ANALYSIS OF MYCOTOXINS FROM CEREALS AND CEREAL PRODUCTS AT IASI COUNTY

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Abstract

Cereals and cereal products are of unique importance because they are consumed by millions of people and are considered, from a nutritional point of view, the main source of carbohydrates for humans and animals. Thus, the microbiological and mycotoxicological safety of cereals is considered very important both for food and for animal feed. The paper presents the results of research on the level of mycotoxin contamination of cereals and cereal products in Iasi County in the 2015-2019 study interval. The aim was to study the incidence of mycotoxins that have a negative influence on the safety of agri-food products, with subsequent negative impact on human and animal health. The obtained results were within the maximum allowed limits provided by the specific legislation in force (EC Reg. 1881/2006).

Key words: mycotoxins, cereals, cereal products, ELISA

Cereal grains, due to their high content of toxins, represent the ideal nutrient medium for the multiplication of micromycetes and the elaboration of their mycotoxins. Mycotoxins produced by fungi are extremely toxic and cause acute or chronic mycotoxicosis in animals and humans consuming feed / food contaminated with such compounds (Pfohl-Leszkowicz *et al*, 2007). It was found that cereals and fodder can be contaminated with mycotoxins from the field, but also after harvest, especially during their conservation period, when storage conditions are favorable for germination of resistance forms of fungi and their multiplication. The extremely heterogeneous distribution of mycotoxins within a batch of agri-food product is demonstrated by the fact that the products contain variable amounts of mycotoxins, which are dependent on the nature of the substrate, storage conditions, but also on the place and manner of sampling to be analyzed. Therefore, sampling must be a reference step in the mycotoxin analysis of agri-food products. The samples taken must be representative of the analyzed batch and ensure fidelity and reproducibility for it (Coman I. *et al*, 2012).

Mycotoxins have toxic effects on both human and animal health, and the level of toxicity of various mycotoxins depends on the amount of toxins, age and time of exposure (Chhonker T. *et al*, 2018). Mycotoxin contamination in animal feed and the potential transfer to animal products for human consumption remains a major problem that alerts the world. Mycotoxins have been found in animal products (eggs, milk and dairy products) obtained from animals which have been fed contaminated feed (Meucci V. *et al*, 2010). It is well established that not all molds are toxic and not all secondary metabolites in molds are toxic. Currently, over 300 mycotoxins have been identified; however, only a few regularly contaminate food and feed. These are: aflatoxins, ochratoxins, zearalenone, fumonisins and trichothechenes (Alshannaq A., Yu J., 2017).

Aflatoxins are mycotoxins produced by the fungal species *Aspergillus sp*., the most toxicogenic being *Aspergillus flavus* and *Aspergillus parasiticus*. They are normally present in the soil and in various organic materials (Liu Y. Wu F., 2010). While *Aspergillus flavus* strains produce only aflatoxins B1 (AFB1) and B2 (AFB2), *Aspergillus parasiticus* strains can produce AFB1, AFB2, G1 (AFG1) and G2 (AFG2) (Bennett J., Klich M., 2003). In fact, AFB1 is considered to be the most potent hepatocarcinogen in mammals and listed as a carcinogen I by the IARC. Aflatoxins are probably the best known and most researched mycotoxins in the world, but they present the highest toxicological risk (the strongest natural carcinogens known). They have been associated with various diseases, such as aflatoxicosis in pets and humans. They received more attention than any other mycotoxin due to

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their strong carcinogenic effect demonstrated in laboratory animals and their acute toxicological effects in humans (Mila et al., 2015). Practically, the only species of non-toxic *Aspergillus* are *Aspergillus niger* and *Aspergillus oryzae*, the latter being the most used in industrial biotechnologies. Ochratoxins were first isolated from *Aspergillus ochraceus* in 1965 in South Africa after corn infected with these fungi was found to cause the death of experimental animals (Luís C. *et al*, 2016). These are secondary, low molecular weight fungal metabolites that are mainly produced by molds belonging to several species of the genera *Aspergillus* and *Penicillium*, especially *A. ochraceus* and *P. verrucosum* (Freire *et al*, 2017). Ochratoxin has been shown to be a nephrotoxic, immunosuppressive, teratogenic and carcinogenic agent (Richard *et al*, 2007). Several types of ochratoxins occur naturally, namely ochratoxin A, ochratoxin B (dechlorinated OTA) and ochratoxin C (ethylated OTA) and are often co-produced.

Ocratoxin A is the most common toxin and is classified by the IARC as a potential human carcinogen of group 2B. It is one of the most relevant ochratoxins, with great public and agroeconomic significance, due to the confirmed nephrotoxic, genotoxic, neurotoxic, immunotoxic, embryotoxic and teratogenic effects and suspected carcinogenicity (Milićević D. *et al*, 2016). Practically, the lack of exposure to ochratoxin is impossible, given that it is constantly found in cereals and cereal products, which are also the major source of contamination of animals or humans. The carcinogenic effect of ochratoxin is manifested by the induction of tumors in the kidneys, liver and urinary tract. Regarding genotoxicity, ochratoxin has been found to break the nucleotide chains of DNA, in vitro, and in vivo disrupt DNA synthesis, causing genetic mutations in cells. People are frequently exposed to OTA due to its ubiquitous presence in a wide variety of foods, including grains (barley, oats, rye, corn and wheat), beans, dried fruit, tea, coffee, cocoa, wine, beer, herbs, poultry, fish, pork, eggs, cheese and milk. OTA as a causative agent of endemic human nephropathy has been highlighted worldwide. Recently, induction of apoptosis in neuronal cells has been reported to be a contributing factor in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Milićević D. *et al*, 2016).

Zearalenone is a mycotoxin with hyperestrogenic effects produced mainly by fungi: *Fusarium graminearum*, *Fusarium culmorum* and *Fusarium sporotrichoides*. Maize, wheat, oats, barley and rye are largely infected with zearalenone-producing molds. It is a non-steroidal estrogen and its main metabolites (alpha zearalenol and beta zearalenol) cause significant estrogenic activity in humans and animals (hepatic, uterine, mammary and hypothalamic estrogens). In humans, toxicity is mainly chronic, while the acute form is quite rare. Zearalenone and its metabolites can effectively stimulate the growth of mammary gland cells. Thus, it has been suggested that it may be involved in the onset of breast cancer. Some cases of early puberty have been reported in adolescents exposed to this mycotoxin. Zearalenone is included in the category of non-carcinogens for humans, group 3, according to IARC. However, it has a strong embryonic toxicity and induces apoptosis and oxidative stress in human embryonic stem cells (Cao H. *et al*, 2019).

Fumonisins are mycotoxins that frequently contaminate cereals, mostly corn. Fumonisins disrupt lipid synthesis in nerve cells, causing damage to the nervous system, liver and lung damage. Consumers of large amounts of corn are frequently exposed to esophageal cancer. Fumonisins cause damage to plants, attacking cell membranes and reducing chlorophyll synthesis.

Trichothecenes are secondary fungal metabolites that are harmful to human and animal health, causing a number of acute and chronic symptoms. They are produced by different species of *Fusarium*, such as: *Fusarium culmorum, Fusarium sporotrichioides, Fusarium tricinctum, Fusarium roseum Fusarium graminearum, Fusarium nivale* and *Fusarium sambucinum*.

Trichothecenes have the same basic structure, all these substances containing an epoxy function placed at C12-C13, which is responsible for their toxic activity; they are classified into two major groups: macrocyclic and non-macrocyclic. Their synthesis takes place with great efficiency at a temperature between 6-24 °C and in extremely humid environment.

Macrocyclic trichothecenes are subdivided into types C and D, and *non-macrocyclic trichothecenes* are subdivided into types A and B, where type A appears to be more toxic than type B. Type A includes: toxin T-2, toxin HT-2, and type B includes deoxynivalenol, as important representatives of these groups.

T-2 toxin causes intoxication, which is manifested by nausea, dizziness, vomiting, necrotic lesions in the mouth and throat, diarrhea and bleeding in various organs. It is also an immunosuppressant, irreversibly attacking the bone marrow and causing a reduction in the number of leukocytes. It has an effect on serum proteins, causes lymphocyte depletion and subsequently stomach necrosis.

Deoxynivalenol (vomitoxin) is formed in plants before harvest, and its presence is highly dependent on temperature and other climatic conditions. This mycotoxin causes general weakening of organisms, necrosis (gangrene) in various tissues (gastrointestinal wall, bone marrow, lymphatic tissue), changes blood parameters and attacks the immune system (Radiana M. *et al*, 2017).

These fungi are found worldwide and are adapted for colonization and growth on a wide range of substrates, with preferences for moisture and nutrient content (Susan *et al*, 2011).

Most mycotoxins have chemical stability, being resistant to temperature (including baking, storage or other food-related biotechnological procedures). The danger of the appearance and increase of the amount of mycotoxins occurs, however, in the improper storage of agri-food products. The toxic effects of mycotoxins depend largely on their chemical structure, but also on the amount accumulated in the body. In Europe, most of the amount of ochratoxin, for example, is taken up by ingesting cereals. Molds that can appear on cereals and cereal products must be carefully monitored, as their toxins act strongly on higher organisms, through synergism, even in small quantities. For this reason, the European Community has regulated maximum permitted limits for mycotoxins in food and feed. The mycotoxin monitoring program for food and feed is mandatory for the following sectors of activity: vegetable farms, grain reception bases, concentrated feed mills, livestock farms, food manufacturing units. In all these types of units, the level of contaminants must be kept as low as possible, by using good hygiene practices (GHP) and production (GMP).

MATERIAL AND METHOD

The determinations were performed in the period 2015-2019, from samples of cereals (wheat, corn, rye) and cereal products (white flour, wheat gluten, semolina, corn, wheat bran), which were taken at random, after an objective sampling strategy, both from grain storage units and from their processing units (mills) in Iasi county.

Sampling was performed based on a pre-established working procedure, in accordance with Annex no. 1 of the EC Regulation no. 401/2006 which establishes the sampling modalities and the analysis methods for the official control of the mycotoxin content in food.

Elemental samples are taken from different points distributed throughout the batch or sublot. Mycotoxins are unevenly distributed in a batch, therefore all necessary measures are taken to ensure that the sample taken is representative of the batch. Therefore, it is necessary to take a large number of elementary samples from various places in the lot, according to the legislation (random sampling = several elementary samples which by joining, form the aggregate sample).

The global sample is obtained by summing the elementary samples. Each aggregate sample shall be placed in a clean container of inert material, which provides adequate protection against the risk of contamination and against damage which may occur during transport. All necessary precautions shall be taken to avoid any change in the composition of the sample which might occur during transport or storage. Each sample shall be sealed at the place of sampling and identified by labeling with the following information: nature of the sample, date and place of sampling, and any other additional information. It must reach the laboratory as soon as possible.

The sampling method is done depending on the size of the batches of cereals and cereal products (*Tables 1 and 2*).

Table 1

Dividing the lots into sublots according to the product and the weight of the lot

Product	Batch weight (tons)	Weight or no. of sublots	No. of elementary samples	Overall sample weight (kg)
Cereals and cereals products	≥ 1 500	500 tons	100	10
	> 300 și < 1 500	3 sublots	100	10
	≥ 50 și ≤ 300	100 tons	100	10
	< 50	-	3-100	1-10

Table 2

The number of elementary samples taken depending on the weight of the lot

Batch weight (tons)	The number of elementary samples	Overall sample weight (kg)
≤ 0,05	3	1
> 0,05-≤ 0,5	5	1
> 0,5-≤ 1	10	1
> 1-≤ 3	20	2
> 3-≤ 10	40	4
> 10-≤ 20	60	6
> 20-≤ 50	100	10

In the case of samples taken in the storage-marketing stage, these being prepackaged in packaging ready for sale, the global sample must be a minimum of 1 kg, and the product is not removed from the original packaging until it is to be analyzed in the laboratory.

The samples that arrive at the laboratory for analysis are each divided into three distinct

samples: the test sample, the additional opinion sample and the reference sample. All these are obtained only in the laboratory, being separated from the previously homogenized laboratory samples.

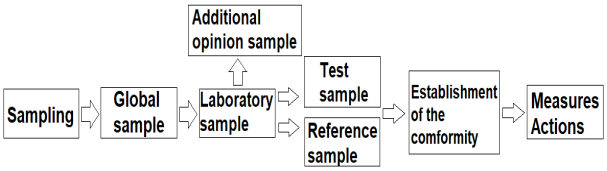

Figure 1 **Sampling and analysis of samples**

The sample for additional opinion can be kept in the laboratory or at the operator. If the sample for additional opinion is kept in the laboratory, the storage time is:

-until the issuance of the analysis bulletin, in case of compliant products;

-1 year when the evidence is non-compliant.

The reference sample is kept in the laboratory for the following period:

-until the release of BA in the case of compliant products;

-1 year when the evidence is non-compliant.

The establishment of the conformity of the samples is made based on the results obtained following the laboratory analysis, to which the correction for recovery and the degree of uncertainty of the measurement are applied, in accordance with the provisions of Reg. CE 401/2006.

Acceptance of lots:

-The lot is considered "accepted" when the sample is compliant, i.e. the value obtained after performing the laboratory analysis is less than or equal to the maximum allowed limit (AML) provided in EC Regulation no. 1881/2006 with the subsequent completions and modifications.

-The lot is considered "rejected" when the sample is non-compliant, i.e. the value obtained from performing the laboratory analysis is higher than the AML provided in EC Regulation no. 1881/2006 establishing the maximum levels for certain contaminants in foodstuffs.

During the study period (2015-2019) a total number of 261 samples consisting of cereals and cereal products were taken and analyzed, as follows: 57 samples of white flour, 7 samples of wheat gluten, 94 samples of wheat grains, 2 samples of wheat semolina, 17 samples of corn, 77 samples of corn grains, 6 samples of rye and 1 sample of wheat bran.

Samples of products taken for the quantitative determination of mycotoxin content were analyzed based on RIDASCREEN determination kits specific to each mycotoxin (aflatoxin B1, total aflatoxins, ochratoxin A, zearalenone, fumonisins and trichotecenes), and for the interpretation of the results was used microELISA photometer.

Sample preparation of cereals and cereal products - grinding, extraction, filtration, dilution

Detection limit: for cereals and cereal products - 1 - 2.5μg / kg.

Reproducibility: 80-100%.

Principle of the test: the test is based on the antigen-antibody reaction. The plaque wells are labeled with specific anti-mycotoxin antibodies. Add standards or samples, enzyme conjugate and anti-mycotoxin antibodies. Free mycotoxin and the enzyme conjugate compete for antibody binding sites (competitive enzyme-linked immunosorbent assay). At the same time, mycotoxin antibodies are bound to immobilized antibodies. The unbound enzyme conjugate is removed by the washing step. Add the substrate-chromogen mixture to the wells and leave to incubate; the bound enzymatic conjugate will convert the colorless chromogen into a blue substance. Adding the stop reagent will cause the color to change from blue to yellow. The measurement is made spectrophotometric at a wavelength of 450 nm. The absorbance is inversely proportional to the mycotoxin concentration in the sample.

Equipment used: microELISA photometer (450nm), mill, shaker, centrifuge, mechanical stirrer, graduated cylinder: 100ml, 1l, funnel and Whatman filter paper no. 1, graduated pipettes, micropipettes: 50μL, 100μL, 1000μL.

Reagents: methanol, dilution buffer, wash buffer, distilled water, etc.

Sample preparation:

Samples should be kept in a cool place, protected from light. The representative sample must be ground and thoroughly homogenized before starting the extraction procedure.

Way of working:

-weigh 5g of ground sample and transfer to a container with a lid over which the specific amount of reagents is added (methanol / distilled water 70/30). The sample size can be increased if necessary, but the volume of distilled water must be adapted accordingly (for example: 25 g of sample in 125 ml of distilled water or 50 g of sample in 250 ml of distilled water):

- shake vigorously for a few minutes (manually or with a stirrer)

-filter the extract using Whatman filter paper no.1

-to dilute the extract

-50l of extract per well is used, in the test.

Protocol:

- Before starting work, bring all reagents to room temperature.

-Insert a required number of wells for standards and test samples into the holder.

-Add 50 μL of standard sample.

-Add 50 μL of enzyme conjugate to each well.

-Add 50 μL of antibody solution to each well.

-Mix manually by rotating the plate and incubate approx. 30 min at room temperature (20-25 ° C).

-Pour the liquid and beat it vigorously face down on an absorbent paper to remove traces of liquid.

-Add 250 μL wash buffer and remove the liquid again. Repeat the washing step 2 times.

-Add 100 μL (2 drops) of substrate / chromogen in each well. Mix by hand, rotating the plate and incubate for 15 minutes. at room temperature (20-25°C).

-Add 100 μL (2 drops) of stop solution in each well.

Mix gently, shaking the plate nicely and measure the absorbance at 450 nm. The reading is done at 10 min. after adding the stop solution.

The average value of the absorbents obtained for standards and samples are divided by the absorbance value of the first standard and multiplied by 100. The zero standard is equal to 100% and the absorbent values are expressed as a percentage:

$$\frac{abs.\ standard}{absorbanta} \times 100 = \%\ abs.\ zero\ standard$$

The values calculated for the standards are entered in a coordinate system on semilogarithmic millimeter paper with respect to the mycotoxin concentration expressed in μg / kg.

The mycotoxin concentration corresponding to the absorbance of each sample can be read using the calibration curve. To obtain the mycotoxin concentration in the sample in μg / kg, the concentration read from the calibration curve must be multiplied by the corresponding dilution factor.

RESULTS AND DISCUSSIONS

Due to the enormous impact of mycotoxins on public health and the economy, it is extremely important to prevent and continuously monitor the incidence of mold infections and associated mycotoxin production (Mia E. *et al*, 2013).

EC Regulation no. 1881/2006 establishes the maximum allowed levels for some contaminants in food products. According to this Regulation, the maximum permitted level of mycotoxins in cereals and cereal products is:

-2 μg / kg for aflatoxin B1;

-4 μg / kg for total aflatoxin;

-750-1750 μg / kg for deoxynivalenol (depending on the product category);

- 75-100 μg / kg for zearalenone;

- 5-10 μg / kg for ochratoxin A;

- 4000 μg / kg for fumonisins.

T2 and HT2 toxins are subject to a monitoring period, before establishing maximum legal limits allowed by the competent authority in the field (EFSA- European Food Safety Authority).

In *table 3* are presented the analytical results of the analyzed samples, which recorded *positive values*, obtained in the study interval 2015-2019, for the characterization of mycotoxins from cereals and cereal products.

Table 3

Analytical results of the analyzed samples

Nr. crt.	Name of mycotoxin	Cereals and cereals products					
		Total number of samples analyzed	Number of negative samples	Number of positive samples	Nature of the sample	Value of the analyzed positive sample (μg/kg)	LMA (μg/kg) according Reg. CE 1881/2006
1.	Aflatoxin B1	40	38	sample no. 1	wheat	1.49	**2**
				sample no. 2	wheat	1.55	
2.	Aflatoxins	44	40	sample no. 1	wheat	1.5	**4**
				sample no. 2	wheat	1.61	
				sample no. 3	wheat	1.97	
				sample no. 4	maize	2.65	
3.	Deoxynivalenol	54	36	sample no. 1	white flour	21.0	**750**
				sample no. 2	white flour	22.0	
				sample no. 3	white flour	23.0	
				sample no. 4	white flour	82	
				sample no. 5	white flour	82	
				sample no. 6	white flour	136	
				sample no. 7	white flour	242.322	
				sample no. 8	white flour	347.353	
				sample no. 9	white flour	491	
				sample no. 10	wheat	636	**1250**

				Sample no. 11	maize	41	**1750**
				sample no. 12	maize	69	
				sample no.13	maize	114	
				sample no. 14	maize	157.916	
				sample no. 15	maize	170.489	
				sample no.16	maize	252.267	
				sample no. 17	maize	324.2	
				sample no. 18	maize	446.956	
4.	Zearalenone	35	31	sample no. 1	maize	2.68	**100**
				sample no. 2	maize	2.68	
				sample no. 3	wheat	3.984	
				sample no. 4	white flour	5.597	**75**
5.	Ochratoxin A	47	41	sample no. 1	rye	1.56	**5**
				sample no. 2	wheat	1.56	
				sample no. 3	wheat	1.56	
				sample no. 4	wheat	1.56	
				sample no. 5	wheat gluten	3.5	**10**
				sample no. 6	wheat gluten	4.556	
6.	Fumonisins	17	9	sample no. 1	maize	45.36	**4000**
				sample no. 2	maize	56	
				sample no. 3	maize	82.233	
				sample no. 4	maize	212.87	
				sample no. 5	maize	299	
				sample no. 6	maize	431	
				sample no. 7	maize	757	
				sample no. 8	maize	854	
7.	Toxin T2/HT2	24	24	-	-	-	**monitoring**
Total number of samples		**261**	**219**	**42**			

CONCLUSIONS

In the studied period (2015-2019) a total number of 261 samples was analyzed, of which, for 219 samples representing a percentage of 83.9%, no mycotoxin values were registered, these being below the detection limit. A number of 42 samples, representing approx. 16.1% of the total studied were positive for mycotoxins, which is a relatively low percentage. None of the samples registered with a positive value for the content of mycotoxins, registered as exceeding the maximum allowed limit provided by the legislation in force (EC Reg. 1881/2006).

Of the total samples found to be positive, the highest frequency of contamination was recorded by deoxynivalenol, with a percentage of about 42.8%, the other mycotoxins having the following incidence: 19% fumonisin, 14.3% ochratoxin A, 9.5% zearalenone, 9.5% total aflatoxin and 4.8% aflatoxin B1.

The maximum levels for mycotoxins, permitted by the legislation in force, shall be set at a level which considers human exposure in relation to the tolerable dose of the toxin in question and which can reasonably be attained for the production, storage and processing of cereals and cereal products. This approach ensures that agri-food operators apply all possible measures to prevent and limit, as far as possible, mycotoxin contamination in order to protect public health.

In general, mycotoxins are unpredictable and unavoidable natural contaminants in agri-food products, posing a permanent serious risk to health for both humans and animals, while contributing to considerable economic losses for agriculture. Particular attention should be paid mainly to prevention measures against mycotoxin contamination of cereals, as it is impossible for other technologies to ensure their complete decontamination. Experts in the food field believe that the most effective way to avoid the effects of mycotoxicosis is to prevent fungal infestation of agricultural crops, by respecting the agrotechnical conditions of cultivation and harvesting, as well as storage conditions, as the processing of contaminated raw materials cannot lead to the removal of mycotoxins.

In order to ensure effective protection of public health, products containing mycotoxins exceeding the maximum permitted levels should not be placed on the market as such or mixed with

other foodstuffs and not will be used as ingredients in other processed products.

The general conclusion is that, based on the results of the analyzes performed for Iasi county in the period 2015-2019, for the selected products, taken "from the base of the food chain", the degree of mycotoxin contamination can be considered acceptable. The general situation is characterized by low contamination, which leads to risks to human health related only to chronic exposure. At the same time, the cumulative toxic effect of mycotoxins encountered simultaneously, especially in cereals, must be considered. Cereals should be ranked first on a hypothetical "warning list" due to their national dietary importance, natural synergism and all possible combinations of major mycotoxins, and the frequent level of contamination.

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