the urine of humans [38], indicating a possible but low level of excretion. However, the analytical challenges associated with the detection of this analyte in biological and food matrices usually make this analyte escape routine surveillance.

Therefore, biomarkers allow for more accurate and objective assessment of exposure at the individual level. So far, advances in analytical techniques in the fields of molecular biology and biochemistry have allowed the development and usage of various biomarkers in human and animal tissue or body fluids, and thus can provide definitive identification of a specific mycotoxicosis [39–41]. Generally, the candidate biomarkers include the excreted toxin or its metabolites, as well as the products of interaction between the toxin and macromolecules such as protein or nucleic acid. However, the choices of biomarkers are subjected to the commercial availability of these compounds, the convenience of its use, and the scientific question to be solved.

### **EFSA principles of risk assessment**

The final aim of risk assessment of contaminants is to establish, based on toxicological and occurrence data, health-based guidance value (HBGV) such as tolerable daily intake. For establishing HBGV a reference point (RP) should be identified from experimental data. EFSA Scientific Committee recommended the use of a benchmark dose lower confidence limit (BMDL) as RP based, if possible, on mathematical modeling of dose-response relationship. Officially BMDL is an estimate of the lowest dose that is 95 % certain to cause no more than a specified change in response over background. In other words this is the exposure level without appreciable health risk in animals if they are exposed to certain compound a whole life. If mathematical modeling of BMDL as RP is not possible due to lack of reliable studies of dose-response relationship, it is possible to use as a RP no-observed-adverse-effect level (NOEL). NOEL is the highest dose not causing a statistically significant adverse effect compared to controls. There are only rare cases when data on toxicity in humans are available, and therefore the establishing of HBGV is based on long-term studies on experimental animals. For this purpose carcinogenicity studies (which are usually combined with chronic toxicity studies) or multigeneration studies are taken into account. In case of genotoxic and carcinogen compounds it is not possible to establish threshold of effect. In such cases EFSA suggests the margin of exposure approach (MOE) which takes into account the fact that carcinogens differ in their potency at a given dose over time. MOE approach uses as RP dose-response-relationship from animal data corresponding to a dose that causes low, but measurable cancer incidence in animals. When RP is established, it should be divided by uncertainty factors to account for extrapolation from animals to humans (usually factor 10) and for particularly sensitive individuals in human population (another factor 10). CONTAM Panel may increase the first uncertainty factor (for extrapolation from animals to humans) when the RP was derived from the study with some faults (i.g. too short observation period of experimental animals in chronic studies).

The other specific EFSA approach is establishing threshold of toxicological concern approach (TTC) which is used for risk assessment of compounds with very low levels in the diet, insufficient toxicological data, but known chemical structure. In this approach the information on human exposure, which should be reliable and not underestimated, are combined with generic human exposure threshold values (TTC values) [42] established for substances grouped according to their chemical structure and likelihood of toxicity. Human exposure threshold values are based on data from extensive testing in animals and when the exposure is lower than threshold, the probability of adverse effects is considered to be very low.

Risk assessment in animals associated with exposure to any compound in feed is complicated with species-specific and inter-species differences in animals. Namely, toxicokinetics are usually different in different species and good example is the reduced absorption of mycotoxins due to forestomach of ruminants. For most of mycotoxins there data on biotransformation are also lacking which could be particularly important for their carry-over.

Risk assessment for animals is based usually on unsatisfactory data which are intrinsic errors. Particular problem are differences in geographic origin, plant stress and climatic conditions that influence feed contamination with mycotoxins. The composition of feed shows considerable variation for the same species in different parts of Europe. CONTAM Panel developed exposure assessment approach which takes into account common standards in animal nutrition. This means that the standard consumption pattern was established for animal species and age of animal. However, animal health risk assessment contains still considerable uncertainties.

## CONCLUSIONS

Mycotoxins pose a significant risk to the health and wellbeing of humans and animals and are a significant food safety issue. Although a lot of efforts to prevent mycotoxin formation have been undertaken, contaminations of those secondary fungal metabolites still occur. Therefore mycotoxin reduction strategies have been developed, including agronomic practices, plant breeding and transgenics, biotechnology, toxin binding and deactivating feed additives, and education of food/feed suppliers and animal producers to reduce mycotoxin contamination and exposure. Nevertheless, it has proven difficult to control exposure of man and animals to these natural environmental compounds. This is a significant issue for both food and feed security globally and we will have to live with some degree of risk. The situation is further complicated when it is appreciated that there are many thousand secondary fungal metabolites, the vast majority of which have not been tested for toxicity or associated with disease outbreaks or reduced animal productivity.

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