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Plant Matrix Reducing Effect of the Object in the Aflatoxin B_1 Defined by Solid-Phase Enzyme Immunodetection

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Abstract.

Plant matrix reducing effect in the aflatoxin B_1 defined by solid-phase enzyme immunodetection in complex plant residues (wheat, corn and silage) is under discussion in this article. Such complex plant residues as wheat, corn and silage were singled out from artificially and naturally contaminated samples by increasing the methanol percentage in the extracting mixture. The study how the methanol percentage (10, 20, 30 and 40%) influences the degree of aflatoxin B_1 extraction in the extracting mixture showed the following. All mixtures provide mycotoxin extraction from the true-positive samples, both in the matrix effect study, and without it. The smallest deviation of 20 and 40% methanol equal to±0.6 % was observed in the study of the mycotoxin content in the control sample containing aflatoxin B_1 at the rate of 10 µg / kg (COB_{10}) without the matrix effect being taken into consideration. However, the maximum matrix effect was observed when 40% methanol was used in the mycotoxin extraction from the plant samples, especially from corn and silage. It is probably due to their coloring pigments maximum extraction. The matrix effect was directly proportional to the methanol amount in the extracting mixture. The less methanol was added, the less the matrix effect was observed. 10 and 20% methanol being used in almost equal deviations in COB_{10} concentration in plant samples, the deviation in COB_{10} was -1.2% and -0.6%, respectively. A minimum matrix effect both in simple plant residues (wheat) and complex (corn, silage) ones was observed when 20% aqueous solution of methanol was used. Mycotoxin aflatoxin B_1 maximum extraction was achieved as well.

Key words: aflatoxin B_1, ELISA, matrix effect.

INTRODUCTION.

Aflatoxin B_1 is a mycotoxin produced by the microscopic fungi Aspergillus flavus and Aspergillus parasiticus. It is referred to highly toxic mycotoxins which belong to one of the dominant groups of biogenic poisons that have been contaminating feed and food lately. Significant evidence has been already summoned up on toxic, carcinogenic, mutagenic, teratogenic and other manifestations of the aflatoxin B_1 biological activity (Allgroft and Carnaghan, 1963; Norred, 1986; Larsson et al., 2003).

Mycotoxins can appear both before and after harvest and accumulate during storage which makes constant monitoring necessary. Food transportation around the world, improper storage and a number of other uncontrolled factors cause mycotoxin contamination. In this regard, many countries have introduced restrictions on the permissible content of mycotoxins in various foods and animal feeds (Off, 2004; Off, 2005 , Off, 2006; Papunidi KKh et al., 2017; Semenov EI et al., 2018).

Chromatography methods including high-performance liquid chromatography with fluorescence detection (Cirlini et al, 2012), especially chromatography in combination with mass spectrometry as the individual aflatoxins (Ventura et al, 2005) or simultaneously with other mycotoxins (Sulyok et al., 2006) are the main methods for aflatoxin B_1 detection (Jaimez et al., 2000; Tutelyan et al., 1987).

Immunoassay methods, including a method of solid-phase immunoassay (ELISA - Enzyme linked immuno-sorbent assay) as the most commonly used format (Raman et al, 2009, Schneider 2003) are much simpler in design implemented with relatively inexpensive equipment. They provide not only high performance due to simultaneous testing in the dozens of samples, but can also detect mycotoxins with high sensitivity and accuracy (Urusov et al., 2010; Mishina et al., 2017).

The 'imperfection' of this method has been revealed in recent studies. The result reliability depends much on the sample matrix, other mycotoxins and their precursors presence which leads to the false positive or false negative results (Wang et al, 2007; Tanaka et al, 1995; Tuzhikova, 2011).

Typically, matrix effects in immunoassays can be controlled by dilution, extracts purification, or by deriving coefficients on matching the matrix effect data with the calibration curve.

A selection of the extractive mixture was carried out in order to study the matrix effect on the maximum toxin detection. The mixture was supposed to provide aflotoxin B_1maximum detection but neither to affect the results of the analysis nor to require significant sample dilution.

MATERIAL AND METHODS.

Conjugate of aflatoxin B_1 - BSA at a concentration of 10 µg/ml in a volume of 150 µl was deluted in 0.1 M carbonate-bicarbonate buffer solution (pH 9.5) for 16-18 hours at a temperature of 4^0C to sensitise plates for setting indirect competitive enzyme linked immunosorbent assay. The plates have been washed three times by phosphate-salt buffer solution (pH 7.4) adding Tween-20 (0.1%). 100 µl of analyzed samples containing toxin in concentrations 0, 5, 10, 20, 50 ng/ml and 100 µl of polyclonal antibody solution in dilution 1:500 were introduced in the wells of the plate. Two wells were used for every concentration. The plate has been incubated at a temperature of 25^0C for 1 hour with constant shaking (250 rpm). 155 µl of horseradish peroxidase conjugate against rabbit JgG ('Sigma') was introduced in a working dilution of 1:5000 into the wells after a three-time washing. A substrate mixture for horseradish peroxidase (3,3', 5, 5'-tetramethylbenzidine) was introduced in the volume of 155 µl after incubation under the same conditions and washing of the plate. It has been incubated for 15 minutes at room temperature in the dark. Then, the color reaction was stopped by adding 1M sulfuric acid solution (H_2S0_4) in the volume of 50 µl. A wavelength of 450 nm on the spectrophotometer 'Multiscan FC' was defined by means of ELISA.

The average characteristics of the optical density measured in the wells with the test solutions were divided by the average value of the optical density measured in the wells with the first (zero) standard. The result was multiplied by 100, this expressed the percentage of signal absorption. The IFA production was performed three times.

The linearity of the calibration line in the analysis carried out on the prepared microplates was the criterion for assessing the advantages of each method.

Digital material processing was carried out by the method of variation statistics using the reliability criterion for the Student on a personal computer using Excel programs.
Phosphate-salt buffer solution (pH 7.4) with methanol addition (from 10-40%) was used to delute the standards for calibration schedule construction (0, 5, 10, 20, 50 ng / ml).
Antibody fractions were prepared in a 10% glycerin solution in the working phosphate-salt buffer solution (pH 7.4) with the addition of Tween-20 (0.1%), BSA (1%), boric acid (1 g). Antispecies conjugate (goat antibodies to rabbit immunoglobulins) was made in a stabilizing solution ‘Immunostab’.
Polyclonal antibodies to mycotoxin were obtained by rabbits’ repeated immunization with aflatoxin B_1-BSA conjugate.
Two samples with true positive ($COB_{10samples}$, relatively clean samples contaminated with mycotoxins at the rate of 10 mg/kg) and true negative ($COB_{10samples}$ - relatively clean samples) were imposed to assess the aflatoxin B_1 ELISA matrix effect in the analysis. The matrix effect was studied on the samples from wheat, corn and silage.
These samples were carried out through a sample preparation scheme: 1.0 grams of ground sample, contaminated with aflatoxin B_1 at the rate of 10 µg/kg, filled with 5.0 ml of aqueous methanol solution (10-40%). It was kept for 15 minutes at room temperature, filtered and mixed 1:1 with a working solution of phosphate-salt buffer solution.
The tests without the studied matrix effect (COB_{10} and COB_0): 5.0 ml of aqueous methanol (10-40%), contaminated with aflatoxin B_1 based 10bмкг/kg were introduced in the analysis to assess the degree of toxin extraction. They were kept 15 minutes at room temperature, filtered and mixed 1:1 with a solution of phosphate-saline buffer solution.

RESULTS AND DISCUSSION.

The results of the methanol percentage influence on the degree of aflatoxin B_1 extraction from true-positive (COB_{10}) and true-negative (COB_0) samples in the extracting mixture and calibration solutions are presented in the table.
The study of the methanol percentage influence on the degree of aflatoxin B_1 extraction in the extracting mixture showed that all mixtures provide the mycotoxin extraction from truly positive samples both in the study of the matrix effect and without it.
The lowest deviation in the study of mycotoxin content in COB_{10} without studying the matrix effect was observed when using 20 and 40% methanol, which was ±0.6 %. However, the maximum matrix effect was observed at 40% methanol in the mycotoxin extraction from plant samples, especially from corn and silage. It is probably due to their coloring pigments maximum extraction. In this case, additional studies to select dilution modes of samples after extraction are required to improve the analysis results. In most cases, the value of the matrix effect is directly proportional to the amount of methanol in the extracting mixture. The less methanol is added, the less the matrix effect is. Almost equal deviations of COB_{10} concentrations in plant samples were obtained at 10 and 20% methanol. The deviation in COB $_{10}$ was -1.2% and -0.6%, respectively.

Table –Results of the Methanol Percentage Influence on the Degree of Aflatoxin B_1 Extraction in the Extracting Mixture from Any Plant Residues.

Calibration solutions and samples	Methanol percentage in the extraction mixture			
	10%	20%	30%	40%
Aflatoxin B_1 0.0 ng / ml	100	100	100	100
Aflatoxin B_1 5.0 ng / ml	84.87±1.6	88.5±2.1	88.85±2.1	88.34±1.8
Aflatoxin B_1 10.0 ng / ml	72.34±1.4	70.2±2.1	70.08±1.4	69.6±1.6
Aflatoxin B_1 20.0 ng / ml	54.56±0.95	55.41±2.8	61.62±2.1	53.20±2.1
Aflatoxin B_1 50.0 ng / ml	33.50±1.9	29.39±1.6	44.97±2.4	36.61±1.8
COB_{10}	73.5±1.2	70.8±3.1	72.11±1.6	68.96±1.6
COB_{10} wheat	73.92±1.8	71.8±1.6	74.79±2.0	65.96±3.2
COB_{10} corn	73.9±1.6	72.5±2.15	74.47±1.6	74.8±1.6
COB_{10} silage	76.2±1.45	74.04±2.6	74.8±1.7	76.02±3.1
The deviation of the mycotoxin concentration COB_{10} , %	-1.2	-0.6	-2.03	0.64
The deviation of the mycotoxin concentration in wheat COB_{10}, %	-1.62	-1.6	-4.71	3.64
The deviation of the mycotoxin concentration in corn COB_{10}, %	-1.6	-2.3	-4.39	-5.2
The deviation of the mycotoxin concentration in silage COB_{10}, %	-3.9	-3.84	-4.72	-6.42
COB_0 wheat	101.8	101.8	104.8	103.9
COB_0 corn	103.8	102.6	105	102.4
COB_0 silage	107.9	108.8	108.1	112.7

CONCLUSION.

The extraction of aflatoxin B_1-BSA from plant samples should be carried out with 20% aqueous solution of methanol taking into account all the above, as well as the fact that the linearity of the calibration line in the analysis is the criterion for assessing the advantages of each method. This ratio achieves a minimum matrix effect in simple plant residues (wheat) and complex (corn, silage) ones, as well as the mycotoxin aflatoxin B_1maximum extraction.

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