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REVIEW

REGULATIONS OF THE EUROPEAN UNION FOR MYCOTOXINS IN FOODS

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Mycotoxins are fungal metabolites that are present in a large part of the world food supply and bear potential threat to food safety. The European Union has been working for several years now on the harmonization of some mycotoxin standards for foods based on toxicological evaluation by its Scientific Committee on Food (SCF). These standards will follow the ALARA (as low as reasonably achievable) principle and will be applied to aflatoxins in the first place followed by ochratoxin A, patulin, and several toxins from *Fusarium* spp. Detailed regulations (limits, methods of sampling, and analysis) for aflatoxins B, G, and M on the commodities at stake have been worked out. At the moment, a variety of interrelated approaches as to the intake and toxicity of ochratoxin A are being discussed to assess the hazards and to determine the associated risk of this toxin. The other toxins like patulin and the ones derived from *Fusarium* spp. such as zearalenon and fumonisins will be discussed in the years to come.

Key words:
aflatoxins, analytical procedures, fumonisins, ochratoxin A, patulin, sampling methods, zearalenon

Molds can produce chemicals known as mycotoxins. There is a range of different types which can contaminate a wide variety of foods. Aflatoxins can be contaminants of cereals such as maize, spices such as paprika, dried fruits such as figs, and nuts such as peanuts and pistachios. Aflatoxins can contaminate animal feed; when given to cattle it can find its way to dairy foods. Ochratoxin A can be found in a variety of cereals; it is also found in coffee beans and will partly survive roasting. Patulin is associated with molds on fruit. If, for example, poor quality apples are used in manufacture, this mycotoxin can contaminate fruit juice products and puree. Mycotoxins present a potential hazard to human health even when present in very low concentrations. The levels of some mycotoxins in food and animal feed are therefore controlled by regulation in more than 90 countries worldwide.

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BACKGROUND OF THE REGULATIONS OF THE EUROPEAN UNION

The European Union (EU) has been working for several years on the harmonization of mycotoxin standards for foods based on toxicological evaluation by its Scientific Committee on Food (SCF). These standards will follow the ALARA (as low as reasonably achievable) principle and will be applied to the major mycotoxins.

AFLATOXINS

Mycotoxins have toxic properties and the most notorious ones, the aflatoxins, are liver carcinogens to humans. They occur on cereals, peanuts, other nuts, dried fruit, milk (aflatoxin M_1), and spices. In 1994, the SCF expressed an opinion on aflatoxins and also on ochratoxin A (OTA) and patulin (1). It was not a surprise that the EU came with a regulation (EC) No. 1525/98 of 16 July 1998 (2) amending Commission Regulation (EC) No. 194/97 of 31 January 1997 (3) setting maximum levels for certain contaminants (in this case aflatoxins) in foodstuffs that was earlier sent as a draft to the World Trade Organization (WTO) and registered as notification (G/SPS/N/EEC/51) by the EU on 8 January 1998.

Beside the low level setting, based on a tolerable exposure of 1 ng/kg b.w./day or less, sampling procedures and criteria for methods of analysis are described in the Commission Directive 98/53/EC (4) of the same date. In its draft, the regulation was highly criticized by the producing countries who seemed to hold laxer views on food safety and consumer protection and rely on the confusing factors the Joint Expert Committee on Food Additives (JECFA) (5) brought about at its 49th meeting in 1997 in which hepatitis B and C were mentioned as confounding factors. The basis of the fundamentally different philosophy of the Europeans is not only the confounding JECFA report that did not take into account that hepatitis virus is prevalent in Eastern Europe and the newly independent states of the former Soviet Union (5–12%). The estimated annual incidence of hepatitis B infection In Central Asia and some Central and Eastern European countries is 520 infections per 100,000 people. These countries will be regarded as having an intermediate or high endemicity, according to the World Health Organization (WHO). Furthermore, a report from 1996 issued by the United States Department of Agriculture (USDA) that summarized the year-by-year (1975 to 1992) aflatoxin distributions for more than 600,000 lots of peanuts tested for aflatoxin has been taken into account. More than 90% of the lots of raw peanuts contained up to 15 μg/kg or less of total aflatoxin and 85% of the lots contained aflatoxins at levels below 10 μg/kg. Blanching and electronic eye color sorting brought in almost all cases the level down to below 5 μg/kg of total aflatoxins. The EU is of the opinion that the ALARA principle can easily be met with some preventive policies such as good agriculture, manufacturing, and storage practices. The levels are more in agreement with those set and practiced for feed and aflatoxin M_1 in milk (0.05 μg/kg) in the Union and now also have been proposed to the Codex Commission.

OCHRATOXIN A

In the case of ochratoxin A, commodities such as cereals, cereal products (including beer), coffee, wine, pork, products containing pig blood/plasma, pulses, and spices are considered. An updated risk assessment on ochratoxin A published in 1996 based on a rat study by the National Toxicology Programme (NTP) (6) showed high tumor and metastasis incidence while the human dietary exposure associated with the Balkan Endemic Nephropathy was also noticed outside the Balkans in countries like Algeria and Tunisia. A recent SCF report (7) underpins the NTP study. A regulatory level of 5 μg/kg for cereals and cereal products intended for direct human consumption is proposed.

PATULIN

JECFA established for patulin a provisional tolerable daily intake (PTDI) of 0.4 μg/kg/b.w. The regulatory level of 25 μg/kg seems to be justified since patulin is found in many types of fresh or processed fruit and vegetables such as juices, sauces, compotes, and jellies. Especially apple-juice and sauce seem to contribute to the problem.

OTHER MYCOTOXINS DERIVED FROM *FUSARIUM*

Regulatory limits for other mycotoxins mainly derived from *Fusarium* fungi such as the trichothecenes T-2 toxin, HT-2 toxin, deoxynivalenol (DON) and nivalenol (NIV) and some other toxins such as zearalenon (ZON), fumonisins, fusarin C, fusarenon-X, diacetoxyscirpenol (DAS), and moniliformin have not reached beyond the discussion stage as yet, although validated methods of a0nalysis have been worked out in several programs of the Standard Measurement and Testing, governed by DG XII and standardized by the European Committee for Standardization (CEN) in its working group on biotoxins (CEN / TC 275 / WG 5 – Biotoxins) within the Technical Committee 275.

MAXIMUM LEVELS

The European Commission services had to stress the need for encouraging preventive action such as good agricultural practice, research into techniques for contamination prevention, improved training, good storage conditions and the use of improved sorting procedures. The ideas from several member states on levels (almost

all different ranging from several μg/kg to 20 μg/kg) and specific sampling procedures (UK, Denmark, Norway, and The Netherlands) encouraged the Expert Committee »Agricultural Contaminants« to come out with the following maximum permitted levels (μg/kg) that have been fixed for aflatoxin B_1 and total aflatoxins (B_1, B_2, G_1, and G_2) in different commodities:

1) Groundnuts, nuts and dried fruit[a] and processed products thereof, intended for direct human consumption or as an ingredient in foodstuffs: 2 (B_1)/4 (total);

2) Groundnuts[a] to be subjected to sorting or other physical treatment before human consumption: 8 (B_1)/15 (total);

3) Nuts and dried fruit[b] to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs: 5 (B_1)/10 (total);

4) Cereals (incl. buckwheat, *Fagopyrum* sp.) and processed products thereof, intended for direct human consumption or as an ingredient in foodstuffs: 2 (B_1)/4 (total);

5) Cereals (incl. buckwheat, *Fagopyrum* sp.) to be subjected to sorting or other physical treatment, before human consumption or use as an ingredient in foodstuffs: no specific limit foreseen before 1 July 1999 other than the ones mentioned under 4);

6) The maximum permitted level for aflatoxin M_1 in milk (raw milk and milk for the manufacture of milk-based products and heat-treated milk) has been fixed at 0.05 μg/L.

Decontamination of products by chemical treatment is forbidden as well as any blending of contaminated products with good quality products in order to reach the admitted level for human consumption. Clear labeling is compulsory for those products to be submitted to sorting or other physical treatment to lower aflatoxin contamination prior to human consumption. The four main aflatoxins (B_1, B_2, G_1, and G_2) usually occur together in varying ratios but normally aflatoxin B_1 is the major component. Because aflatoxin B_1 is by far the most toxic compound of all aflatoxins, setting a separate (lower) level for aflatoxin B_1 offers an extra guarantee for public health (the two-tier approach taken from EEC foodstuffs regulation). Aflatoxin M_1 is believed to be significantly less carcinogenic than aflatoxin B_1. However, the intake of milk and milk products by humans can be considerable, particularly among infants and young children.

METHODS OF SAMPLING AND SAMPLING PREPARATION

The homogeneity of the concentration of mycotoxins in products is a key factor in establishing regulatory sampling criteria. Distribution can be uneven, as is the case with aflatoxins in peanuts. The number of contaminated peanut kernels in a lot is usually very low, but the contamination level within a single kernel can be very high. If insufficient care is taken in representative sampling, the mycotoxin concentration in an inspected lot may, therefore, easily be wrongly estimated.

Also, consumption of peanuts may lead to accidental ingestion of a high single dose of aflatoxins instead of chronic intake at relatively low levels. The situation is similar with most other products susceptible to mycotoxin contamination such as

[a] on edible part
[b] reconsidered before 1 July 1999 after technological justification

cereals in which the order of scale (bulk) plays an important role. The risk to both consumer and producer must be considered when establishing sampling procedures and it has been in the focus of international concern for a long time. Ceaseless discussions have sought to find a harmonized international approach to the problem. The heterogeneous distribution of mycotoxins in relevant commodities can lead to real problems of observing established levels unless adequate sampling plans are used.

A crucial point in the development of sampling plans seems to be the balance between the consumer's and the producer's risk. With this respect, importing/consuming and exporting/producing countries may take a different view. The US plans seek to balance such risks (compare weight and measure regulation). The Europeans have a different approach not only with their weight and measure regulation within the Community, but more philosophically, by applying the Pareto principle (the principle of the »vital few and the trivial many«), well known in quality improvement programs. If the Europeans want to protect their consumers, it is obvious that a balance of risk is out of the question and that instead of a 50/50 risk balance a risk ratio 20/80 is more appropriate. The UK aflatoxin regulation on sampling (8), the Dutch »Code of Practice for Peanuts« (9), the existing legislation on sampling in Denmark and Norway, and the views on sampling in Germany formed the basis for the sampling procedure in the EU Directive 98/53/EC (4). On the other hand, Europeans adopted none of the technical recommendations published in the FAO Food Nutrition Paper No. 55 »Sampling plans for aflatoxin analysis in peanuts and corn« (10) in 1993. The evaluation of sampling plans used in the US, UK, and The Netherlands found the producer-friendly approach not stringent enough in testing raw shelled peanuts for aflatoxin (11). The EU proposed sampling plans for different commodities with an aim to encompass heterogeneous distribution, reach the performance level of less complicated sampling procedures, and respond to requirements related to the dependence of a sample size on the lot size. The EU gives quite detailed definitions as to what is meant under: »(sub)lot«, »incremental, aggregate, and laboratory sample«, and gives general and specific provisions shown in Table 1 for aflatoxin in groundnuts and in Table 2 for aflatoxin in groundnuts and other involved commodities.

Table 1 *Number of incremental samples to be taken depending on the weight of the lot*

GROUNDNUTS	
Lot weight (tonnes)	**Number of incremental samples**
<0.1	10
≥0.1 – <0.2	15
≥0.2 – <0.5	20
≥0.5 – <1.0	30
≥1.0 – <2.0	40
≥2.0 – <5.0	60
≥5 – <10	80
≥10 – <15.0	100

Table 2 *Subdivision of lots into sublots depending on product and lot weight for sampling*

Commodity	Lot weight (tonnes)	Weight or No. of sublots	No. of incremental samples	Aggregate sample weight (kg)
Dried figs and other dried fruit	≥15	15–30 t	100	30
	<15	–	10–100	≤30
Groundnuts, pistachios, Brazil nuts, and other nuts	≥500	100 t		
	>125 and <500	5 sublots	100	30
	≥15 and ≤125	25 t	100	30
	< 15	–	10–100*	≤30
Cereals	≥1500	500 t	100	30
	>300 and <1500	3 sublots	100	30
	≥50 and ≤300	100 t	100	30
	<50	–	10–100*	1–10

*depending on the lot weight

Sampling procedure

- Each sublot must be sampled separately;
- Number of incremental samples: 100. In the case of lots under 15 tonnes, the number of incremental samples depends on the weight of the lot, and ranges from a minimum of 10 to a maximum of 100 samples;
- Weight of the aggregate sample is 30 kg. It has to be mixed and divided in three equal subsamples of 10 kg before grinding (the division in three subsamples is not necessary if the equipment which is able to homogenize a 30 kg sample is available). In cases where the aggregate sample weights are under 10 kg, the aggregate sample must not be divided into three subsamples;
- Laboratory sample: a subsample of 10 kg (each subsample must be separately and finely ground and mixed thoroughly to achieve complete homogenization);
- Provisions for acceptance of a lot or sublot: acceptance if the aggregate sample or the average of the subsamples conforms to the maximum limit; rejection if the aggregate sample or the average of the subsamples exceeds the maximum limit.

Sample preparation

Several precautions have to be taken: shielding from ultra-violet light; calculation of proportion of shell/kernel; treatment of sample as received in the laboratory (mix, homogenize); subdivision of samples for enforcement and defense purposes.

Sampling procedure for milk and milk products

Sampling of these products has to be carried out according to the Commission Decision 91/180/EEC of 14 February 1991 (12) laying down certain methods of analysis and testing of raw milk and heat-treated milk and with Commission Directive 87/524/EEC of October 6, 1987 (13) laying down Community methods of sampling for chemical analysis for the monitoring of preserved milk products. The minimum

number of incremental samples is 5 and a lot is accepted if the aggregate sample conforms to the maximum limit.

METHODS OF ANALYSIS

In order to apply control on mycotoxins and to set standards for trading purposes it is necessary to have validated methods for the analysis of mycotoxins. Validated methods are those which are to be tested by collaborative studies. It is important to establish performance characteristics such as repeatability and reproducibility, recovery, and limit of detection (14). The methods must be proven effective at levels below the regulatory limits and appropriate for the target foodstuff. The EU asked the European Committee for Standardization (CEN) in the early nineties to establish methods of analysis in the field of mycotoxins within the »Technical Committee 275 – Food analysis – Horizontal methods«. The working group »CEN/TC 275/WG 5 – Mycotoxins« was established in 1992. Five years later it expanded the scope to include phycotoxins (algal toxins) and is now to be named »CEN/TC 275/WG 5 – Biotoxins«. Since the mandate of the working group was to select and elaborate validated methods as horizontally as possible in the field of mycotoxins, it was not an easy task to fulfill these desires, the more so because no harmonized legislation in the EU existed at that time and matrices varied. The working group first established a set of criteria for analytical methods for mycotoxins and laid them down in a technical paper of CEN similar to the general criteria in CC/MAS and directive 85/591/EEC (15). A limited modified format based on this paper as shown in Table 3 appeared recently in Annex II of directive 98/53/EC 4.

Table 3 *Specific criteria in Commission Directive 98/53/EC (4)*

Criterion	Concentration range (µg/kg)	Recommended value	Max. permitted value
Blanks	All	Negligible	
Recovery AFM_1	0.01 – 0.05 >0.05	60 to 120 % 70 to 110 %	
Recovery AFB_1 and AFT	<1.0 1 – 10 >10	50 to 120 % 70 to 110 % 80 to 110 %	
Precision RSDR	All	from Horwitz equation	2 x value from Horwitz equation

Precision RSDR may be calculated as 0.66 times the precision of RSDR at the concentration of interest.
Values to apply to both aflatoxin B_1 (AFB_1) and sum of $B_1+B_2+G_1+G_2$ (total aflatoxin or AFT).
If Σ aflatoxins $B_1+B_2+G_1+G_2$ are to be reported, the response of each analytical system is required.
Detection limits that are not stated as RSDR values are given at concentration of interest.
The precision values are calculated from the Horwitz equation, i.e.: $RSDR^{**} = 2^{(1-0.5 \log C^{*})}$
* C is the concentration ratio (i.e. 1 =100g / 100g, 0.001 = 1,000 mg/kg).
** RSDR is RSD calculated from reproducibility conditions [(SR/x) × 100].

The working group reported to the EC that it was necessary to develop validated methods in the field since most methods reported in literature were invalid for all kinds of reasons. The EC responded adequately to CEN's desire by publishing in the Official Journal an »open call for proposals« for validation of analytical methods to determine the content of aflatoxins, ochratoxin A, and patulin in food of vegetable origin, and of aflatoxin M_1 in liquid milk. It remains unclear why the EU at that moment did not consider other mycotoxins such as *Fusarium* (several trichothecenes, fumonisins, and zearalenone) and other commodities such as corn and green coffee. Nevertheless, the establishment of validated methodology at lower limits and for a wider range of matrices was already a task that required enormous effort and funds. The close cooperation between CEN, the SMT, and scientists and experts in the field in the EU member states eventually brought to the contract SMT-CT96-2045 (16) between SMT and the principal Co-ordinator (Ministry of Agriculture, Fisheries and Food, CL Food Science Laboratory, Norwich, UK) Government Laboratory which was signed after the International Union of Pure and Applied Chemistry (IUPAC) conference in Rome in 1996. Eight partners (3 government, 1 industry-funded, 3 small/medium enterprises, and the EC's Joint Research Center in Ispra, Italy) and about 50 participating laboratories from all over the EU took part in intercomparison studies which have recently been completed. They now entered the phase of drawing up the methods in the language of CEN (prEN's). The validated methods of analysis to be standardized through this funded 4th framework SMT Program are HPLC methods with mainly immunoaffinity clean-up for:

1) aflatoxins B and G in peanut butter, pistachios, figs, and paprika;
2) aflatoxin M_1 in liquid milk;
3) ochratoxin A in barley and roasted coffee;
4) patulin in apple juice and apple puree.

Fusarium toxins still lack official reference methods recommended by international organizations. Both HPLC (with fluorescence detector) and TLC (with fluorodensitometry) are in use. Calls for validation of analysis of trichothecenes and fumonisins have been negotiated quite recently. For the trichothecenes, which can be divided in types A an B, the dominating method with relatively low limit of detection and good selectivity is the capillary gas chromatography with electron capture detection (GC-EC).

Calls for analysis validation for fumonisins (B_1 and B_2) expired on 15 May 1997, the contract (SMT 4-CT97-2193) (17) was signed in February 1998. The commonest method is based on HPLC determination. No call for zearalenone has been published in the Official Journal of the EU, although an open call has been granted recently as EC-SMT project Contract No. SMT 4-CT98-2228 (18) and JECFA will evaluate it at the fifty-third meeting in June 1999.

Elisa kits are available for some of the *Fusarium* toxins. In general one can say that thin layer chromatography has been replaced by HPLC that has become the reference method of choice. This technique rapidly expands to the analysis of other discovered mycotoxins and secondary metabolites of fungi. As mycotoxins were discovered in a variety of matrices, the need for cleanup procedures to eliminate interfering substances prior to analysis became more necessary for the variety of expanding technologies for mycotoxins. New techniques for screening purposes such as

Elisa and biosensor techniques such as Surface Plasma Resonance might replace HPLC because they are easier to use and need less qualified labor.

CONCLUSIONS

Mycotoxins have a negative impact on food safety, food trade and consumer's health. A firm stand on mycotoxins is in line with the EU's policy on food safety. The EU is well on its way to establish a firm, strict, and complete policy on mycotoxins based on sound scientific and risk assessment principles. The regulations and the methods of analysis and sampling have been improved and updated to the state of the art and have become suitable for the Codex purposes.

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Sažetak

UREDBE EUROPSKE UNIJE U SVEZI S MIKOTOKSINIMA U HRANI

Mikotoksini su potencijalno opasni gljivični metaboliti koji su prisutni u mnogim prehrambenim proizvodima. Europska unija već nekoliko godina radi na usklađivanju normi za mikotoksine u hrani koje se zasnivaju na toksikološkoj procjeni Znanstvenog odbora za hranu (engl. *Scientific Committee on Food*). Ove će norme slijediti načelo razumnog minimuma te će se odnositi prije svega na aflatoksine, okratoksin A, patulin i nekoliko drugih toksina iz vrsta *Fusarium*. Upravo se donose precizne uredbe (glede ograničenja, metoda uzorkovanja i analize) u svezi s aflatoksinima B, G i M. Trenutno se raspravlja o različitim međusobno povezanim pristupima unosu i toksičnosti okratoksina A kako bi se procijenila opasnost i rizici vezani za ovaj toksin. Tek slijedi rasprava o patulinu odnosno toksinima iz vrsta *Fusarium* kao što su zearalenon i fumonizini.

Ključne riječi:
aflatoksini, analitički postupci, fumonizini, metode uzorkovanja, okratoksin A, patulin, zearalenon

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