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Food safety assessment of the mycotoxin and pesticide residue contamination of our foods, Part 2. Mycotoxins

Keywords: mycotoxins, Codex Alimentarius, consumer's exposure, quality control, sampling

1. SUMMARY

The occurrence, legal regulation, quality requirements for sampling and analysis of mycotoxins occurring in food and feed in Hungary are presented. Furthermore, the current practice is evaluated. To complement the test results of NÉBIH, the WESSLING Hungary Ltd. and the University of Kaposvár provided detailed analytical results for the assessment of consumers' exposure. Besides, the BIOMIN Ltd. and the SGS Hungária Ltd. shared their annual summary data, the Gabona Control Ltd. made available partial test results for preparing this paper. Based on the available data and information, the exposure of Hungarian consumers to Aflatoxin M1 and DON is estimated, and recommendations are made for facilitating the actions aiming to reduce the contamination of our food.

Taking into account the extensive national test results and international information, we concluded that:

- the exposure of consumers to Aflatoxin M1 and DON may exceed the toxicological reference values from time to time, posing a risk to consumers' the health;
- there is a need for coordinated comprehensive actions by all interested parties for the reduction of *Aspergillus* and *Fusarium* fungi infections in cereals and the resulted toxin exposure.

1.1. Abbreviations used in this paper:

ADI: Acceptable Daily Intake
ALARA: As Low As Reasonably Achievable
Bw (tt): Bodyweight [kg]
CAC: Codex Alimentarius Commission
CCCF: Codex Committee on Contaminants in Food
CCFA: Codex Committee on Food Additives
DNA: deoxyribo nucleic acid
EC: European Commission
EDI: Estimated Daily Intake
EFSA: European Food Safety Authority
EPC: European Parliament and Council
EU: European Union
FAO: Food and Agriculture Organization of the United Nations
HBV: Hepatitis-B virus
HPLC: High Pressure (Performance) Liquid Chromatography
IARC: International Agency for Research on Cancer
ISO: International Organization for Standardization
JECFA: FAO/WHO Joint Expert Committee on Food Additives and Contaminants
LOQ: Limit of Quantification

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ML: Maximum Limit [mg/kg]
MS/MS: Tandem Mass Spectrometry
NOAEL No Observed Adverse Level [ppm in feed expressed also in mg/kg bw per day]
NOEL: No Observed Effect Level
OECD: Organisation for Economic Cooperation and Development
PMTDI: provisional maximum tolerable daily intake
QC: Quality Control
SFC: European Commission Scientific Committee on Food
TDI: tolerable daily intake (it is used for agents that are not deliberately added to food)
USA: United States of America
US FDA: US Food and Drug Administration
UV: ultraviolet.

2. Introduction

The National Population Roundtable urged the development of a strategic action plan at Governmental level to reduce the adverse effect of agricultural chemicals and toxins on human health and fertility. The call identified mycotoxins as one of the major sources of contamination.

The opinion poll conducted by the European Food Safety Authority (EFSA) in 2019 **[1]** revealed that 10-29% of the population of the Member States is concerned about the mycotoxin contamination in food. Further details are published in Part 1 of our paper.

In this article, we present data on occurrence and toxicological effects of mycotoxins, introduce the testing system of mycotoxin contamination of food and summarise the results of laboratory analyses. Based on the results, the exposure of consumers to mycotoxins is evaluated and recommendations are made to protect the health of consumers.

2.1. Characterization of mycotoxin contamination and the regulation of their permissible maximum concentrations

Mycotoxins are secondary metabolites of various fungi infecting the plants. They may occur not only during the growing season, but further propagate throughout shipping and storage under unfavourable conditions. The hot, dry weather, inappropriate agricultural and storage practices provide favourable conditions for the formation of aflatoxins and mycotoxins, in general **[2, 3]**.

The aflatoxins, deoxinivalenol (DON), zearalenone (F-2 toxin), T-2 and HT-2 toxins are the most significant ones from a practical point of view. They are produced by *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nominus, Fusarium graminearum, F. verticillioides, F. proliferatum, F. culmorum*). The group of trichothecenes consists of over 50 structurally related compounds **[4]**.

Until the end of the 19th century, the contamination due to Fusarium toxins, and aflatoxins had posed a risk mainly present under tropical and Mediterranean climatic [5]. However, in recent years the aflatoxins have appeared in Central European countries, namely in Serbia **[6, 7]** and Hungary as well.

Mycotoxins are generally persistent and heat resistant compounds with various complex chemical structures. Aflatoxins and other mycotoxins present in raw agricultural commodities and feed **[8]** are transferred into the food chain and they are detectable in milk **[9, 10]**, eggs, meat and edible offal **[11]**. The metabolite of AFB_1, namelyAFM_1 concentrates during the cheese production process **[12]** and is present in mother milk at a similar concentration as in the cow milk **[6, 13-15]**. The degradation of mycotoxins to less toxic derivatives is practically negligible during food processing. The change of mycotoxin concentration during food processing and their distribution in the processed food products are discussed in numerous publications and reviews **[16-28]** and are not repeated in this article.

In addition to the 17 toxins regularly tested in food and feed (12 of which are regulated by the European Union (EU) or national legislations), the researchers identified over several hundred mycotoxins. The most frequent potential human toxic impacts comprise of carcinogenic effects (aflatoxins, ochratoxin A, fumonisines, patulin), developmental disorders (zearalenon (F-2 toxin), ochratoxin A), infertility (zearalenone, trichothecenes), decreased resistance, immunosuppression (trichothecenes), neurodegenerative diseases (ochratoxin A, fumonisines) **[29, 30]**.

Several international organisations (e.g. JECFA, IARC, SFC and EFSA) deal with the toxicological **[31-35]** evaluation of mycotoxins. The current acceptable daily intake reference values are listed in **Table 1**. The references provide detailed information on the adverse health effects of the listed toxins. The exposure of Hungarian consumers was evaluated in several publications **[4, 36-39]**. The earlier publications were summarised by Kovács **[29]**.

The permitted maximum concentrations of mycotoxins in food in the EU are listed in the regulations No. 1881/2006 **[46]** and 165/2010 **[47]**, while the maximum limits in feed are specified in the 64/2012 (VII.3) VM decree [48] based on the 2002/32/EK directive. Various maximum limits are set for food consumed directly by infants and young children, as well as for feed intended for various animal species and young animals. Taking into account the local circumstances and the ALARA principles, the national authorities may establish different maximum limits. For instance, the ML for AFM_1 in cow milk and baby food is 50 ng/kg and 25 ng/kg, respectively in the EU, while Austria and Switzerland set 10 ng/kg for baby food.

Nearly one hundred countries issued guidance values or maximum limits for different mycotoxins until 2003 [49]. The Codex Alimentarius Commission published the recommended maximum limits for food in international trade **[50]**. Additional limits are published by the Codex Committee on Contaminants in Food (CCCF) [51]. The US FDA guidance documents emphasise that the "action levels and tolerances are established based on the unavoidability of the poisonous or deleterious substances and do not represent permissible levels of contamination where it is avoidable. The blending of a food or feed containing a substance in excess of an action level or tolerance with another food or feed is not permitted, and the final product resulting from blending is unlawful, regardless of the level of the contaminant" **[52-55]**. Similar principles are included in the EU regulations, for example in 1881/2006 **[46]**.

The distribution of mycotoxins is very heterogeneous within the fields or in the harvested crops. One thousand times higher concentration can be measured in close vicinity of infected seeds, while it is possible that hundreds of thousands of seeds do not contain detectable contamination **[56-58]**. Whitaker determined the AFB_1 concentrations of a lot by taking 16 independent samples of 1.1 kg each **[59]**. **Figure 1** illustrates the results. The distribution of aflatoxins in corn grains, nuts, peanuts and soybeans could be best modelled with negative binomial distribution **[57, 60]** which also gave the best fit for fumonisines in corn grains **[61]**. The lognormal function described best the distribution of ochratoxin A in wheat and coffee beans, and DON in barley, corn and wheat grains **[63, 64]**. Normal distribution could be used to characterize the distribution of aflatoxins and OTA **[65]** in ginger powder. Based on their research for decades, Whitaker and coworkers developed an Excel worksheet, which can be used to determine the operation characteristic curves for sampling and analyses of 29 commodity-toxin combinations with various input parameters **[66]**.

The evaluation of the testing results clearly shows that the sampling is the major contributor (>90-97% of total variance) to the combined uncertainty (random error) of the whole determination process (from sampling to quantitative determination) **[67-70]**. The total variance is the function of mycotoxin concentration **[71]**. The importance of representative sampling is emphasized in several publications **[62, 67, 68, 72-76]**. Considering these findings, the European Union **[77, 78]** and several national authorities strictly regulate the method of sampling and issue guidance documents for their correct implementation **[79, 80]**. **Figure 2** shows the division of the aggregate sample into replicate samples **[79]**.

Due to physical constraints, it is not possible to take representative samples from bulk materials stored in large stores or silos. In such cases, samples should be taken preferably with an automatic sampler at the time of discharging of the product. A representative sample can also be obtained by withdrawing cross-section portions from the conveyor belt at regular intervals and combining the sub-samples **[77,78,81,82]**.

The uncertainty of the measured values also includes the effects of sample size reduction, comminution and quantitative determination. The error of sample size reduction can especially be significant (90-94% of total variance excluding sampling), as in cases of lots over 1 tons the 10 kg aggregate sample obtained from 100 primary samples cannot be properly homogenised manually at the sampling site. Furthermore, the particles of the sample material can be segregated during sample size reduction, shipping and storage. Therefore, to obtain reliable results, the whole aggregate sample should be transported to the laboratory, where the whole sample can be grounded with a suitable equipment to < 2-3 mm diameter and after applying proper sample divider can be further grounded to ∅ < 1 mm. The 25-50 g test portions to be extracted should also be obtained by passing the ground material through suitable sample divider [85-87]. The newer models of grinders (e.g. Retch, Romer, Dickens) can be used to process the 10 kg sample in one step. The slurry mixing proved to be very efficient for the comminution of granular materials. The Silverston mills can accommodate 10-30 kg grains and produce a statistically well-mixed matrix **[69, 75, 88-90]**.

We consider it as a serious professional error, when the performance characteristics of analytical methods are determined based on spiking 5-30 g test portions, and the repeatability as well as the reproducibility of the method is reported based on these results. Furthermore, some authors even claim, based on the recovery tests, that the method is suitable for sensitive detection of mycotoxins from the extraction of 1-2 g test portions without proving that they properly represent the whole laboratory sample. Some publications report the sensitivity of the method in ng/ml extract **[91-93]**. It is obvious that such results do not provide any reliable information about the practical applicability, accuracy and uncertainty of the measurements. Such methods cannot be used for testing compliance with legal limits or assessment of consumer's exposure.

3. Testing the compliance of marketed products

The maximum limits (ML) defined by legal documents refer to the average concentrations of the contaminants in the samples taken according to the corresponding official sampling procedures. If the measured average concentration, taking into account the measurement uncertainty, does not exceed the legal limit the commodity can be marketed. Though no realistic conclusion can be drawn regarding the average contamination of the sampled lot based on a single sample. If replicate samples are taken from a

lot, there may be large differences in the results of the analyses as shown in **Figure 1**. If the mycotoxin concentration measured in one sample is equal to the legal limit, a substantial proportion of additional samples may contain the contaminant at higher concentrations due to the heterogeneous distribution and the uncertainty of the measurements.

Figure 3 shows the concentration distribution of AFB_1 and the probability of compliance of the sampled lot containing an average of 5 μg/kg AFB_1. The figure illustrates the importance of the mass of laboratory sample. When 1, 2 or 10 kg sample is ground and 30 g test portion is extracted, the probability of acceptance of the lot would be 27% (100-73), 33%, or 37%, respectively. Under the same conditions, if the same lot was resampled, the probability of detection of 10 μg/kg AFB_1 would be 48%, 40% és 27%, respectively. However, further 1 kg samples containing 20 μg/kg AFB_1 could be found with a 19% probability!

Recognising the limitations of sampling, taking the responsibility for the quality of their products in some countries certain manufacturers or distributors set their internal acceptance limit for incoming products at a much lower level than the legal limits for assuring compliance of their goods when they are marketed. If a corn sample of 10 kg is analysed as described above and 2 μg/kg accept limit is applied, the marketed produce will satisfy the 5 μg/kg ML with a 66% probability. It means that 66% of 10 kg portions of the lot will contain ≤ 5μg/kg AFB_1 contamination. We point out that in case of a pre-marketing control, one 10 kg sample taken from the lot should contain ≤ 0.3 μg/kg AFB_1 to assure 95% compliance. This strict precondition can be "softened", if 3 independent 10 kg replicate samples were taken and none of them would contain AFB_1 above 2.5 μg/kg (**Figure 4.**).

Note: storing lots with different contamination levels can significantly increase the heterogeneity of chemical substances and the uncertainty of sampling, moreover facilitate the propagation of fungi infection, therefore it should be avoided.

The NÉBIH laboratories, working in compliance with the quality assurance provisions of ISO/IEC 17025 Standard (ISO17025 in the followings), perform the official control of mycotoxin contamination of food and feed for which ELISA, Biochip Array Technology, HPLC-fluorimetry, UV detection, and HPLC-MS/MS methods are used. Similar methods were also used by the other laboratories which provided their results.

The specialised national reference laboratories of NÉBIH regularly take part in European proficiency tests. The samples to be tested are prepared from naturally contaminated materials. Some additional toxins may be added to the test samples. For instance in 2017, one of the samples was corn semolina (grit) in which deoxynivalenol, zearalenone, fumonisin B_1, fumonisin B_2, (sum of B_1 + B_2), T-2 toxin, HT-2 toxin, (sum of T-2 + HT-2), aflatoxin B_1, B_2, G_1, G_2 (B_1 + B_2 + G_1 + G_2), enniatin B, enniatin B_1 and beauvericin had to be identified and quantitatively determined with both chromatographic and immunochemical methods. The reported results were evaluated separately for each toxin according to ISO 13528:2015. The robust statistical method used for the evaluation is described in the report [94]. The number of toxins identified by the participating 28 laboratories depended on the laboratories and the methods used. The calculated Z-values (see part 1) widely varied (-5 – >+5).

The methods used for the determination of mycotoxins have been reported in several thousand publications. They were summarised by several authors based on different criteria **[95-99]**. The operating principles and performance characteristics of various detection methods are presented in a separate publication **[93]**.

3.1. Results and their evaluation

The mycotoxins in food and feed are determined by several institutes and laboratories. Responding to our call detailed results were provided by NÉBIH and WESSLING Hungary Ltd., summary data were given by BIOMIN Ltd., SGS Hungária Ltd. and the Gabona Control Ltd. offered only limited information. The latter laboratories, accredited according to ISO 17025 standard, carry out the tests on the samples provided by their clients.

NÉBIH conducted 43,480 tests for 22 toxins including their combinations from 2008 to 2018. The results are summarised in **Table 2**. Those used for risk assessment are given in **Table 3**.

The WESSLING Hungary Ltd. performed 59,888 tests for 18 toxins and their combinations between February 2017 and March 2018. Some of them are summarised in **Table 4**.

The 'Mycotoxins in food chain research group' of University of Kaposvár made available the results of 122 tests for AFM_1 in milk. Ten samples contained AFM_1 contamination above the limit of quantification with the highest contamination of 31.6 ng/kg.

The results of tests carried out in corn and winter wheat by SGS Hungária Ltd. are given in **Table 5**. For example, the occurrence of aflatoxin and DON contamination in Hungary are shown in **Figures 5-7**.

In 2017, 67% of 39 wheat samples tested by BIOMIN Ltd. contained total aflatoxin above the limit of quantification. The results of the analyses of 54 wheat samples tested in 2019 are summarized in **Table 6**.

The concentrations of DON and AFB_1 reported by the laboratories are of similar magnitude, but the average concentrations reported by NÉBIH are slightly higher.

Generally, the concentration of mycotoxins is very low or below the LOQ in most of the samples. While high concentration occurs at low frequency in a very wide range.

In Hungary, there are large differences in the *Aspergillus* and *Fusarium* infection depending on the location and year.

3.1.1. Evaluation of the results of the estimation of the consumers' exposure

The exposure of consumers was calculated for DON in white flour and for AFM_1 in cow milk from the results presented in the previous section and the consumption data obtained during the dietary intake survey conducted in 2009 [100]. Only those recorded as white flour and/or milk consumers during the survey were considered. The non-detected contamination was calculated with 0.5 LOQ value in both cases.

The DON exposition from white flour was calculated from the sum of white four and bakery products. The flour equivalents of bakery products were taken as 70%, while in the case of homemade cakes the proportion of flour was 50% by dry weight. Though the wheat-based products are the major source, the DON content of other cereal products may substantially increase the total exposure.

The AFM_1 exposition was calculated from the combined consumption of milks of different fat contents. The contribution of various processed milk products (cheese, curd, etc.) was not considered, as there were not sufficient measurement results available.

Since AFM_1 is carcinogenic, acceptable daily intake cannot be defined. The health impact of AFM_1 intake can be characterised with the frequency of liver cancer cases. There is no official data published for the frequency of HBV cases in Hungary. According to some estimates, the infection rate of the adult population is between 0.5 and 1% **[101]**. We consider 0.7% to be the best estimate **[102]**. According to FAO/WHO JECFA (**1. Table**), the annual average cancer cases for 100,000 persons can be calculated as:

$$Ri_{ave} = (0{,}03 \times 0{,}007 + 0{,}001 \times 0{,}993) \times \bar{C}_{AFM1} \quad (1)$$

The upper 95% confidence limit is:

$$Ri_{P0,95} = (0{,}0562 \times 0{,}007 + 0{,}0049 \times 0{,}993) \times \bar{C}_{AFM1} \quad (2)$$

where is the average AFM_1 concentration in milk.

Naturally, the AFB_1 contamination (1000 times more toxic than AFM_1) of wheat- and corn-based products substantially increases the risk of liver cancer. The exposure derived from different sources add up. However, it could not be considered due to the lack of relevant contamination data of baked or cooked products made of wheat or corn.

The exposure calculation can only be considered to be preliminary. The actual exposition is likely higher because mycotoxins may occur in several food items consumed within one day. The transfer of mycotoxins from raw materials to ready to eat products depends on the preparation methods (fermentation, baking, cooking etc.). Taking them into account will only be possible after systematic evaluation of the partly contradicting or controversial scientific literature.

In addition to the exposure through food, the workers dealing with products infected with *Aspergillus* or *Fusarium* species (for instance during harvest, storage, sorting, milling, production of animal feed) can be exposed to further significant doses **[103-106]**, unless wearing suitable protective clothing.

3.2. Evaluation of the current situation

The presence of Fusarium fungi infection of cereals in Hungary and the consequently high exposure of consumers to Fusarium toxins have been known for a long time. Several publications called attention to this problem **[28, 33-36]**. Furthermore, guidance documents were published on the appropriate agrotechnology **[107,108]** and effective plant protection **[109-113]** aiming to reduce the infection. Nevertheless, there has been no progress in controlling the infection of cereals and decreasing the mycotoxin contamination of our food **[115]**. There are resistant hybrids, species and strains available, and the efficient pesticide application (spraying) technology for protecting cereals has been developed **[116]**. Their practical use should be promoted.

Further to Fusarium infection, the Aspergillus species are also present all over the country resulting in notable aflatoxin contamination and food safety and health risk. Due to the Global warning, the *Fusarium* and *Aspergillus* infections will increase during the coming years unless effective control measures are not implemented. For assessing the actual situation, it would be necessary to calculate the exposure of consumers to mycotoxins at regular intervals based on the food consumption data obtained with the ongoing dietary intake survey applying the unified EU methodology **[117, 118]** and the results of up-to-date laboratory control measurements.

4. Summary and recommendations

In addition to the *Fusarium* infection, the *Aspergillus* fungi and the consequent aflatoxin contamination also occurred in food and feed produced in Hungary during the last decade. The dry and warm weather, inappropriate cultivation, handling and storage practice provide favourable conditions for the infection of cereals, especially of corn and wheat, and the conse-

quent aflatoxin contamination of food and feed. The mycotoxins present in raw agricultural commodities are carried over to the food chain and can be detected in mother milk, milks and milk products, eggs, meat, liver and kidney.

The mycotoxin contamination of marketed food and feed is tested in a large number of samples by the official laboratories of NÉBIH based on a complex risk-based sampling plan. Up-to-date analytical procedures are applied for analysing the samples taken according to the relevant official sampling protocols. Also, several laboratories, such as BIOMIN Ltd., Gabona Control Ltd., SGS Hungária Ltd. and WESSLING Hungary Ltd., carry out the determination of mycotoxins in samples provided by their clients.

For obtaining reliable results, it is inevitable that the samples are taken and processed for analyses according to the methods described in relevant regulations or directives. Samples provided by the owners of the sampled commodity cannot be used for certifying the compliance of the lot to legal limits if their mass is much smaller than the minimum required. Sampling should be carried out by properly trained specialists applying accredited methods. Furthermore, the whole laboratory sample must be properly processed to obtain a representative portion for analysis.

We did not have sufficient data for the comprehensive risk assessment of the mycotoxin contamination of food based on the official control carried out by NÉBIH. The large number of test results of private laboratories could not be used, though they indicated substantial mycotoxin contamination, because they were either provided in summary form or the samples analysed were not representative. In some cases, it was not clear whether the sampled commodities were intended for food or feed.

Our preliminary estimates, based on the results of NÉBIH tests, indicate that some segments of the population (especially babies, toddlers, young children and adolescents) may be exposed from time to time to AFM_1 and DON above the ground risk level or acceptable daily intake, respectively. These signal significant human health risk and raise concern. The EFSA evaluations and European surveys confirm our conclusions.

Unless effective preventive measures are implemented, as a result of Global warming, the tendency of Aspergillus and Fusarium infection will increase with yearly varying intensity depending on the actual weather conditions and fungi species. This will increase the mycotoxin concentration in food and feed and result in growing health risk.

The mycotoxin contamination of food primarily threatens the health of pregnant women, breastfeeding mothers, babies and children in developing age. Therefore, attention should be paid to keep the contamination of their food at the lowest possible level. The food basket should be diversified and composed preferably of many various fruits and vegetables. The purchased products should be fresh and of good quality. Food should not be prepared from mouldy or rancid raw materials.

In addition to the general guidance or warning documents, it seems necessary to introduce monetary and economic incentives, together with their regular official control, for implementing effective measures for reducing fungi infections of cereals by proper plant production and protection, storage and processing practices.

Furthermore, it would be important to harmonise and financially support the research and testing activities of institutes dealing with food safety and the health impact of toxin contaminations of food.

Moreover, it is recommended to carry out regularly comprehensive assessment of consumers' exposure to toxins contamination of food based on the test results of the last 4-5 years and food consumption data obtained from the ongoing national dietary survey. The results can be used to evaluate the effect of preventive measures and to define further targeted actions.

It is pointed out that the health of the Hungarian population is not only affected by the chemical contaminants of the food. The adverse effects of environmental contaminants, especially the alarmingly high air pollution in certain areas of the country, can cause a similar or higher health risk. The adverse effects of various factors can be additive or amplify each other.

The combined effects can only be quantified with the targeted health surveys and by monitoring the levels of various environmental and food contaminants. The targeted control measures can only be done based on their results.

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The average mycotoxin concentration values given in the Hungarian county maps of Figures 5, 6 and 7 were taken with the characters of the number plots (integer and decimal values) published in the source work with the kind permission of the data owner.

(The Editor)