

## Occurrence, Health Risks and Methods of Analysis for Aflatoxins and Ochratoxin A

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### ABSTRACT

The most frequent toxigenic fungi in the Europe are *Aspergillus*, *Penicillium* and *Fusarium* species. They produce aflatoxin B1 transformed into aflatoxin M1 found in the milk, as well as Ochratoxins and Zearalenone, Fumonisin B1, T-2 toxin, HT-2 toxin and deoxynivalenol (vomitoxin), which are of increasing concern in human health. These mycotoxins are under continuous survey in the Europe, but the regulatory aspects still need to be set up and/or harmonised at the European level. They are found in foodstuffs and are not destroyed by normal industrial processing or cooking since they are heat-stable. Some of their metabolites are still toxic and may be involved in human diseases. Their toxic effects (liver, kidney and haematopoietic toxicity, immune toxicity, foetal toxicity, teratogenicity, and mainly carcinogenicity) are mostly known in experimental models. Chemical assays are of major importance for the determination of mycotoxins. Generally, all chemical methods for the analysis of mycotoxins include the basic steps of extraction, clean-up, separation, detection, quantification and confirmation of identity. The various approaches that exist for the determination of nephrotoxic mycotoxins, and in particular the ochratoxins are discussed below. This paper gives an overview of chromatographic methods used for the determination of aflatoxin and ochratoxin A (OA) in animal and human tissues and fluids. These methods are needed for monitoring studies of OA occurrence in the food chain and for studies dealing with the OA carry-over. The review includes sampling, sample storage, extraction, spiking procedures, clean-up, detection and determination, and confirmation procedures.

**Key Words:** Reviews; Occurrence, Sample preparation; Health risk, Ochratoxins; Aflatoxins; Mycotoxins.

### INTRODUCTION

Mycotoxins are microbial agents which cause food or feed-borne intoxications and are therefore considered as undesirable substances in feed and food. Mycotoxins are compounds often found in cereal grains and forages (Sweeney and Dobson, 1998). These toxins are produced by saprophytic fungi during storage or by endophytic fungi during plant growth. Mycotoxins are secondary

metabolites of certain fungi, mostly *Penicillium* and *Fusarium*. They are produced in cereal grains as well as forages before, during and after harvest, in various environmental conditions. Due to the diversity of their toxic effects and their synergetic properties, mycotoxins are considered as risks for consumers of contaminated foods and feeds (Yiannikouris and Jonany, 2002). They can cause acute and chronic toxic effects in animals and can be

transferred into products, or may affect human health directly. They are relatively small molecules with highly diverse chemical structures and biological activity.

The production of mycotoxins is not essential for the fungal growth or reproduction, but could be a “virulence factor” for some plant diseases and act against other microorganisms and higher organisms (Desjardins et al., 1993; Puschner, 2002). Plant stressors such as draught or over-irritation, insect damage and pesticide exposure result in a higher susceptibility to fungal infection, whereas the production of mycotoxins may be due to stress or altered conditions for the fungus (Oldenburg et al., 2000).

Mycotoxin production does not only depend on the genotype of a certain strain alone, but also on a range of environmental factors including humidity, temperature, water activity, processing-errors and insect damage, which have an influence on fungal growth and metabolism (CAST, 2003). Most mycotoxins are stable chemical compounds and cannot be destroyed by processing and heat treatment of feed and foodstuffs. When the fungal metabolites present in foods in sufficiently high levels, they can have toxic effects, either acute (for example, liver or kidney deterioration), chronic (for example, liver cancer), mutagenic, or teratogenic. The resulting symptoms range from skin irritation to immunosuppression, birth defects, neurotoxicity, and death (Murphy et al., 2006).

No region of the world escapes the problem of mycotoxins and according to Lawlor and Lynch (2005) mycotoxins are estimated to affect as much as 25 percent of the world's crops each year. Whether grain is produced in temperate, sub-tropical or tropical climates, if rainfall and humidity are experienced in the harvest season, infection of the grain by fungi is likely. In most European countries aflatoxins are not

considered to be a major problem in domestic crops or forages. In contrast, vomitoxin, ochratoxin, zearalenone are found more frequently. Aflatoxins are common in humid climatic conditions like those existing in Asian and African countries and certain parts of Australia.

Apart from direct effects, the economic consequences of mycotoxin-induced poor performance and productivity are additional important factors in animal husbandry and the multiplier effect this has on other industries as a result of the reduced spending power of producers. Costs of chemical analyses, quality control and regulatory programs, research and development, extension services, law suits, and the cost of human illnesses must all be borne by the national economy.

The value of the losses encountered depends on grain, animal, and animal product prices, interest rates, degree of contamination, and other economic variables. Even during favourable seasons it is likely that millions of dollars are lost as a result of the contamination of crops with mycotoxins (Charmley et al., 1995). Problems associated with mycotoxin contamination and the economic losses resulting will continue to be seen in food and agriculture industries.

Therefore, it can be predicted that food and feed are always contaminated with toxins to a greater or less extent, and with increasing accuracy of analysis (lower detection limits) toxins can be detected in more and more cases.

### **Food Borne Mycotoxins**

Deoxynivalenol, Nivalenol, Zearalenone, Ochratoxin, Aflatoxins and Fumonisin occur quite often in food (Table 1). T 2 toxin is also found in a variety of grains but its occurrence is less frequent than the other mycotoxins.

The food-borne mycotoxins are of greatest significance for human health in tropical

developing countries is aflatoxins and the fumonisins. Fumonisin has been found as a very common contaminant of maize-based food and feed in Africa, China, France, Italy, and the USA (Figure 1, 2, 3, 4, 5, and 6).

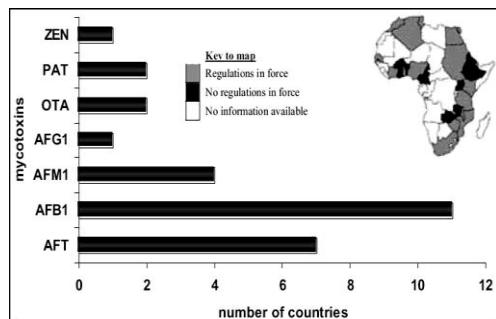


Figure 1 Mycotoxins regulated in food in Africa (FAO 2004)

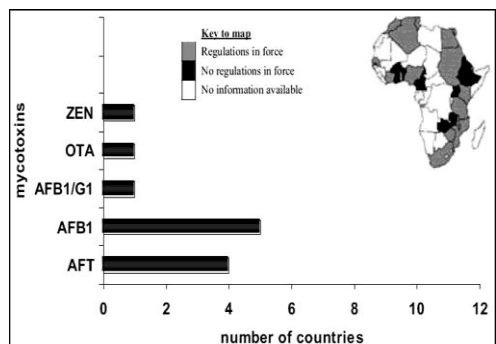


Figure 2 Mycotoxins regulated in feed in Africa

**Which are the relevant mycotoxins to be analysed?**

According to the survey, the most important mycotoxins are the following: aflatoxins (AFB1, B2, G1, and G2), ochratoxin A (OA), tricothecenes, zearalenone and fumonisins (B1, B2). Of the tricothecenes, attention is paid mainly to deoxynivalenol (DON or vomitoxin), nivalenol and T2-toxin. Some laboratories also focused on HT2 toxin, 3-Ac-DON, DAS, Fusarenon-X, Neosolaniol and 15-Ac-DON. Surveys were published in the brewing world recently describing the most important mycotoxins, the producing agents and the toxicological

effects and giving guidelines to avoid or reduce the level of mycotoxins.

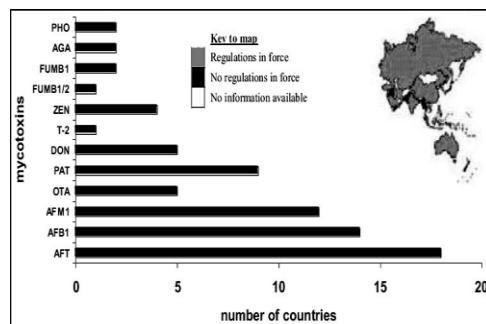


Figure 3 Mycotoxins regulated in food in Asia/Oceania

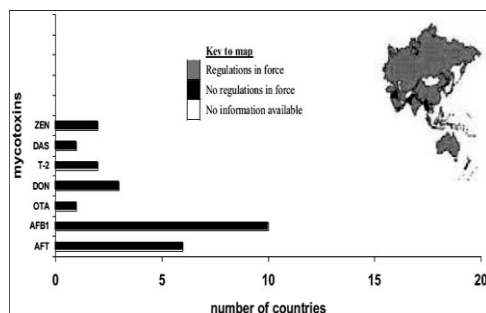


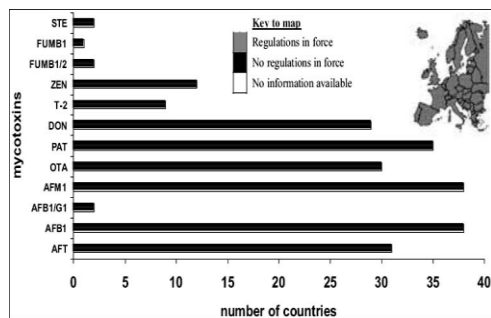
Figure 4 Mycotoxins regulated in feed in Asia/Oceania

**Aflatoxins**

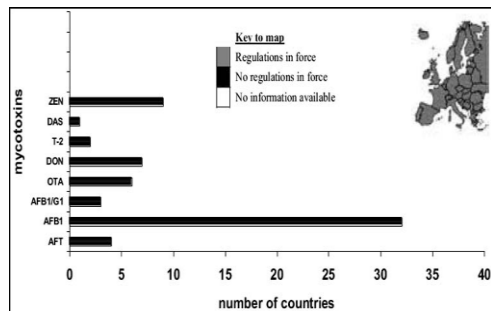
There is to our knowledge very little evidence of the occurrence of aflatoxins in the European cereals but a regulation exists in the EC and in some other countries. Therefore, the analysis of aflatoxins has to be included in mycotoxins management.

Aflatoxins are toxic metabolites produced by certain fungi (*Aspergillus* species) in/on foods and feeds predominantly in hot and humid regions. Aflatoxins have been associated with various diseases, such as aflatoxicosis, in livestock, domestic animals and humans throughout the world. They were the first mycotoxins that were identified as potential health hazard, when approximately 100,000 turkeys died from an

acute necrosis of the liver after consuming groundnuts infected by *Aspergillus flavus*.



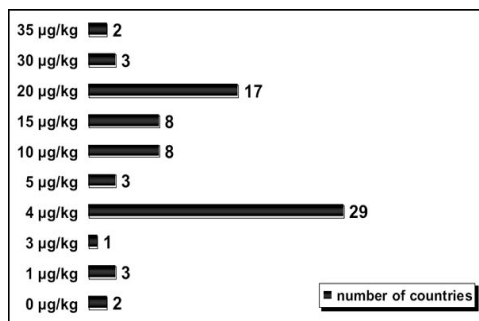
**Figure 5** Mycotoxins regulated in food in Europe



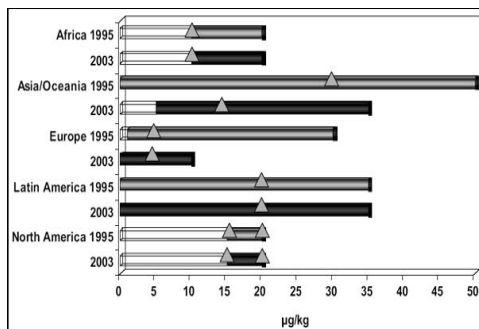
**Figure 6** Mycotoxins regulated in feed in Europe

Occurrence of aflatoxins is influenced by certain environmental factors; hence the extent of contamination will vary with geographic location, agricultural and agronomic practices, and the susceptibility of commodities to fungal invasion during pre-harvest, storage, and/or processing periods. Aflatoxins have received a greater attention than any other mycotoxins because of their carcinogenic effects in laboratory animals and acute toxicological effects in humans. Due to carryover in food and feed, they are considered to have the most severe impact on human health amongst all mycotoxins. Maximum residue levels (Figures 7 and 8) have been set down to the ppb range in a wide variety of agricultural commodities (Table 2), food, feed and milk. For instance a maximum level of 0.01 µg/kg of aflatoxin

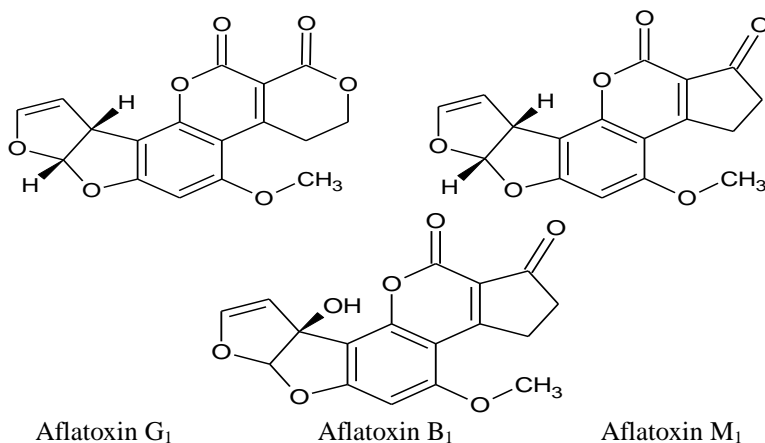
M1 (AFM1) is acceptable concentration in milk for infants. The others important aflatoxins are B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>, and M<sub>1</sub> (Figure 9) found in a wide variety of foodstuffs including nuts, cereals, dried fruit and milk. As complete elimination of these toxins from the food or feed is difficult to attain, however, many countries attempted to limit exposure to aflatoxins by imposing regulatory limits on food and feed commodities. It appears that in Africa, Latin America and North America, no observable changes have occurred. On the contrary, Asia/Oceania and the Europe, where a downward trend to limit total aflatoxins is visible (Figure 10). Regulations have been imposed in more than 70 countries to restrict the intake of mycotoxins (FAO, 1997). However, the legal limits vary significantly both from country to country and by mycotoxin type and matrix.



**Figure 7** Worldwide limits for total Aflatoxins in food



**Figure 8** Ranges and medians of limits for total Aflatoxins in food per world region



**Figure 9** Chemical structures of Aflatoxins.

The frequent occurrence of aflatoxins led to temporary ban on certain “high-risk” foods imported to the Europe.

### Methods of Analysis

A reliable risk assessment of mycotoxin contamination for humans and animals relies on their identification and accurate quantification in food and feedstuff. In general, fast and easy-to-use ELISA based aflatoxin screening kits are commercially available for all major types of aflatoxins. Quantification is predominantly done with (LC-FL). Due to the excellent native fluorescence activity of aflatoxins, detection limits in the low ppb range can easily be achieved when iodine is added post-column to enhance method sensitivity. In addition, immunoaffinity sample clean-up has shown a great potential to increase method specificity and sensitivity by selective enrichment and isolation of the target aflatoxins.

A limited number of quantitative methods have been published to determine major aflatoxins and the structurally related sterigmatocystin in milk, herbs, and urine. Most screening methods for mycotoxins are based on immunoassays; analyte confirmation can be easily achieved with

mass spectrometric methods, like gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/mass spectrometry (LC/MS). Analytical methods used by enforcement laboratories for the implementation of legislation must be subject to validation procedures so that methods produce reliable results (EC, 1993). These methods need to provide accurate and reproducible results both within and between laboratories. This is especially important in view of legal actions and trade specifications as well as monitoring and risk-assessment studies.

### Sample preparation

Sampling and sample preparation remain a considerable source of error in the analytical identification of aflatoxins.

There is also variability in the sub-sampling procedure used to grind and mix the sample. Sub-sampling variance is not as large as the sampling variance due to the larger number of particles after comminution. Both sampling and sub-sampling, variances are a function of the sample size, mycotoxin concentration and matrix. Aflatoxins continue to be the only mycotoxins for which theoretical and practical studies of these variabilities have been carried out

particularly on shelled corn, cottonseed, peanuts and peanut products, other nuts, and palm kernels.

The production of mycotoxins is not homogeneous. For instance, Ochratoxin A contamination often occurs in spots during storage in silos. In consequence, a poor sampling procedure of the batch can miss the highly infected grains. Therefore, 10 to 100 incremental samples up to a total of 10 kilos have to be milled before analyses.

Thus, systematic approaches to sampling, sample preparation, and analysis are absolutely necessary to determine aflatoxins at the parts-per-billion level. In this regard, many plans have been developed and tested for some commodities such as corn, peanuts, and tree nuts. 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.

Extraction and clean-up are one of the most critical steps in analytical procedures. Clean-up of the sample extract is generally necessary when the detection limit is low. They can be performed in different ways:

### **Solid-Phase Extraction**

All analytical procedures include three steps: extraction, purification, and determination. The most recent improvement in the purification step is the use of solid-phase extraction. Test extracts are cleaned up before instrumental analysis (thin layer or liquid chromatography) to remove co-extracted materials that often interfere with the determination of target analytes.

### **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 as the AOAC official method of choice to

identify and quantitate aflatoxins at levels as low as 1 ng/g. The TLC method is also used to verify findings by newer and more rapid techniques.

### **Liquid Chromatography**

Liquid chromatography (LC) is similar to TLC in many aspects, including analyte application, stationary phase, and mobile phase. Liquid chromatography and TLC complement each other. 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.

### **Immunochemical Methods**

Thin layer chromatography and LC methods for determining aflatoxins in food are time consuming. These techniques require knowledge and experience of chromatographic techniques to solve separation and interference problems. Through advances in biotechnology, highly specific antibody tests, based on affinities of the monoclonal or polyclonal antibodies for aflatoxins are now commercially available that identifies and measure aflatoxins in food in less than 10 minutes. The three types of immunochemical methods are radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and immunoaffinity column assay (ICA).

### **Mycosep® columns**

The multifunctional clean-up columns consist of packing material containing a variety of adsorbents, e.g. charcoal, celite, ion-exchange resins, and others (Krska & Josephs, 2001). The packing material is housed in a plastic tube between filter discs with a rubber flange on the lower end containing a porous frit and a 1-way valve. When the column is inserted into the culture tube the flange seals tightly, thus forcing the

extract through the packing material of the column to the top of the plastic tube. The Mycosep column enables rapid sample purification within 10 to 30 seconds.

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).

### **Aflatoxicosis and Animal Health**

No animal species is immune to the toxic effects of aflatoxins including humans. However, humans have an extraordinarily high tolerance for aflatoxin exposure and rarely succumb to acute aflatoxicosis. Aflatoxin is a potent toxin. It binds to nucleic acids and also impairs protein formation in the body. There prolonged exposure may cause organ damage and/or cancer. Low levels of aflatoxins in feed - sometimes less than 1 part per million (PPM) - can cause poor growth, interfere with the immune system and result in liver damage and bleeding. High dosages cause acute loss of appetite, depression, hemorrhage, diarrhea and death.

Aflatoxicosis is primarily a hepatic disease. The susceptibility of individual animals to aflatoxins varies considerably depending on species, age, sex, and nutrition. Aflatoxins cause liver damage, alteration in digestion, absorption and/or metabolism of nutrients, decrease milk and egg production, immunosuppression (eg. salmonellosis), in addition to embryo toxicity in animals consuming low dietary concentrations. While mostly young species are susceptible; all ages may be affected but in different degrees for different species. Clinical signs of aflatoxicosis in animals include

gastrointestinal dysfunction, reduced reproductivity, reduced feed utilization and efficiency, anemia, and jaundice.

Animal susceptibility varies with species and age. In general, young animals (pre-weaning to early adolescence) are more affected than adult animals. Species that are highly sensitive are trout, ducks, turkey poult and pre-weaning pigs. Animals that are moderately sensitive include swine, growing turkeys, broiler chicks, pre-ruminant calves, dogs and horses. Animals most resistant are beef feedlot cattle, open cows, and sheep. Aflatoxin generally does not interfere with fertility or cause abortions. However, nursing animals may be affected as a result of the conversion of aflatoxin B1 to the metabolite aflatoxin M1 excreted in milk of dairy cattle. The induction of cancer by aflatoxins has extensively been studied. Aflatoxins B1, M1, and G1 have been shown to cause various types of cancer in different animal species. However, only aflatoxin B1 is considered by the International Agency for Research on Cancer (IARC) as having produced sufficient evidence of carcinogenicity in experimental animals.

The clinical effects of aflatoxins may also be produced by some other diseases or toxins. Veterinary examination for differential causes of disease should always accompany a suspected aflatoxin poisoning case. Characteristic changes occur in liver which can be confirmed by microscopic examination. Aflatoxin metabolites (Aflatoxin M1) can be detected in liver, kidney, urine and milk to confirm exposure and to determine if residues are a problem. Aflatoxin is excreted rapidly from the body, so detectable levels may vanish within a few days to one to two weeks. Complete laboratory submission for diagnosis should include suspect grain or feed, fresh liver and kidney, urine if available, serum for laboratory tests of liver function and gastrointestinal contents. A portion of liver,

kidney and other organs may be examined for histopathological changes.

### Ochratoxin A

Ochratoxin A is mainly produced by *Penicillium verrucosum* and several species of *Aspergillus*. Ochratoxin A (OA) has nephrotoxic and immunosuppressive properties (Murphy et al., 2006) and has been classified by the IARC as a Class 2B carcinogen (IARC, 1993).

Contamination is most commonly associated with cereals, pulses coffee and wine. Unlike other aflatoxins, the organisms which produce ochratoxin A can thrive in the temperatures and humidity.

The intake of OA by contaminated feed may lead to residues in the blood, kidney, livers

of pigs and poultry, and to a lesser extent in muscle tissue, adipose and eggs (Krogh, 1987). Transfer to milk has been demonstrated in rats, rabbits and humans, but little OA is transferred to the milk of ruminants owing to its metabolism by the rumen microflora. Thus, products of animal origin can contribute to the OA- intake of humans (Table 3).

For the quantification of ochratoxin A, the use of thin layer chromatography or HPLC-FLD method for corn and barley using a clean-up of the extract on a C18 column, or by HPLC-FLD and a clean up by IA. AOAC has recently adopted a method for OA in barley. The detection limits reported by HPLC ranged from 0.1 to 2.0 ppb in barley.

**Table 1** Mycotoxins in grains

Mycotoxin	Commodity	Fungal source (s)	Effects of ingestion
Aflatoxin B1, B2	Maize, Peanuts, and many other commodities	<i>Aspergillus flavus</i>	Aflatoxin B1, and naturally occurring mixtures of aflatoxins, identified as potent human carcinogens by IARC. Adverse effects in various animals, especially chickens
Aflatoxin B1, B2, G1, G2	Maize, Peanuts	<i>Aspergillus parasiti</i>	Especially chickens
Ochratoxin A	Barley, Wheat, and many other commodities	<i>Aspergillus ochraceus</i> <i>Penicillium verrucosum</i>	Suspected by IARC as human carcinogen. Carcinogenic in laboratory animals and pigs
Fumonisin B1	Maize	<i>Fusarium moniliforme</i> plus several less common species	Suspected by IARC as human carcinogen. Toxic to pigs and poultry. Cause of equine leucoencephalomalacia (ELEM), a fatal disease of horses
Deoxynivalenol / nivalenol	Wheat, Maize, Barley	<i>Fusarium graminearum</i> <i>Fusarium crookwellense</i> <i>Fusarium culmorum</i>	Human toxicoses India, China, Japan, and Korea. Toxic to animals, especially pigs
Zearalenone	Maize, Wheat	<i>F. graminearum</i> <i>F. culmorum</i> <i>F. crookwellense</i>	Identified by the International Agency for Research on Cancer (IARC) as a possible human carcinogen. Affects reproductive system in female pigs



**Table 2** World volume of export of major agricultural products (Figures in thousand metric Tonnes)

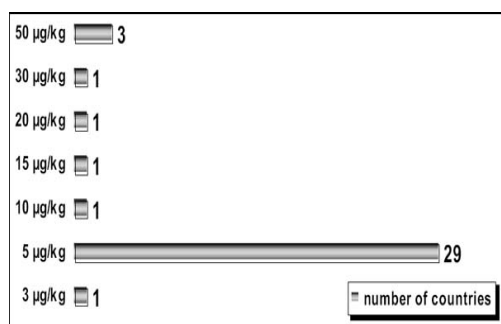
Feedstuff	World	Developing countries
Wheat	102 234	4 622
Rice	11 986	6663
Barley	18203	1 807
Maize	70001	9 352
Millet	215	131
Sorghum	13 653	6 050
Potatoes	5 135	585
Soybeans	28 916	2 906
Groundnuts	790	353
Copra	436	266
Palm nuts	137	122
Oil seed cake & meal	27063	11 301

Source: State of Food and Agriculture 1983, FAO,

**Table 3** Average ochratoxin A (OA) contamination levels of relevant food category and number of samples\*

	n	mean (µg/kg)	n	mean (µg/kg)
Cereals and products	1538	0.20 (a)	5180	0.29
Beer	975	0.02	496	0.03
Wine	1828	0.32	1470	0.36
Grape juice	87	0.39	146	0.55
Cocoa	171	0.55	547	0.24
Pork (edible offal)	3603	0.17	1860	0.20
Roasted coffee (b)	2085	0.62	1184	0.72
Instant coffee (c)	767	0.76	-	-

\*Modified from EU (2002), FAO (1997)



**Figure 10** Worldwide limits for ochratoxin A in cereals and cereal products

In conventional procedures, clean-up is usually achieved by liquid-liquid extraction or adsorption column chromatography, followed by thin-layer chromatography or HPLC. The recent introduction of methods

based on immunochemical principles has had a large impact on analytical methodology for mycotoxins, including the ochratoxins in both purification and determination steps. The ELISA approach for screening and (semi-)quantitative determination and the immunoaffinity column approach for rapid clean-up followed by a conventional instrumental analysis are rapidly gaining attention. The advantages of this approach are the possibility to obtain very clean extracts and to simplify the extraction and clean-up procedures. However, in case of OA analysis in tissues and fluids, further improvements in the methodology are required.

Contamination of cereal commodities with mycotoxins represents a significant hazard to consumer health and has thus received increasing attention from food safety authorities and legislators. Knowledge of the pre- and post-harvest stages in the cereal production chain and particular information regarding the implementation of preventive strategies is being used to develop quality assurance systems for improving food safety. Additional information like ecology of fungal species, breeding for resistance, effective fungicidal, potential for biological control, are also helpful to develop effective preventive strategies for minimising consumer exposure to mycotoxins.

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