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Mycotoxins incidence in the Romanian malting barley (harvest 2007)

Mihaela Begea[a], Liliana Bălăuță[a], Elena Baron[a], Liliana Vasilescu[b]

[a] *Institute of Food Research, Bucharest, Romania*
[b] *Research and Development Institute for Agriculture, Fundulea, Calaraşi, Romania*

Abstract
The paper presents information regarding the contamination with mycotoxins of barley varieties experimentally cultivated in Romania for brewing by the Research and Development Institute for Agriculture. There are presented data regarding the incidence of the main mycotoxins (fusariotoxins (DON) and ochratoxins) of barley from 2007 harvest. The presence of these mycotoxins in barley has a negative influence on the food safety of malt and beer produced from the contaminated barley. The sdudy was performed in order to evaluate the contamination level and to select the best malting varieties. Romania adopted the European Commission regulations concerning the maximum levels for mycotoxins in different raw materials and products for human or animal consumption. The method used for the study is a direct immunoenzymatic competitive test (CD-ELISA), which allow us to obtain accurate concentrations in parts per billion (ppb). The results obtained were into the admissible limits of the present national legislation.

Keywords: Micotoxins, ochratoxin, DON, ELISA, malting barley

1. Introduction

Fungal and mycotoxins contamination of food and feed represents a major risk for consumers' safety. Mycotoxins represent secondary metabolits produced by different specia of filamentous fungi, especially those of Aspergillus, Penicillium and Fusarium genera. Between the most outspread mycotoxins are aflatoxins, fusariotoxins, ochratoxins, etc. European Commission elaborated regulations concerning maximum levels for aflatoxins and ochratoxins in different raw materials and products for human or animal consumption. The regulations are adopted also in Romania.

At European Commission level, they are working to diminish maximum admissible levels for mycotoxins in products for human and animal consumption.

Mycotoxins occurrence in food and feed can drive to acute intoxications, at high levels contaminations; it is necessary to mention that mycotoxins are toxic at extremely low concentrations and they have cumulative, immunosuppressive and immunotoxic effects into the human body.

Deoxynivalenol (DON) is part of trichothecenes (group B) family and represents the widespread secondary metabolites of different specia of Fusarium, produced mainly in the field.

When compared to other trichothecene mycotoxins which can form in grains and forages, DON is relatively mild. Reduced feed intake, and the accompanying decrease in performance, are the only symptoms of DON toxicity livestock producers will likely encounter. This response to DON appears to occur through the central nervous system. DON belongs to trichothecenes which are strong protein inhibitors. Inhibition of protein synthesis following exposure to DON causes the brain to increase its uptake of the amino acid tryptophan and, in turn, its synthesis of serotonin. Increased levels of serotonin are believed to be responsible for the anorexic effects of DON and other trichothecenes.

* Corresponding author: *e-mail address*: ela_begea@yahoo.com

Irritation of the gastrointestinal tract may also play a role in reducing feed intake [1].

Between toxicological effects of DON we can mention: nauseas (vomiting), feed refusal, gastroenteritis, diarrhoea, immunosuppression, blood disorders (red blood cells hemolysis, etc).

DON occurrence also produces DNA and RNA synthesis inhibition.

Ochratoxins are metabolites produced by different species of *Aspergillus* and *Penicillium* genera of fungi. These types of mycotoxins are produced mainly between (+4...+37°C), having a wide incidence on cereals (barley, maize etc.), green coffee, different dried fruits, etc. They are storage mycotoxins, but can be produced also during the malting process [2].

Ochratoxin A is potentially carcinogenic to humans (Group 2B). Ochratoxin A has been shown to be weakly mutagenic, possibly by induction of oxidative DNA damage.

A peculiar aspect of these mycotoxins: they can arrive in animal blood via contaminated feed and, from there, in humans which consumed meat products contaminated with that blood.

Ochratoxin may be present in conjunction with aflatoxins, between the most potent naturally-occurring carcinogens (aflatoxin B_1). Ochratoxins are suspected to be carcinogenic.

They affect kidneys in animals exposed to naturally-occurring levels of these mycotoxins. Turkeys or other poultries exposed to such toxins by feed exhibited lower productivity levels during field outbreaks of ochratoxicosis. Symptoms include retarded growth and decreased feed conversion; they also affect egg production in laying hens.

There are two different ways of mycotoxins access in human body:

- directly, by consuming the products that contain mycotoxins (vegetal origin of mycotoxins sources - vegetables, fruits, processed products like: bread, cereal flakes, pasta, soy products, jams, etc., or animal origin products, like milk, eggs, meat, poultry meat, fish, etc);
- indirectly, by consuming food processed from animals feeded with fodder with mycotoxins content, dairy products, meat products, etc.

The best protection against mycotoxins presence in food and feed and their effects on humans and animals is represented by their attentive monitoring by testing their presence all along the pathway from pre-harvest of grains to the finished product.

The purpose of this work was to quantify DON and ochratoxins concentrations in barleys of 2007 crop from Fundulea Agricultural Research Institute.

2. Materials and Method

There are presented data regarding the incidence of the main mycotoxins - fusariotoxins (DON and ochratoxins) of barleys from 2007 crops. The presence of these mycotoxins in barley has a negative influence on the food safety of malt and beer produced from the contaminated barley.

There were analysed 31 barley samples of 2007 crops, produced at Fundulea Agricultural Research Institute for the DON and ochratoxins contamination, by immunoenzymatic CD-ELISA Veratox method.

This is a rapid, precise and easy-to-use method. The test is based on an antigen-antibody reaction.

DON (vomitoxin, deoxynivalenol) determination

Method of analysis: Veratox for DON HS, CD-ELISA - Quantitative high sensitivity competitive-direct enzyme-linked immunosorbent assay [3].

Assay principles: Veratox for DON HS is an immunoenzymatic method which allows the user to obtain exact concentrations of DON, in parts per billion (µg/kg). Free DON in the samples and controls is allowed to compete with enzyme-labelled DON (conjugate) for the antibody binding sites.

After a wash step, substrate is added, which reacts with the bound conjugate to produce a blue colour. The more blue colour means less DON. The optical densities of the controls form the standard curve, and the sample optical densities are plotted against the curve to calculate the exact concentrations of DON (by Neogen Log/Logit software).

The controls and samples optical densities are read at 650 nm into a microwell reader type Neogen Stat Fax 303 Plus, from Awareness Technology Inc., SUA.

*Sample preparation and extraction:*The sampling method is critical. Contamination by mycotoxins can occur in the field, during storage or during the malting process. 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, the European Commission has recommended a procedure for a sampling according to the size of the batch. This includes 10 to 100 incremental samples up to a total of 10 kilos that have to be milled before analyses [4]. All results obtained from samples not taken by this method are generally underestimated.

The samples are collected according to the accepted sampling methods, depending on the dimension of the batch in order to obtain a representative sample and they are maintained at 2...8°C until analyzing.

1. Grind the entire sample so that at least 75% of the grinded material passes through a 20 mesh sieve, the particle size of a fine instant coffee.
2. Blend 10 g of ground sample with 50 ml of distilled water for 3 minutes in a high speed blender. Alternative: add 5 g of ground sample in 25 ml of distilled water and shake vigorously for 3 minutes.
3. Let to settle the material 2 or 3 minutes.
4. Filter the extract by pouring at least 5 ml, through a Whatman no.1 filter and collecting the filtrate as a sample.
5. The sample is ready for testing.

Test procedure:

1. Allow all reagent to warm to room temperature (18-30^{0}C) before use.
2. Place 100µl of conjugate in each mixing well.
3. Transfer 100µl of controls/ samples to the mixing wells.
4. Homogenize. Transfer 100µl in antibody-coated wells. Incubate for 10 minutes.
5. Shake out the contents of the antibody wells.
6. Wash the wells with distilled water 5 times. Shake out the contents of the antibody wells.
7. Place 100µl of substrate into the wells. Incubate 10 minutes.
8. Place 100µl stop solution into the wells.
9. Read, during next 20 minutes, with a Neogen microwell reader, at 650 nm and calculate the results using a Neogen Log/logit software.

Performance characteristics

Quantitative limit: 25 ppb (determined by the mean average of 10 DON free samples plus 2 standard deviation).
Range of quantitation: 25-250 ppb.

Ochratoxins determination

Method of analysis: Veratox for ochratoxins - Quantitative high sensitivity method, CD- ELISA [3].

Assay principles: Veratox for ochratoxins is a CD-ELISA method, which permits to the utiliser to obtain exactly concentrations of ochratoxins, in parts per billion (µg/kg). Free ochratoxins from samples and standards are in competition with the conjugate (ochratoxins-enzyme) for the antibody binding sites. After a washing step, add the substrate, which reacts with the bounded conjugate producing a blue colour. The more blue is the colour, a less quantity of total ochratoxins in the sample is detected. The optical densities of the controls from the standards curve, and the sample optical densities are plotted against

the curve to calculate the exact concentration of ochratoxins (by Neogen Log/Logit software).

Sample preparation and extraction:

The samples are collected according to the accepted sampling methods, function of the dimension of the lot and they are maintained at 2...8°C until analysing. Obtain a representative sample.

1. Prepare a methanol solution of 50% with distilled water.
2. Grind the entire sample so that at least 75% of the milled material passes through a 20 mesh sieve, the particle size of a fine instant coffee.
3. Blend 25 g of ground sample with 100 ml of 50% methanol for 2 minutes in a high speed blender. Alternative: add 10 g of ground sample to 40 ml of 50% methanol and shake vigorously for 5 minutes.
4. Filter the extract by pouring at least 5 ml, through a Whatman no.1 filter and collecting the filtrate as a sample.
5. The sample is ready for testing.

Test procedure
Allow all reagent to warm to room temperature (18-30^{0}C) before use.

1. Place 100 µl of conjugate in each mixing well.
2. Transfer 100 µl of controls / samples to the mixing wells.
3. Homogenize. Transfer 100µl in antibody-coated wells. Incubate for 10 minutes.
4. Shake out the contents of the antibody wells.
5. Wash the wells with distilled water 5 times. Shake out the contents of the antibody wells.
6. Place 100 µl of substrate into the wells. Incubate 10 minutes.
7. Place 100 µl stop solution into the wells.
8. Read, during next 20 minutes, with a Neogen microwell reader, at 650 nm and calculate the results using a Neogen Log/logit software.

Performance characteristics
Limit of detection: 1 ppb (determined by the mean average of 10 ochratoxins free samples plus 2 standard deviation).

Quantitative limit: 2 ppb (Described as the lowest concentration point on the calibration curve that this test can reliably detect ochratoxins).
Range of quantitation: 2-25 ppb

3. Results and Discussion

Regulation no. 1881/2006/CE [5] establishes maximum admissible levels for some contaminants in various food products. Maximum admitted level for ochratoxin A in raw cereals must be 5 ppb, according to this Regulation.

According to Regulation no.1126/2007/CE [6], maximum admitted level for DON in raw cereals must be 1250 ppb.

In Table 1, there are presented the analytical results obtained for the characterization concerning DON and ochratoxins contamination of some barley varieties of 2007 harvest from Fundulea Agricultural Research Institute.

Table 1: DON and ochratoxins contamination of barley varieties (2007 harvest) cultivated by Fundulea Agricultural Research Institute

Sample number	Description	DON concentration (ppb)	Ochratoxins concentration (ppb)
1	COMPACT/2007	0.5	0.2
2	ORIZONT/2007	0.0	0.1
3	TOCADA/2007	0.0	0.0
4	DN148-11- 2002/2007	44.8	0.0
5	MAURITIA/2007	0.0	0.0
6	ADI/2007	12.3	0.0
7	DANA/2007	35.0	0.0
8	ANABEL/2007	19.2	0.0

9	DN32-26-2002/2007	20.1	0.0
10	F8-127-2001/2007	19.5	0.0
11	ANDRA/2007	39.5	0.0
12	F8-105-2001/2007	23.4	0.0
13	F8-110-2002/2007	17.7	0.0
14	04-07/2007	22.9	0.0
15	01-10/2007	8.1	0.0
16	DN 99-12- 2002/2007	12.2	0.0
17	DN-94-4-2002/2007	18.2	0.0
18	F8-113-2002/2007	0.9	0.1
19	F8-43-2006/2007	34.3	0.3
20	F8-54-2001/2007	7.6	0.6
21	GERMINA/2007	11.4	0.0
22	MADALIN/2007	18.1	0.0
23	SCARLET/2007	0.6	0.0
24	BEATRIX/2007	25.4	0.0
25	XANADU/2007	14.3	0.1
26	SEBASTIAN/2007	15.7	0.0
27	LILIANA/2007	13.8	0.0
28	PASADENA/2007	23.1	0.2
29	ANDREI/2007	11.9	0.0
30	UNIVERS/2007	0.9	0.0
31	LAURA/2007	10.1	0.1

4. Conclusion

- None of barley samples analysed exceed maximum admitted levels, neither for DON, nor for ochratoxins.
- All of the results were situated under the detection limit of the method for ochratoxins (1 ppb).
- The limit of quantitation for ELISA Veratox method for DON is 25 ppb; only the values for samples no. 4, 7, 11, 19 and 24 exceeded the quantitative limit.
- No correlation was observed between the higher concentration in DON and that in ochratoxins for the same sample of barley.
- The immunoenzymatic CD-ELISA Veratox method represents a rapid and safe quantitative method for determination of mycotoxin contamination on the entire food chain.
- It is obvious necessary to monitory the presence of mycotoxins in agri-food raw materials, in order to prevent the contamination of food and feed products.

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