STUDY ON THE CONTENT OF ZEARALENONE FROM WHEAT AND DERIVATIVES

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Abstract: *In food and fodder naturally contaminated with fungus there are in high concentrations seven groups of mycotoxins: aflatoxins, ochratoxin, trichothecenes, zearalenone, patulin, citrinine and acid penicilic. Mycoestrogens are estrogens produced by fungi. The most important mycoestrogen is zearalenone, produced by Fusarium species of fungi. Zearalenone and its metabolites exert their estrogenic effects through binding to estrogen receptors[1]. Zearalenone is the main phyto-oestrogen and it is the primary toxin causing infertility, abortion or other breeding problems, especially in swine. Chickens fed with contaminated feed showed zearalenone residues in muscles and liver. The concentrations of zearalenone and its metabolites, α- and β-zearalenol, were determined quantitatively by high-performance liquid chromatography (HPLC), in placenta, in maternal liver and spleen rats, α- and β-zearalenol were transferred into the foetus [2]. The paper analyzed raw materials used in the bakery industry, samples of grain, flour and bran in order to determine the content of zearalenone Specific limits for zearalenone have been regulated in 2007 by the European Union, ranging from 20 µg/kg for infants and small children foods to 75 µg /kg for maize flour (EU Commission Regulation (EC No 1126/2007). Amounts of mycotoxins have been highlighted by ELISA. In analyzed samples were detected exceeded limits for a number of one sample of wheat and one sample of bran, the other samples containing zearalenone below the maximum allowable limit, the analysed raw materials considered as safe for bakery.*

1.Introduction

Mycotoxins are secondary metabolites of fungi which contaminate feed and food. Mycotoxins presented in food products and animal feeds are an important problem concerning food and feed safety and significant economic losses are associated with their impact on human and animal [3]. Vegetable products are convenient substrates for fungi development and mycotoxins production[4]. During storage they are favorable substrates if they have a high moisture content above 80% and a temperature above 20°C. The main types of fungi producing mycotoxins are: *Aspergillus, Penicillium, Fusarium* şi *Alternaria.* It should be noted that a fung can produce more mycotoxins and that a particular mycotoxin may be produced by several fungi; it can lead to phenomena of synergism and potentiation of toxic action. Zearalenone is heat-stable and is found worldwide in a number of cereal crops, such as maize, barley, oats, wheat, rice, and sorghum and also in bread.

Zearalenone has a chemical structure similar to steroid hormones and it is produced by fungi of the genus Fusarium (F. graminearum, F. avenacum F. Equisetti). Zearalenone belongs to the group of phytoestrogens have an estrogen-mimetic effect, but it is not a true, it is not a plant product (soy, alfalfa, clover) but a fungical one.

Zearalenone is a lactone of resorcylate acid, it is carcinogenic, mutagenic and genotoxic in a concentration 1.5 mM/L.

Zearalenone induces cell proliferation in estrogen-dependent tissues (carcinogenesis), and contributes to endometrial hyperplasia and adenocarcinoma formation. 2.

2. Materials and methods

A method for determination of zearalenone in cereal flour has been developed applying pressurized liquid extraction (PLE) using methanol/acetonitrile (50:50 v/v) as the solvent extraction[5]. The extracted samples is analyzed with liquid chromatography coupled to mass spectrometry (LC–MS) with an electro spray ionisation interface (ESI).

Conventional chromatographic methods are generally intensive time and capital consuming and therefore have been developed and marketed a series of rapid analysis methods mostly based on immunological principles. Method used in paper is based on immunoenzymatic reactions in heterogeneous system (ELISA - Enzymes Linked Immuno Sorbent Assay) that used for determination both of antigen and of antibody.

Antigenic structures, usually in the form of liquid suspension can have microbial origin (bacteria, viruses, fungi). Antibodies can be represented by non-immune sera or purified polyclonal immunoglobulins.

In the technique used, the specific antigen or antibody is attached to a solid support that can bind antigen or antibody by passive adsorption or covalent binding. Passive adsorption of macromolecules consists of immobilization on a surface of glass or plastic.The vast majority of protein diluted (1-10 mg / ml) in an alkaline solution (pH - 9.6) are adsorbed on plastic in few hours. As a solid support can be used propylene, polystyrene, polycarbonate or latex. In the tests were used microplates of polystyrene, with fully automated work advantage.Covalent binding allows irreversible connections between reactants and solid support so that quantitative assessments have a maximum precision. Calitatea fixării structurilor proteice pe suportul solid depinde de concentraţia proteinei, de natura suportului utilizat şi de intervenţia unor agenţi chimici de fixare. Quality of protein structures are fixed on solid support depends on protein concentration, the nature of substrate used and the occurrence of chemical fixation. Enzyme conjugate there should be a couple antigen - enzyme or antibody - enzyme, the latter being the most frequently used form.

2.1 Materials

The sample must be kept cold, avoid light. A quantity of 5 g of ground sample is mixed with 25 ml of methanol / water (70/30); shaked vigorously for 3 minutes; centrifuged 10 minutes at 3500 rpm. at room temperature or filter; the supernatant or filtrate are diluted 1: 7 with a buffer for diluting the sample and using 50 µl per well.

Reagents. It works with reagents at room temperature, prepared before use .

Microtitre plate. Wells are coated with antibodies.

Conjugate solution. Enzyme conjugate is concentrated and for reconstitution the enzyme conjugate is diluted 1: 11 in buffer (eg. 200 µl concentrate + 2.0 ml of buffer solution, sufficient for four wells). After use, the vial is kept at 2°C to 8°C in the dark.

Solution substrate / chromogen. Solution substrate/chromogen is ready to use and tends to precipitate at 4°C. It brings the bottle to room temperature and kept in the dark, stirring before pipetting.. Chromogen staining is an indicator of deterioration and reagents should be discarded.

Wash buffer. Wash buffer is diluted before use. Samples are needed to achieve the next steps.

1. Pipette 100 µl standard dilution buffer, which is the reagents blank (wells A_1 and A_2).

2. Pipette 50 µl standard S_0 (wells B_1 şi B_2).
3. Pipette 50 µl standard, in duplicate (S_1…S_6).
4. Pipette 50 µl sample solution in duplicates, in the remaining wells (P_1, P_2 ....P_{40}).

Tabel 1

Order in which pipette standards and samples in microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	P1	P1	P9	P9	P17	P17	P25	P25	P33	P33
B	S0	S0	P2	P2	P10	P10	P18	P18	P26	P26	P34	P34
C	S1	S1	P3	P3	P11	P11	P19	P19	P27	P27	P35	P35
D	S2	S2	P4	P4	P12	P12	P20	P20	P28	P28	P36	P36
E	S3	S3	P5	P5	P13	P13	P21	P21	P29	P29	P37	P37
F	S4	S4	P6	P6	P14	P14	P22	P22	P30	P30	P38	P38
G	S5	S5	P7	P7	P15	P15	P23	P23	P31	P31	P39	P39
H	S6	S6	P8	P8	P16	P16	P24	P24	P32	P32	P40	P40

5. Add 50 µl enzyme conjugate into each well. Cover the plate with aluminum foil, stir the stirrer of the microplate (60 rpm) and incubated for two hours at room temperature in the dark.
6. Discard the liquid and beat with power plate face down on absorbent paper to remove traces of liquid. Add 250 µl washing solution (PBS and distilled water) and discard the liquid. Repeat washing step twice.
7. Add 50 µl substrate and 50 µl chromogento each well. Mix rotating plate and incubated 30 min at room temperature (20-25 ° C), from darkness. Blue color intensity obtained is inversely proportional to the concentration of mycotoxins in the sample or standard.
8. Add 100 µl stop solution to each well. Mix gently rotating plate and measured at 450 nm against air blank site. Not to exceed 60 min after adding stop solution.

Using the optical densities (OD) of the standard, the calibration curve is plotted against the concentrations of other standards, and the amount of of mycotoxin in the sample is extrapolated from standard curve.

Results and discussion

Mycotoxicological samples analysed by ELISA were represented by samples of wheat flour, samples of bran and samples of wheat.

Table 2

Zearalenone concentrations for each analysed sample

No.	Sample	Zearalenone ppb	No.	Sample	Zearalenone ppb	No	Sample	Zearalenone ppb
1	Wheat	33.40	1	Bran	21.12	1	Wheat flour	18.12
2	Wheat	42.07	2	Bran	38.53	2	Wheat flour	16.22
3	Wheat	53.56	3	Bran	58.10	3	Wheat flour	19.36
4	Wheat	23.60	4	Bran	43.67	4	Wheat flour	22.02
5	Wheat	47.11	5	Bran	52.13	5	Wheat flour	14.21
6	Wheat	44.63	6	Bran	40.49	6	Wheat flour	16.41
7	Wheat	46.34	7	Bran	44.88	7	Wheat flour	11.12
8	Wheat	48.19	8	Bran	54.41	8	Wheat flour	13.14
9	Wheat	61.45	9	Bran	24.62	9	Wheat flour	10.84
10	Wheat	111.76	10	Bran	46.42	10	Wheat flour	19.41
11	Wheat	27.75	11	Bran	53.53	11	Wheat flour	52.63
12	Wheat	39.39	12	Bran	41.43	12	Wheat flour	28.41
13	Wheat	45.35	13	Bran	31.11	13	Wheat flour	18.74
14	Wheat	68.29	14	Bran	37.88	14	Wheat flour	31.02

15	Wheat	33.98	15	Bran	51.63	15	Wheat flour	24.51
16	Wheat	67.22	16	Bran	42.64	16	Wheat flour	15.47
17	Wheat	28.20	17	Bran	76.87	17	Wheat flour	25.74
18	Wheat	82.22	18	Bran	19.41	18	Wheat flour	33.43
19	Wheat	76.14	19	Bran	32.01	19	Wheat flour	57.96
20	Wheat	51.13	20	Bran	51.07	20	Wheat flour	12.64
			21	Bran	48.96			
			22	Bran	54.11			

Analyzing the data presented in Table 2 derived from zearalenone determination it found that:

- in one wheat sample, (5%) of the total number of samples, zearalenone is presented in an amount greater than the maximum limit permitted by applicable law (100 ppb);
- in two wheat samples (10% of total samples), zearalenone is presented in a quantity of 76-100 ppb and falls below the maximum allowed by the applicable law;
- the five wheat samples (25% of total samples), zearalenone is presented in an amount between 51-75 ppb and falls below the maximum permitted by applicable law;
- the nine wheat samples (45% of total samples), zearalenone is presented in an amount between 31-50 ppb and falls below the maximum permitted by applicable law;
- the three wheat samples (15% of the total number of samples), zearalenone is presented in an amount between 1-30 ppb and falls below the maximum permitted by applicable law;

The frequency of wheat samples with zearalenone values is shown in Fig .1

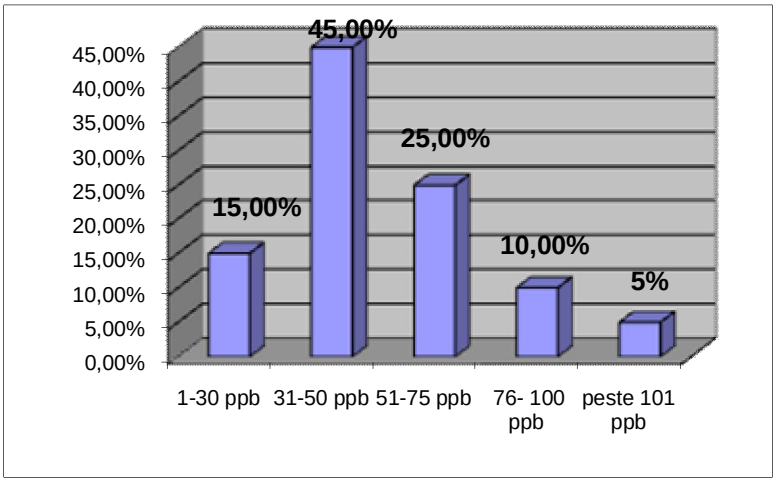

Fig.1. Graphical representation of samples of wheat contamination with zearalenone

- From the analyzing samples of bran is resulted the following values for zearalenone content
- in a single sample of bran (4.54%) of the total number of samples, zearalenone is presented in an amount greater than the maximum permitted by applicable law (ppb 75);
- in seven samples of bran (31.81% of total samples), zearalenone is presented in an amount of 51-75 ppb and falls below the maximum permitted by applicable law;
- în 11 probe tărâţe (50% din numărul total de probe) ZEA este prezentă într-o cantitate cuprinsă în intervalul 31 – 50

ppb şi se încadrează sub limita maximă admisă de legislaţia în vigoare;

- in eleven samples of bran (50% of the total number of samples), zearalenone is presented in an amount of 31-50 ppb and falls below the maximum permitted by legislation;
- in three samples of bran (13.65% of total samples), zearalenone is presented in an amount of 1-30 ppb and falls below the maximum permitted by applicable law;

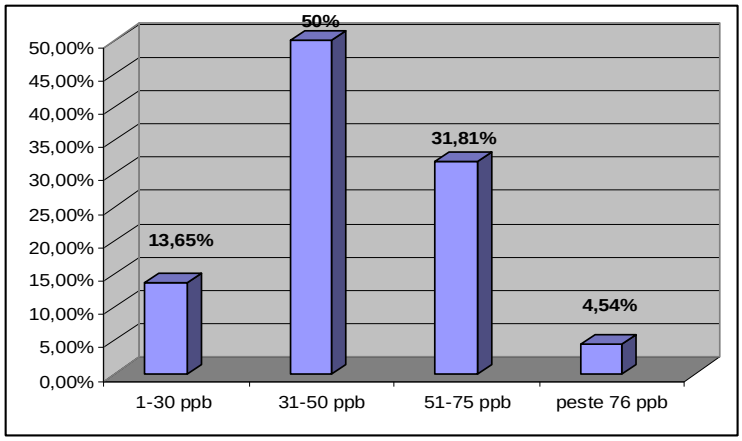

Fig. 2. Graphical representation of samples of bran contamination with zearalenone

The results of analysis of flour samples are these:

- in two samples of wheat flour (10% of total samples), zearalenone is presented in an amount of 31-50 ppb and falls below the maximum permitted by applicable law;
- in eighteen samples of wheat flour (90% of total samples), zearalenone is presented in an amount of 1-30 ppb and falls below the maximum permitted by applicable law

Conclusions

From the analysis of samples represented by wheat flour, bran and wheat using ELISA method was found that a single sample of wheat, and one of bran contained zearalenone amount over the limit while the vast majority of samples (90% flour wheat, 63.65% samples of bran and 35% samples of wheat) are below the permissible content of zearalenone and other samples are well below the maximum allowed. It can be concluded that these raw materials can be used to obtain reliable bakery food.

References

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