tehnologija mesa
Osnivač i izdavač: Institut za higijenu i tehnologiju mesa, Beograd

UDK: 637.05/.07 : 579.67/.87
BIBLID: 0494-9846

MYCOTOXINS IN THE FOOD CHAIN – OLD PROBLEMS AND NEW SOLUTIONS*

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A b s t r a c t: Mycotoxins are toxic compounds, produced by the secondary metabolism of toxigenic molds in the Aspergillus, Penicillium, Fusarium, Alternaria and Claviceps genera occurring in food and feed commodities both pre- and post-harvest. Adverse human health effects from the consumption of mycotoxins have occurred for many centuries. When ingested, mycotoxins may cause a mycotoxicosis which can result in an acute or chronic disease episode. Chronic conditions have a much greater impact, numerically, on human health in general, and induce diverse and powerful toxic effects in test systems: some are carcinogenic, mutagenic, teratogenic, estrogenic, hemorrhagic, immunotoxic, nephrotoxic, hepatotoxic, dermotoxic and neurotoxic.

Although mycotoxin contamination of agricultural products still occurs in the developed world, the application of modern agricultural practices and the presence of a legislatively regulated food processing and marketing system have greatly reduced mycotoxin exposure in these populations. However, in the developing countries, where climatic and crop storage conditions are frequently conducive to fungal growth and mycotoxin production, much of the population relies on subsistence farming or on unregulated local markets. Therefore both producers and governmental control authorities are directing their efforts toward the implementation of a correct and reliable evaluation of the real status of contamination of a lot or food commodity and, consequently, of the impact of mycotoxins on human and animal health.

***Key words**: mycotoxins, human and animal health, risk analysis*

Mikotoksini u lancu ishrane−stari problemi i nova rešenja

***S a d r ž a j:** Mikotoksini su toksična jedinjenja, proizvod sekundarnog metabolizma plesni iz roda Aspergillus, Penicillium, Fusarium, Alternaria i Claviceps, koja mogu da kontaminiraju hranu za ljude i životinje, kako u poljima tako i u skladištima. Štetni efekti upotrebe plesnive hrane zabeleženi su još od davnina. Alimentarnim unošenjem toksina plesni nastaju intoksikacije tzv. mikotoksikoze koje, s obzirom da su vezane za hranu, mogu da poprime akutne i hronične razmere. Hronični efekti nastali upotrebom hrane kontaminirane mikotoksinima imaju veoma veliki uticaj na zdravlje ljudi i rezultuju kancerogenim, mutagenim, teratogenim, estrogenim, hemoragičnim, imunotoksičnim, nefrotoksičnim, hepatotoksičnim, dermotoksičnim i neurotoksičnim efektima.*

Iako je problem kontaminacije hrane za ljude i životinje mikotoksinima još uvek prisutan u razvijenim zemljama, primenom novih dostignuća u poljoprivrednoj proizvodnji i odgovarajućom zakonskom regulativom, značajno je smanjena izloženost ljudi i životinja mikotoksinima. U zemljama u razvoju u kojima su klimatski faktori i uslovi skladištenja hrane, često povoljni za kolonizaciju plesni i sintezu mikotoksina veliki deo stanovništva orjentisan jena poljoprivrednu proizvodnju ili na snabdevanje iz neuslovnih objekata prodaje. Iz tog razloga proizvođači hrane i organi državne uprave su svoje aktivnosti usmerili ka implementaciji tačne i pouzdane procene stvarnog stanja kontaminacije hrane mikotoksinima, a u cilju dobijanja relevantnih podataka o uticaju mikotoksina na zdravlje ljudi i životinja.

***Ključne reči**: mikotoksini, zdravlje ljudi i životinja, analiza rizika*

Introduction

Mycotoxins are a structurally diverse group of mostly small molecular weight compounds, produced by the secondary metabolism of some filamentous fungi or molds of the *Aspergillus, Penicillium, Fusarium, Alternaria and Claviceps* genera, which, under suitable temperature and humidity conditions, may develop on various foods and feeds, causing serious risks for human and animal health. In structural complexity, mycotoxins vary from simple C_1 compounds, e.g. moniliformin, to complex substances such as the phomopsins (*Culvenor,* 1989) and the tremorgenic mycotoxins (*Steyn,* 1985). Although currently more than 300 mycotoxins are known, scientific attention is focused mainly on those that have proven to be carcinogenic and/or toxic. Human exposure to mycotoxins may

**Plenary paper on International 55th Meat Industry Conference held from June 15-17th 2009 on Tara mauntain*
**Plenarno predavanje na Međunarodnom 55. savetovanju industrije mesa, održanom 15-17. juna 2009. na Tari*

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result from consumption of plant derived foods that are contaminated with toxins, the carryover of mycotoxins and their metabolites into animal products such as milk, meat and eggs or exposure to air and dust containing toxins (*Jarvis,* 2002; *CAST,* 2003). Human food can be contaminated with mycotoxins at various stages in the food chain and the three most important genera of mycotoxigenic fungi are *Aspergillus, Fusarium and Penicillium*. The principal classes of mycotoxins include a metabolite of *Aspergillus flavus* and *Aspergillus parasiticus, a*flatoxin B_1, the most potent hepatocarcinogenic substance known, which has been recently proven to be genotoxic; ochratoxin A, produced by *Penicillium verrucosum* and *Aspergillus ochraceus,* which is known to be carcinogenic in rodents and nephrotoxic in humans. Although its genotoxic power has so far not been definitively established; zearalenone, produced by various species of *Fusarium,* in particular *F. graminearum* and *F. culmorum,* which has an estrogenous action and is significantly toxic to the reproductive system of animals. The tricothecens, a group of numerous metabolites produced by *Fusarium, Stachyobotris,* and *Cephalosporium* species, cause mainly dermotoxicity, immunotoxicity, and gastrointestinal disturbances; and the fumonisins, produced mainly by *Fusarium moniliforme,* may induce leukoencephalopathy in equines as well as hepatotoxicity in rats (*Pohland,* 1987).

The impact of mycotoxins on health depends on the amount of the mycotoxin consumed, the toxicity of the compound, e.g. acute or chronic (e.g. carcinogenic) effects, the body weight of the individual, the presence of other mycotoxins (synergistic effects) and other dietary effects (*Kuiper-Goodman,* 1991). The incidence and extent of mycotoxin contamination are strictly related to geographic and seasonal factors as well as cultivation, harvesting, stocking, and transport conditions (*WHO,* 1979). The evaluation of the incidence and extent of contamination of foodstuffs is crucial and has, in fact, been taken into account for many years by the various disciplines that concur in the definition and management of the risk associated with these toxins and its management (*Gleadle, in press*).

Chemistry of mycotoxins

Aflatoxins

The aflatoxins, a group of closely related hepatocarcinogenic bisdihydrofurano metabolites, produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus,* led to the resurgence of interest in all aspects of mycotoxicology. Aflatoxin B_1 (AFB_1) is the most carcinogenic of the aflatoxins, also, it is the most commonly occurring aflatoxin and has been said to be the most potent hepatocarcinogen to rats and mice. AFM_1 excreted in the milk of lactating cows has toxic properties similar to AFB_1; it is therefore of great public concern, particularly with regards to young children. The potent hepatocarcinogenicity of the aflatoxins led to extensive studies of their carcinogenic properties; detailed information was obtained on their world--wide occurrence in foods and feeds, and their putative role as causal factors for human PLC (primary liver cancer). IARC declared the aflatoxins in 1987 as human carcinogens; the classification was confirmed by re-evaluation in 1992. The need to control aflatoxin exposure is based on 2 major concerns: the adverse short and long-term effects of aflatoxin-contaminated commodities on human and animal health and the presence of aflatoxin residues or metabolites in animal tissues and milk used as human food.

Ochratoxins

Ochratoxin A (OTA) is a pentaketide-derived dihydroisocoumarin moiety linked via the 12-carboxy group by a peptide bond to L-phenylalanine. There are several OTA analogues, ochratoxins B, C, and alkyl esters of ochratoxins that have similar structure but are less toxic. OTA was the first mycotoxic compound isolated from Aspergillus ochraceous, and later it was found in other *Aspergillus and Penicillium* species such as *Penicillium verucosum*. OTA is a main contaminant of cereals (corn, barley, wheat) and to some extent beans (coffee, soy, and cocoa). *Aspergillus* species are associated with OTA production in tropical areas, whereas OTA producing *Penicillium* species thrive and can produce OTA in a colder climate with temperatures as low as 5°C. The toxicity of OTA involves several mechanisms. OTA inhibits protein synthesis by competing with the phenylalanine aminoacylation reaction catalyzed by Phe-tRNA synthase (*Creppy,* 1984). This results in inhibition of protein as well as DNA and RNA synthesis. OTA also disrupts hepatic microsomal calcium homeostasis by impairing the endoplasmic reticulum membrane via lipid peroxidation (*Omar,* 1991). OTA became regarded as a very important mycotoxin since it plays a major role in the nephropathy occurring both in human and animal, particularly in swine (Danish porcine nephropathy) and poultry.

Trichothecenes

The *Fusarium* fungi are probably the most prevalent toxin-producing fungi of the northern

temperate regions and are commonly found on cereals grown in the temperate regions of America, Europe and Asia. A variety of *Fusarium* fungi, which are common soil fungi, produce a number of different mycotoxins of the class of trichothecenes: T-2 toxin, HT-2 toxin, deoxynivalenol (DON) and nivalenol and some other toxins zearalenone and fumonisins. The trichothecenes are a family of related cyclic sesquiterpenoids, which are divided into four groups (types A–D) according to their characteristic functional groups. Type-A and –B trichothecenes are the most common. Type A is represented by HT-2 toxin and T-2 toxin and type B is most frequently represented by DON, 3-acetyl-DON (3-Ac--DON), 15-acetyl-DON (15-Ac-DON), nivalenol (NIV), and fusarenon X (FUS-X). Whereas type-B trichothecenes possess a carbonyl functionality at C-8, type-A trichothecenes lack the keto group at that position and have other oxygen functions at C-8 instead. This chemical characteristic and the fact that type-A trichothecenes generally have fewer hydroxyl groups makes the type-A trichothecenes less polar, which affects analytical procedures from extraction and clean-up up to separation and detection. Toxinogenic *fusaria* have been implicated in human health diseases such as ATA (*Yagen,* 1977), Kashin-Beck disease, akakabibyo (scabby grain intoxication) and esophageal cancer, as well as in a number of animal diseases such as skin toxicity, bone marrow damage, haemorrhagic and estrogenic syndrome (zearalenone), and equine leukoencephalomalacia (ELEM, fumonisins).

Tremorgenic mycotoxins

A brief survey of the structural properties of the fungal tremorgens, namely penitrems, janthitrems, lolitrems, aflatrem, paxilline, paspaline, paspalicine, paspalinine and paspalitrems A and B, reveals their close biogenetic relationship. In the case of aflatrem and paspalitrems A and B, a unit is attached to the paspaline-type structure (*Steyn,* 1985). Tryptophan (Trp) is a common constituent of many secondary metabolites, several affecting the central nervous system, such as the ergot alkaloids. Trp is the biogenetic precursor of the cyclopiazonic acids (*Steyn,* 1975), tremorgenic substances such as fumitremorgens A and B (*Yamazaki,* 1971) and verruculogen (*Fayos,* 1974). In the structurally related metabolites, the brevianamides and austamides (*Steyn,* 1973), Trp and proline contribute the dioxopiperazine part of the molecules. Trp is again a building block of the tetrapeptide metabolites, the tryptoquivalines, which contain in addition anthranillic acid, valine and methylalanine. L-Trp and L-histidine are the precursors of the dioxopiperazines, oxaline (*Nagel,* 1976) and roquefortine (*Gorst-Allman,* 1982), metabolites of *Penicillium oxalicum* and *Penicillium roqueforti,* respectively. Roquefortine, a compound which affects the central nervous system, is also produced by *Penicillium camemberti* and is as such a frequent contaminant of some cheeses.

Fumonisins

Fumonisins are water-soluble mycotoxins that are produced by several species of *Fusarium*, but primarily *F. verticillioides* and *F. proliferatum*. At least 28 different FBs have been reported (*Rheeder et al.,* 2002). Three groups of FBs (A–C) have been identified based on structural similarities. Groups A and B are characterized by the presence of an amide and amine group, respectively. Group C is similar to the B-group, except for the absence of the methyl group at the C1-terminal (*Cole et al.,* 2003). Of all the FBs identified to date, the fumonisin B_1 (FB_1), fumonisin B2 (FB_2) and fumonisin B3 (FB_3) are the most important. FB_1 usually constitute about 70% of the total FBs content found in naturally contaminated foods and feeds. These molecules differ by lacking one of the free hydroxyl groups at either C-10 position (FB_2) or C-5 (FB_3). In addition, FBs analogues have been identified in some processed foods, following hydrolysis (e.g. nixtamalization) or reaction with food components (sugar, starch and proteins).

Zearalenone

Zearalenone (ZEA), 6-(10-hydroxy-6-oxo--*trans*--1-undecenyl)-β-resorcylic acid lactone; CAS 17924-92-4), is produced as a secondary metabolite by a number of *Fusarium* species including *F. culmorum*, *F. graminearum* (*Hestbjerg et al.,* 2002; *Glenn,* 2007), as well as *F. equiseti* and *F. crookwellense* (*Bennett and Klich,* 2003). These species are known to infest wheat, barley, rice, maize, and some other crops (*Yamashita et al.,* 1995; *Jimenez and Mateo,* 1997). Despite its non-steroidal structure, ZEA activates estrogen receptors resulting in functional and morphological alteration in reproductive organs. ZEA interacts not only with both types of estrogen receptors but is also a substrate for hydroxysteroid dehydrogenases, which convert it into two stereoisomeric metabolites, α-zearalenol and β-zearalenol. A second reduction step yields the two minor metabolites α-zearalanol and β-zearalanol. Alpha-hydroxylation results in an increase in estrogenic potency as compared to the parent compound, and the species-specific rate of alpha-hydroxylation may account for the

susceptibility of certain animal species, including pigs, towards ZEA exposure.

The topic of conjugated or masked mycotoxins first caught attention in the mid-1980s because in some cases of mycotoxicoses, clinical observations in animals did not correlate with the low mycotoxin content determined in the corresponding feed. The unexpected high toxicity could, for instance be attributed to the occurrence of undetected, conjugated forms of mycotoxins that hydrolyze to the precursor toxins in the digestive tracts of animals (*Gareis,* 1994). It was shown that plants can reduce the toxicity of mycotoxins either by chemical modification and/or by inclusion into the plant matrix (*Wallnööfer et. al.,* 1996). This detoxification process includes the conjugation of mycotoxins to polar substances such as sugars, amino acids, or sulfate (*Schneweis et. al.,* 2002) and subsequent storage of the conjugates in vacuoles. So far, the natural occurrence of a zearalenone glucoside in wheat has been reported (*Langseth et. al.,* 1998). High-performance liquid chromatography (HPLC) combined with tandem mass spectrometry (MS/MS) offers a powerful tool for identification and characterization of mycotoxin conjugates (*Berthiller et. al.,* 2005).

Mycotoxin exposure and effect on human and animal health

A wide range of commodities can be contaminated with mycotoxins (Table 1) both pre- and post-harvest. (*CAST,* 2003). Aflatoxins are found in maize and peanuts as well as in tree nuts and dried fruits. Ochratoxin A is found mainly in cereals, but significant levels of contamination may also occur in wine, coffee, spices and dried fruits. Fumonisins are found mainly in maize and maize based products. Tricothecenes are chiefly associated with grain, as is zearalenone. Available evidence suggests that tissue accumulation of mycotoxins, or their metabolites, is very low and that residues are excreted in a few days. The hydroxylated metabolite of aflatoxin B_1, aflatoxin M_1, is excreted into milk from 1 to 6% of dietary intake. (*Van Egmond, 1989, Veldman,* 1992) Ochratoxin A has been detected in blood, kidneys, liver and muscle tissue from pigs in several European countries. (*Leistner,* 1984, *Van Egmond,* 1994, *Milićević,* 2008). Residues of cyclopiazonic acid (CPA), a co-contaminant with aflatoxin, have been found in meat, milk and eggs. (*Bryden,* 2001). After an extensive review of the literature, Pestka (1995) concluded that trace levels of mycotoxins and their metabolites may carry over into the edible tissue (meat) of food producing animals. However, he concluded that to date there is no evidence to suggest that the levels of transmitted mycotoxins pose a threat of acute toxicity.

AFB1 has been extensively linked to human primary liver cancer (*PLC*) in which it acts synergistically with HBV infection and was classified by the International Agency for Research on Cancer (IARC) as a human carcinogen (group 1 carcinogen), (*IARC,* 1993a). This combination represents a heavy cancer burden in developing countries. A recent comparison of the estimated population risk between Kenya and France highlighted the greater burden that can be placed on developing countries (*Shephard,* 2006). Based on respective estimates for aflatoxin exposure of 133 and 0.12 ng kg^{-1} body weight day^{-1} and respective HBV prevalence of 25 and 1%, the liver cancer risk would be 11 vs. 0.0015 cancers per year per 100.000 population, respectively. Given recently published liver cancer incidence rates in the European Union of 10.0 per 100.000 for males and 3.3 per 100.000 for females (*Bray et al.* 2002), it is clear that aflatoxin plays a significant role in liver cancer in developing countries, but not in the developed world where other risk factors such as cirrhosis are more important. Fumonisins have been implicated in one incident of acute food-borne disease in India in which the occurrence of borborygmy, abdominal pain, and diarrhea was associated with the consumption of maize and sorghum contaminated with high levels of fumonisins (*Bhat et al.* 1997). Fumonisin B_1, the most abundant of the numerous fumonisin analogues, was classified by the IARC as a group 2B carcinogen (possibly carcinogenic in humans), (*IARC,* 2002). Studies in the former Transkei region of South Africa and in Linxian and Cixian counties, China, have demonstrated an association between fumonisin exposure in rural subsistence farming areas and a high incidence of oesophageal cancer as well as with field outbreaks of ELEM in many countries such as Egypt, South Africa and the United States of America (*Marasas,* 1988) and pulmonary oedema in swine (*Ross,* 1990). ELEM is a fatal neurological disease of horses, characterized by liquefactive necrosis of the white matter of the brain. ELEM has been experimentally induced in horses by, either supplementing their diets with *F. moniliforme-con*taminated corn, or by the oral administration of fumonisin B, (FB_1), a toxin produced by *F.moniliforme* (*Kellerman,* 1990). Fumonisins, which inhibit the uptake of folic acid via the folate receptor *(Stevens and Tang,* 1997), have also been implicated in the high incidence of neural tube defects in rural populations known to consume contaminated maize, such as the former Transkei region of South Africa and areas of Northern China (*Marasas et al.* 2004). The other three agriculturally

important mycotoxins have also been associated with various outbreaks of human disease, mostly in developing countries. A number of occurrences of acute food-borne illness in India and China involving gastrointestinal symptoms have been attributed to the consumption of DON-contaminated cereals (*Luo,* 1988; *Bhat et al.* 1989). OTA has long been associated with Balkan endemic nephropathy (BEN), a fatal renal disease with histopathological similarities to OTA-induced nephropathy in swine and has been associated with the incidence of epithelial tumours of the upper urinary tract (*Benford et al.* 2001; *Castegnaro et al.* 2006). OTA was classified by the IARC as possibly carcinogenic to humans (group 2B carcinogen), (*IARC,* 1993b). ZON is a naturally occurring endocrine-disrupting chemical and has been associated with clinical manifestations of hyper-oestrogenism in humans and animals, including an outbreak of precocious pubertal changes in young children in Puerto Rico in the Caribbean (*Saenz de Rodrigues et al.* 1985) and gynecomastia with testicular atrophy in rural males in southern Africa (*Campbell,* 1991).

The role of sampling and analysis in mycotoxin contamination

The correct evaluation of mycotoxin contamination in foodstuffs depends principally on the degree of accuracy associated with the single steps by which this information is obtained. Because of the highly heterogeneous distribution of mycotoxins in a lot, taking a representative sample is the most critical stage. In Fig. 1 are presented the errors associated with sampling for Aflatoxins analysis (expressed as a coefficient of variation). From this it can be seen that the error associated with sampling procedures is notably higher than that associated with subsampling or analysis.

The most prominent reason for collecting food samples for the investigation of contaminants, such as mycotoxins, is to protect consumer health,

Table 1. Some human diseases in which mycotoxins have been implicated
Tabela 1. Neka oboljenja ljudi povezana sa mikotoksinima

Disease	Mycotoxin source	Fungus
Akakabio-byo	Wheat, barley, oats, rice	*Fusarium spp.*
Alimentary toxic aleukia	Cereal grains (toxic bread)	*Fusarium spp.*
Balkan nephropathy	Cereal grains	*Penicillium spp.*
Cardiac beriberi	Rice	*Aspergillus spp., Penicillium spp.*
Celery harvester's disease	Celery (Pink rot)	*Sclerontinia*
Ergotism	Rye, cereal grains	*Claviceps purpurea*
Hepatocarcinoma	Cereal grains, peanuts	*Aspergillus flavus, A. parasiticus*
Kwashiorkor	Cereal grains	*Aspergillus flavus, A. parasiticus*
Neural tube defects	Maize	*Fusarium verticillioides, F.proliferatum*
Oesophageal tumors	Corn	*Fusarium verticilloides, F.proliferatum*
Onyalai	Millet	*Phoma sorghina*
Reye's syndrome	Cereal grains (grain dust)	*Aspergillus*
Stachybotryotoxicosis	Cereal grains, (grain dust)	*Stachybotrys atra*

The evaluation of mycotoxins in biological fluids can provide useful indications of the dietary intake of mycotoxins. This approach can also constitute a valid, although indirect, evaluation of mycotoxin contamination in foodstuffs. This methodology, in fact, can somehow give a better estimate of the exposure of humans to mycotoxins than that obtained by monitoring food data since the latter may, as stated, be affected by sampling, subsampling, and analysis errors.

mainly verifying the compliance of food and feed with acceptable safety standards. Sampling is one of the most crucial, but underestimated parts of the multifaceted and complex bulk of activities aimed at addressing and managing food issues. In practice, the overall objective of good sampling is to provide reliable samples to be analyzed that can represent the

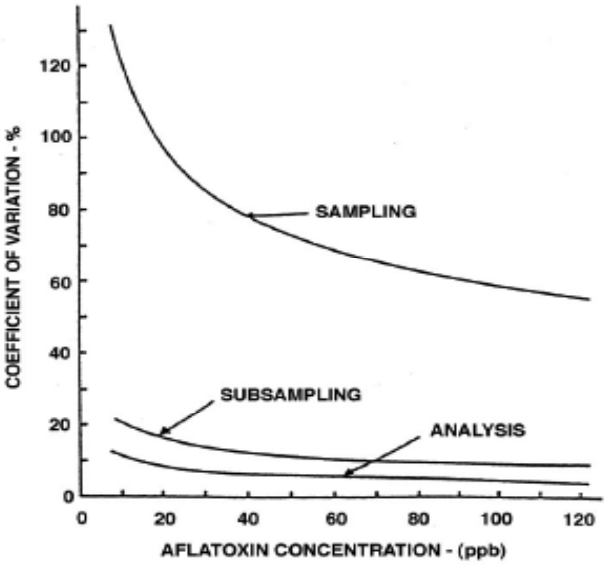

Figure 1. Coefficient of variation characterizing sampling, subsampling and analysis as a function of Aflatoxins concentration

Slika 1. Koeficijent varijacije koji karakteriše uzorkovanje, poduzorkovanje i analizu u funkciji koncentracije alfatoksina

basis for ‘‘fit for purpose’’ investigations. In most cases, meaningful sampling is a process comprising two very dissimilar steps:

(1) The first step (hereafter referred to as ‘‘primary sampling’’) consists in taking the decision on ‘‘why, where and when’’ to collect the samples. In other words, the process of ‘‘statistically’’ locating the sites (populations) from which food samples should be taken;

(2) The second step (hereafter referred to as ‘‘secondary sampling’’) consists of establishing how samples should be collected in order to be representative of the lot under investigation. For both steps the quality and the consequent reliability of the data are strongly dependent on the available resources and on the skill of the people involved.

For this class of contaminants, the need for statistically-based planning is particularly relevant for: (i) The multifaceted implications of mycotoxin contamination (health, trade, ethical issues related to developing countries' difficulties), and (ii) the largely inhomogeneous distribution of the toxins within food commodities, with the consequent need for careful secondary sampling. Appropriate sampling plans are essential to ensure that the analytically-derived mean concentration of a sample is representative of the true mean concentration of a lot. Sampling plans are particularly relevant in the area of mycotoxins where it is known that the contamination of a commodity can be heterogeneously distributed. Good primary sampling schemes have so far been developed for several classes of contaminants, such as dioxins and pesticides (*South et al.* 2004), in contrast to the very few valid ones so far proposed for mycotoxins. In contrast, a large number of papers have appeared, related to secondary sampling schemes for aflatoxin B_1 (particularly on its distribution in a lot and on related sampling plans), (*Whitaker et al.* 1979, 1994), but only a few studies deal with some Fusarium toxins (*Hart and Schabenberger* 1998; *Whitaker et al.* 1998; *Whitaker et al.* 2000). Conversely, specific studies focused on the distribution of OTA-contaminated units are not yet available, apart from the vague assumption that ‘‘representative sampling’’ for aflatoxins is more difficult than sampling for other known mycotoxins in food products. Sampling procedures recommended for aflatoxins should thus be adequate for other mycotoxins (*Dickens and Whitaker* 1982). Nevertheless, the European legislation dealing with sampling and methods of analysis of mycotoxins for official control was recently adopted (*EC,* 2006).

In conclusion, the analysis of sources of errors in evaluating the impact of mycotoxins on human health should be carefully performed taking into account many aspects such as planning and accomplishment of monitoring programs, consumer's health protection, economic, political and commercial considerations.

Analysis

Legislation calls for monitoring methods. Reliable analytical methods must be available to enable enforcement of the regulations in daily practice. In addition to reliability, simplicity is desired, as it will affect the amount of data generated and the practicality of the ultimate measures taken. The reliability of mycotoxin analysis data can be improved by use of interlaboratory-validated methods of analysis (e.g. the methods of AOAC International and methods standardized by CEN). These methods have been largely developed in response to planned regulations for mycotoxins or regulations that came into force. The requirements for these methods were dictated by the needs, i.e. they had to be suitable for the (planned) regulated mycotoxin–matrix combination(s). The limits of determination of the methods had to be demonstrated to be low enough for precise and accurate determination of the mycotoxins of interest at regulatory levels. Methods were also developed and validated for toxin–matrix combinations for which there were no regulations (yet), but for which the scientific community saw a need, e.g. for surveillance purposes. These developments eased

the establishment of specific mycotoxin regulations. AOAC currently has approximately 45 analytical methods for determination of mycotoxins (*AOAC,* 2005). All have undergone extensive testing in interlaboratory validation studies, and subsequent review by the AOAC's rigorous approval process. AOAC methods are referred to as official methods in mycotoxin legislation in a few dozen countries (*FAO,* 2004). In Europe, CEN methods are becoming increasingly important. Ten mycotoxin methods have been standardized by the CEN, and this number will grow substantially in the years to come. Although CEN mycotoxin methods are not mandatory for official food control in the EU, all CEN mycotoxin methods can be used in the EU for official food-control purposes because their performance characteristics fulfill the criteria laid down in the EU regulation for sampling and analysis *(EC,* 2006). One of them, high performance liquid chromatography (HPLC) with different detectors, is frequently used both for routine analyses and as a confirmatory method for novel or screening techniques. For some mycotoxins, e.g. trichothecenes, gas chromatography (GC) is the method more often used (*Krska,* 2001). Except for direct mass spectrometric methods, all the other analytical methods used for mycotoxin determination are, either immunoassay based, or otherwise fall into the category of direct or indirect screening methods. The use of good, validated methods of analysis is no guarantee that reliable analytical results will be obtained in mycotoxin determination. Analytical quality assurance (AQA) is another prerequisite for adequate food-law enforcement. AQA includes, where possible, the use of (certified) reference materials (e.g. CRMs supplied by the European Commission's Joint Research Centre/Institute for Reference Materials and Measurements; JRC/IRMM, see http://www. irmm.jrc.be).

Factors affecting the mycotoxin regulations

Regulations relating to mycotoxins have been established in many countries to protect the consumer from the harmful effects of these compounds. Different factors play a role in the decision-making process of setting limits for mycotoxins. These include:

- the availability of toxicological data on mycotoxins,
- the availability of exposure data on mycotoxins,
- knowledge of the distribution of mycotoxins concentrations within commodity or product lots,
- the availability of analytical methods,
- legislation in other countries with which trade contacts exist,
- the need for sufficient food supply.

The first two factors provide the information necessary for hazard assessment and exposure assessment, respectively, the main bases of risk assessment. Risk assessment is the scientific evaluation of the probability of occurrence of known or potential adverse health effects resulting from human exposure to food-borne hazards. It is the primary scientific basis for promulgation of regulations. The third and fourth factors are important factors enabling practical enforcement of mycotoxin regulations through adequate sampling and analysis procedures. The last two factors are merely socio-economic in nature but are equally important in the decision-making process to establish meaningful regulations and limits for mycotoxins in food and feed. Risk assessment regulations are primarily based on known toxic effects. For the mycotoxins currently considered most significant (aflatoxins B_1, B_2, G_1 and G_2; aflatoxin M_1; ochratoxin A; patulin; fumonisins B_1, B_2 and B_3; zearalenone; T-2 and HT-2 toxins; and deoxynivalenol), the Joint Expert Committee on Food Additives (JECFA—a scientific advisory body of the World Health Organization WHO and the Food and Agriculture Organization FAO has evaluated their hazard in several sessions (*WHO,* 1999, 2000, 2002). In February 2001 a special JECFA session was devoted to entirely mycotoxins. Two reports have appeared on this session, a longer version (*FAO,* 2001) and a shorter version (*WHO,* 2002). These reports provide good and detailed insight into the process of risk assessment of mycotoxins. The reports addressed several concerns about the mycotoxins considered—their properties and metabolism, toxicological studies, and final risk evaluation. With the mycotoxin evaluations the Committee discussed general considerations on sampling, analytical methods, associated intake issues and control. Risks associated with mycotoxins depend on both hazard and exposure. The hazard of mycotoxins to individuals is probably, more or less, the same all over the world (although other factors are, sometimes, also important, e.g. hepatitis B virus infection in relation to the hazard of aflatoxins). Exposure is not the same, because of different levels of contamination and dietary habits in various parts of the world. Risk analysis framework for food safety is illustrated in Fig. 2.

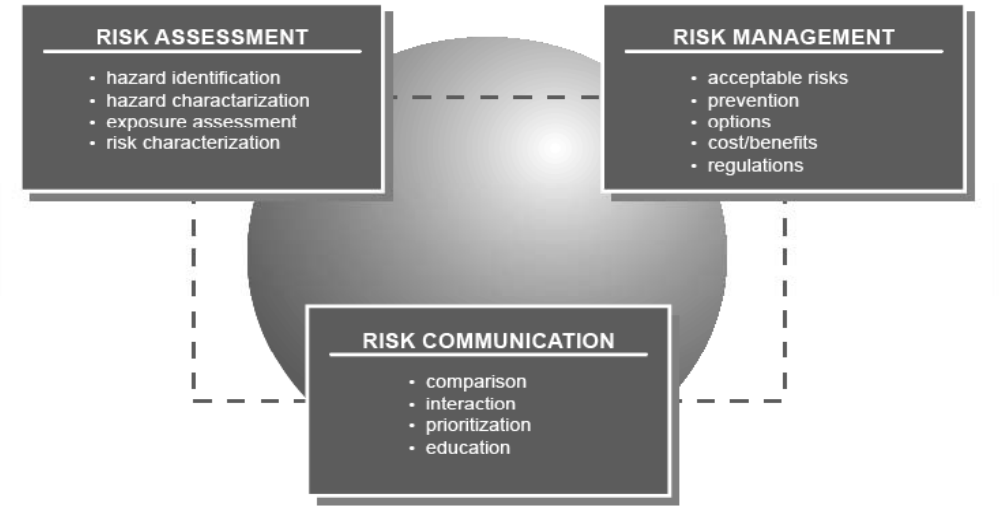

Figure 2. Risk analysis framework for food safety
Slika 2. Okvir analize rizika za bezbednost hrane

The international mycotoxin regulatory situation

Since the discovery of the aflatoxins in 1960 and subsequent recognition that mycotoxins are of significant health concern to both humans and animals, regulations gradually developed for mycotoxins in food and feed. In the early days of mycotoxin regulations these measures focused mainly on the aflatoxins. They were established by industrialized countries and limits often had an advisory or guideline character. Over the years, the number of countries with known specific mycotoxin regulations has increased from 33 in 1981 (*Schuller,* 1983) to 56 in 1987 (*Van Egmond,* 1989), 77 in 1995 (*FAO,* 1997), and 100 in 2003 (*FAO,* 2004). Current regulations encompass 13 different mycotoxins or groups of mycotoxins and specific limits have been established for many food and feed commodities and products. Until the late 1990s setting of mycotoxin regulations was mostly a national affair. Gradually, several economic communities e.g. EU, European Union; MERCOSUR Mercado Cómún del Sur; Australia and New Zealand harmonized their mycotoxin regulations, thereby overruling existing national regulations. Current regulations are increasingly based on scientific opinions of authoritative bodies, for example the FAO/WHO Joint Expert Committee on Food Additives of the United Nations (JECFA) and the European Food Safety Authority (EFSA). At the same time, requirements for adequate sampling and analytical methods put high demands on other professional organizations, for example AOAC International and the European Standardization Committee (CEN).

Economic impact

Mycotoxin contamination of the food chain has a major economic impact. However, the insidious nature of many mycotoxicoses makes it difficult to estimate incidence and cost (*CAST,* 1989). In addition to crop losses and reduced animal productivity, costs are derived from the efforts made by producers and distributors to counteract their initial loss, the cost of improved technologies for production, storage and transport, the cost of analytical testing, especially as detection or regulations become more stringent, and the development of sampling plans (*Whitaker,* 1995). There is also a considerable cost to society as a whole, in terms of monitoring; extra handling and distribution costs, increased processing costs and loss of consumer confidence in the safety of food products. It is estimated that in developing countries, the greatest economic impact is associated with human health. (*Miller,*1998). Delineating economic impact reflects the complexity of a mycotoxin contamination within the food chain. There is a clear need to protect consumers through regulations but

at what cost? A comprehensive risk and economic analysis of lowering the acceptable levels for fumonisins and aflatoxin in world trade demonstrated that the United States would experience significant economic losses from tighter controls (*Wu,* 2004). The developing countries, China and Argentina, were more likely to experience greater economic losses than sub-Sahara Africa. The disturbing outcome of this detailed analysis was that tighter controls were unlikely to decrease health risks and may have the opposite effect (*Wu,* 2004). In other words, very stringent international trade regulations could lead to the situation where exporting countries, especially developing countries, would retain higher risk commodities which would subsequently be available for their own populations; communities which are already exposed to higher levels of mycotoxins than consumers in developed countries.

Strategies to Prevent Mycotoxin Contamination of Food and Animal Feed

Many strategies to prevent mycotoxin contamination of food and animal feed have been developed (*Rustom,* 1997; *Yılmaz,* 2001). It is clear that mycotoxins can contaminate agricultural produce, both in the field as well as during storage. The use of pre-harvest control strategies for such resistance varieties, field management, the use of biological and chemical agents, harvest management and post-harvest applications, including improving drying and storage conditions, together with the use of natural and chemical agents and irradiation have clearly been shown to be important in the prevention of mycotoxigenic mould growth and mycotoxin formation (*CAC,* 2002). The importance of drying and moisture control during storage is generally well understood by the industry, in terms of the importance of prevention of fungal contamination. Interesting results have been reported on the potential use of biocompetitive agents in different biological control strategies to prevent the pre-harvest aflatoxin contamination of crops, such as peanuts, rice, maize, and cottonseed. It is clear that much more work must be conducted to identify various crop genotypes which are resistant to mycotoxigenic fungus infection and subsequently mycotoxin formation. It is also clear that a combination of the development of crop species with resistance to toxigenic fungi and biocompetitive non-mycotoxigenic strain technologies may yield one of the most effective strategies for prevention of mycotoxin contamination (*Gendloff*, 1986; *Reid,* 1994). Several natural plant extract and spice oils of eugenol, cinnamon, oregano, onions, lemongrass, (*Yin,* 1998*, Juglal,* 2002) tumeric, mint, and chemical compounds (fungicide, herbicide, and surfectant) are known to prevent both mycotoxigenic mould growth and mycotoxin formation during post-harvest season. In addition to application of plant extracts and chemical agents as well as antagonistic microorganisms, such as lactic acid bacteria with their antifungal properties, seem to be potentially very effective in the prevention of mycotoxin formation. The precise antifungal properties of lactic acid bacteria are still largely unresolved but may involve microbial competition (*El-Gendy,* 1981), as well as extracellular metabolites which are heat-stable and of low molecular weight. Again, further investigations are clearly needed to gain a better understanding of this antifungal action. Various physical and chemical strategies have also been developed to help prevent mycotoxin contamination, including physical separation, extraction with sorbents, and adsorption (*Sinha,* 1998). The fluorescence sorting of maize, cottonseed and figs by examination under UV light is known to be the cheapest and the simplest acceptable way for the screening of aflatoxins. It is clear that no single currently available physical or chemical detoxification method will be suitable for all foods and animal feeds. The effectiveness of a method in the detoxification of mycotoxins depends on the nature of the food, environmental conditions such as moisture content, temperature, as well as the type of mycotoxin, its concentration and the extent of binding between mycotoxin and constituents. While a range of chemical compounds, including hydrochloric acid, ammonia, hydrogen peroxide, O_3, sodium bisulfite, and chlorine seem to hold great potential in the detoxification of mycotoxins, unfortunately their use significantly decreases the nutritional value of the foods or produces toxic derivatives in the treated product with undesirable sensory properties. This will severely limit their widespread use. At the same time it should be noted that chemical treatment is not allowed within the EC for commodities destined for human consumption. Recently, there has been an increasing interest in the use of bacteria, yeast, and fungi to help reduce the toxic effect of mycotoxins (*Bata,* 1999). While most studies to date on mycotoxin detoxification by microorganisms have been undertaken under laboratory conditions, there is data on the effective use of *F. aurantiacum* in the detoxifying AFB_1 from various food products, including milk, peanuts, maize, and red pepper without leaving toxic end products. One potential drawback here is the production of a bright orange pigment by the organism which restricts its use in the detoxification of food and in feed fermentations.

The most recent approach to the problem has been the use of mycotoxin-binding agents in the diet that sequester the mycotoxin in the gastrointestinal tract thus reducing their bioavailability. Although AC, HSCAS, aluminosilicate, zeolite, and bentonite have shown good potential for use in the animal feed to help overcome aflatoxicosis, the future *in vivo* investigations must focus on other problematic mycotoxins. Interestingly lactic acid bacteria and bifidobacteria have been shown to bind AFB1, but mechanistic studies need to be conducted on the precise binding mechanism, while the conditions favoring the release of bound toxin molecules need to be investigated as well.

Concluding comments

Mycotoxins are a food safety risk globally. International risk assessments have been performed by JECFA (1998, 2001) for aflatoxin B_1, aflatoxin M_1, DON, fumonisins, ochratoxin A, T-2 toxin and HT-2 toxin. These analyses indicate that health risks from mycotoxins are generally orders of magnitude lower in developed countries that for populations from developing regions. The scope of the mycotoxin problem is readily understood when it is appreciated that there are many thousand secondary fungal metabolites (*Cole,* 2003), the vast majority of which have not been tested for toxicity or associated with disease outbreaks. In developing countries it is likely that consumers will be confronted with a diet that contains a low level of toxin and in many cases, there may be other toxins present. For example, aflatoxins, fumonisins, DON and zearalenone may occur together in the same grain; many fungi produce several mycotoxins simultaneously, especially *Fusarium* species (*Cole,* 2003). Co-occurrence of mycotoxins is of special concern, for instance, in the case of fumonisins (apotent cancer promoter) and aflatoxin (a potent human carcinogen) where a complimentary toxicity mechanism of action occurs (*Riley,* 1998). In Africa and Asia the co-occurrence of these mycotoxins is common and a significant percentage of the population is infected with Hepatitis B or C which leads to the conclusion that mycotoxins in these regions can have devastating human health effects. Implicit with these conclusions are the existence of syndromes of apparently unknown aetiology and epidemiology that may involve mycotoxins and the difficulty of establishing „no effect“ levels for mycotoxins.

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Paper recieved: 23.03.2009.