Determination of Aflatoxins in Food Products by the ELISA Method

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Abstract

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To determine the total content of aflatoxins, aflatoxin B_1 and aflatoxin M_1 in food the ELISA method was used. Milk, dairy products and cereal samples were mainly investigated. A few samples were found to be contaminated with aflatoxins. A great usability of the ELISA method for aflatoxin determination in food was established. Selectivity and sensitivity of the method is reported.

Keywords: aflatoxins; ELISA; immunoenzymatic method

Mycotoxins are secondary metabolites of microscopic fungi which are not indispensable to the fungi's life but show toxic effects on human beings, animals, plants and microorganisms. The toxin-producing fungi include: *Penicillinum*, *Aspergillus* and *Fusarium* being developed on agricultural products (CVETNIC & PEPELJNJAK 1995; YAMASHITA *et al*. 1995).

The aflatoxin content in food and fodder has been determined so far mostly by conventional analytical techniques such as: thin layer, gas or liquid chromatography, spectrofluorometry and spectrophotometry (BARBIERI *et al.* 1994; LEBIEDZIÑSKA & GANOWIAK 1995; BRADBURN *et al.* 1995; PEI-YIN WU *et al.* 1995; SABINO *et al.* 1995; ESPINOZA *et al.* 1996). These methods require special and expensive apparatus and instruments as well as laborious and time consuming preparation of samples connected with the separation of the given component from a mixture.

As shown in our previous studies (LESZCZYŃSKA *et al.* 1997, 1998), the immunochemical methods with the use of antigen-antibody reactions belong to selective, sensitive and fast techniques, being relatively inexpensive at the same time. Recently, they are more frequently used for the determination of food components (DESPHANDE 1994; PESTKA *et al.* 1995). These methods have also found application in the examination of the level of aflatoxins (BACIGALUPO *et al.* 1994; PESTKA *et al.* 1995; ABOUZIED *et al.* 1991; BRADBURN *et al.* 1995; DRAGACCI *et al.* 1995; JOANNON-KAKOURI *et al.* 1995).

The aim of the present study was to examine the level of aflatoxins (total and B_1 and M_1) in selected commercially available food products: milk, cheese, yoghurt, kefir, cereal products and to compare these results using Ridascreen-Aflatoxin tests. The usability of the three commercially available tests for the determination of aflatoxins in food samples was evaluated.

MATERIAL AND METHODS

Materials

Aflatoxins were determined in food products available in retail outlets. Aflatoxins were extracted from food samples with a methanol-water mixture (7:3 or 4:1) and solvents such as chloromethane or heptane.

Reagents and Tests

The aflatoxins-total test included the following components: a 96-well plate coated with antibodies to mouse antibodies; standard solutions of aflatoxin B_1 in methanol with concentrations: 0, 0.5, 1.5, 4.5, 13.5 and 40.5 ng per cm^3; a conjugate of peroxidase and aflatoxin B_1; a solution of monoclonal mouse antibodies to aflatoxin; a substrate: urea peroxide; a chromogen: tetramethylbenzidine; a reagent for process termination: sulphuric acid (1 mol/dm^3); a buffer for dilution (PBS 0.1 mol/dm^3, pH 7.4).

A set for the determination of aflatoxins B_1 consisted of a 96-well plate coated with antibodies to aflatoxin B_1; standard solutions of aflatoxin B_1 in methanol containing: 50, 100, 200, 500, 1000 and 5000 ng/dm^3; a buffer for diluting standard solutions; a solution of aflatoxin-peroxidase conjugate; a buffer for the conjugate; a solution of urea peroxide; a chromogen containing tetramethyl-benzidine; a reagent for process termination:

0.25 mol/dm^3 sulphuric acid; a buffer for washing (PBS 0.1 mol/dm^3, pH 7.4).

A set for the determination of aflatoxin M_1 included a 96-well plate coated with antibodies to aflatoxin M_1; standard solutions of alfatoxin M_1 in milk with concentrations: 0, 5, 10, 20, 40, and 80 ng/dm^3, a solution of peroxidase-aflatoxin M_1 conjugate; a substrate containing urea peroxide; a chromogen containing tetramethylbenzidine; sulphuric acid (1 mol/dm^3) as a reagent for process termination; a buffer for diluting the solutions of examined and standard samples; a buffer for diluting the conjugate.

The experiments included three special tests: Ridascreen Aflatoxin Total, Ridascreen Aflatoxin B_1 and Ridascreen Aflatoxin M_1, all of them of R-Biopharm (Darmstadt, Germany).

Apparatus and Equipment: The moisture content of the examined samples was determined by means of a WPE 30s balance of Radwag (Poland). The samples were disintegrated by means of a high-speed CAT x-120 homogeniser of Cole-Palmer (USA) (speed 4, 10 min) and the precipitates were centrifuged with a 310 centrifuge of Mag-Pol (Wrocław, Poland) (3500 rpm, 10 min.). The plates were washed using a special washing machine (Biotek Instruments INC, USA). Absorbances of the examined and standard solutions were measured by means of a reading apparatus for ELISA tests. Multi-channel pipettes of Sigma and automatic pipettes with various volumes were also used.

Total Aflatoxin Content: About 2 g of a disintegrated sample was weighed to ±0.0001 g and extracted with 10 ml of methanol–water mixture (7+3) to separate aflatoxin. To that end, the rest was homogenised for 10 min at room temperature and then the resultant deposit was centrifuged. An aliqout (100 µl) of the supernatant was diluted with 600 µl of phosphate buffer at pH = 7.2. An aliquot of this solution (50 µl) or standard solution (50 µl), 50 µl of the aflatoxin-peroxidase conjugate and 50 µl of the mouse antibody solution against alfatoxin were added to each well of the used plate. The determination was repeated three times. The samples were incubated for 30 min at room temperature in the darkness. The free and peroxidase-combined aflatoxins compete for the combining site with antibodies to mouse antibodies immobilised on the plate. Next, the plate was emptied and washed five times with phosphate buffer at pH = 7.2. Then, 50 µl of tetramethylbenzidine and 50 µl of urea peroxide were added and incubated again for 30 min in darkness. The reaction was terminated by adding 100 µl of the stop reagent. The absorbance of solution was measured at a wavelength of 450 nm, using an ELISA reading apparatus. The content of aflatoxins was calculated using the previously prepared standard curve.

Aflatoxin B_1 Content: About 2 g of sample weighed to ±0.0001 g was extracted and homogenised simultaneously for 10 min in a homogeniser with 6 ml of methanol–water mixture (4+1) and centrifuged (10 min at 3500 rpm). An aliquot (100 µl) of the supernatant was diluted with 700 µl of phosphate buffer and the resultant solution was used for determinations.

An aliquot (100 µl) of the examined sample solution or the standard solution and 50 µl of the aflatoxin-peroxidase conjugate solution were added to each plate well. The rest was stirred and incubated at room temperature for 1 h. Next, the wells were emptied and washed five times with 0.1 mol/l phosphate buffer (pH = 7.2). Then, 150 µl of the chromogen (tetramethylbenzidine)-substrate (urea peroxide) mixture was added to each well and the whole solution was incubated at room temperature for 30 min in darkness. Once the reaction was terminated by adding the stop reagent, the absorbance was measured at the wavelength of 450 nm by using the ELISA reading apparatus. The aflatoxin B_1 content was calculated from the reference curve.

Aflatoxin M_1: The tested samples (5 ml) of milk were cooled to 10°C and centrifuged (10 min at 3500 rpm) to separate fat. To weighed cheese samples (2.0000 ± 0.0001 g), 40 ml of dichloromethane was added and they were extracted for 2 min by means of a homogeniser. After filtration, the oily residue was dissolved in 0.5 ml of methanol and 0.5 ml of phosphate buffer at pH = 7.3 and then, 1 ml of heptane was added. Next, the deposit was centrifuged for 15 min at 3500 rpm and 15°C. After removing the heptane layer, 100 µl of the methane phase was mixed with 400 µl of phosphate buffer to obtain a solution sample for the determination of aflatoxin M_1. An aliquot (100 µl) of the tested sample solution or the standard solution was added to each well of 96 well plate coated with antibodies to aflatoxin M_1 and incubated for 60 min at a room temperature. Once the plate was emptied and washed five times, 100 µl of the aflatoxin M_1-peroxidase conjugate solution was added and the whole solution was incubated for 60 min at room temperature. The plates were washed five times and then 50 µl of the substrate and 50 µl of the chromogen were added. After 30 min of incubation at a room temperature, the reaction was terminated and the absorbance of solution was measured by means of the ELISA reading apparatus at the wavelength of 450 nm. The incubation process was carried out in darkness. The aflatoxin M_1 content was calculated from the standard curve. The stages of the complete determination of aflatoxin M_1 are the same as those of aflatoxin B_1.

RESULTS AND DISCUSSION

The preparation procedure in the immunoenzymatic methods is very simple as it consists mainly in extraction and there is no need for purification or isolation of the tested component.

In the present study, the usability of the three commercially available tests for the determination of aflatoxins in food samples was evaluated. Despite the fact that the sensitivity of the total aflatoxin content test is rather low, amountin to about 25 ng/kg, its use for testing real samples is considered reasonable since it allows one to be selected samples containing aflatoxins. The selected samples and those which give uncertain results can be additionally tested by means of more sensitive tests for aflatoxins M_1 and B_1. It has been shown that the sensitivity of the aflatoxin B_1 test is 12.5 ng/kg and that of aflatoxin M_1 – 2.5 ng/kg.

Competitive ELISA methods were used in the tests. The antibodies anti-B_1 give cross reaction with aflatoxins G_1 (21%) and B_2 (14%). In the case of this determination, an error can be made: an overstated result due to the presence of aflatoxins G_1 and B_2. However, the antibody to aflatoxin M_1 is more specific. It gives cross reactions with aflatoxin B_1 (12.4%) and aflatoxin M_2 (9.5%), so considerably lower than in the case of the antibodies anti-B_1. The antibodies used in the Aflatoxin Total test give positive cross-reactions with almost all the antibodies – aflatoxin B_1 100%, B_2 200%, G_1 15%, G_2 16%, M_1 63%. Of the several dozens of tested samples, only one of milk (Table 1) and two of dairy products (Table 2) show the aflatoxin content – 0.062 ng/g of aflatoxin M_1 in yoghurt and 0.260 ng/g of aflatoxin B_1 in cheese. Similar quantities of aflatoxins were detected in food products by other researchers, for example LEBIEDZIŃSKA and GANOWIAK (1995) detected 745 μg of aflatoxin B_1 and 50 μg of aflatoxin G_1 per 1 kg of nuts. ABOUZIED *et al*. (1991) determined from 5 to 100 mg/kg of aflatoxins in cereal products. In the years 1983–1986, SCHATZKI (1995) showed that pistachio nuts were contaminated with 3 to 12% of aflatoxins. In some samples (Table 1 and 3) total aflatoxin content was found but neither aflatoxin B_1 nor M_1 were found. It might be caused by the presence of other aflatoxin or the products of their metabolism, or some cross-reactions.

Table 1. The content of aflatoxin in milk determined by the ELISA method (6 determinations)

Foodstuff		Aflatoxin content	
		total [μg/dm^3]	M_1 [ng/dm^3]
Goat milk	1	<0.15 ± 0.03	<75 ± 5
	2	0.38 ± 0.03	<75 ± 5
Cow milk from foil 2%	1	0.28 ± 0.16	<75 ± 5
	2	1.08 ± 0.07	<75 ± 5
	3	1.50 ± 0.11	<75 ± 5
Milk 0.5%	1	1.65 ± 0.12	<75 ± 5
	2	0.99 ± 0.07	<75 ± 5
	3	1.56 ± 0.11	300 ± 21
UHT milk	0.5%	0.38 ± 0.03	<75 ± 5
	3.2%	1.50 ± 0.11	<75 ± 5

Despite the fact that aflatoxins have been detected only in some food products, the obtained results point to the necessity of food inspection for contamination with mildew and mycotoxins. Due to their toxicity, aflatoxins should not be present in food in quantities detectable by available methods. According to HAMILTON (1984), "in the present state of knowledge, one cannot be relied upon safe dosages of mycotoxins in foods", hence any dosage presents a serious hazard. The contamination of the human organism with bacteria brings about an immediate intoxication and its diagnosis is relatively simple, with an appropriate treatment being immediately applied. On the other hand, the consumption of food products contaminated with mildew results in no immediate symptoms but only in the future in the form of tumors as well as mutagenic and teratogenic effects.

Food covered with mildew must not be consumed at all. However, mildew can be invisible. Therefore, a systematic inspection of food for mildew contamination is necessary. Microbiological methods are laborious and time-consuming. The most advisable is the determination

Table 2. The content of aflatoxin in selected dairy products

Product	Total		B_1		M_1	
	[ng/g]	[ng/g$_{dm}$]	[ng/g]	[ng/g$_{dm}$]	[ng/g]	[ng/g$_{dm}$]
Yellow cheese	<0.15 ± 0.01	<0.21 ± 0.01	<0.50 ± 0.01	<0.21 ± 0.01	<0.075 ± 0.005	<0.060 ± 0.007
Smoked cheese	<0.15 ± 0.01	<0.24 ± 0.02	<0.15 ± 0.01	<0.24 ± 0.02	<0.074 ± 0.005	<0.121 ± 0.007
Cottage cheese	<0.14 ± 0.01	<0.36 ± 0.03	<0.14 ± 0.01	<0.36 ± 0.03	<0.069 ± 0.005	<0.177 ± 0.012
Cheese	2.49 ± 0.17	7.63 ± 0.53	0.26 ± 0.02	0.80 ± 0.07	<0.050 ± 0.005	<0.153 ± 0.011
Yoghurt	<0.13 ± 0.01	<0.56 ± 0.03	<0.13 ± 0.01	<0.56 ± 0.04	<0.067 ± 0.005	<0.288 ± 0.020
Kefir 2%	<0.11 ± 0.01	<0.98 ± 0.07	<0.11 ± 0.01	<0.98 ± 0.08	<0.053 ± 0.005	<0.473 ± 0.033
Yoghurt	4.22 ± 0.30	18.30 ± 1.30	<0.10 ± 0.01	<0.43 ± 0.03	0.062 ± 0.005	0.260 ± 0.018
Yoghurt	<0.11 ± 0.01	<0.49 ± 0.03	<0.11 ± 0.01	<0.49 ± 0.03	<0.055 ± 0.005	<0.243 ± 0.017

Table 3. The results of aflatoxin determination in cereals and some other samples

Product	Total		B_1		M_1	
	[ng/g]	[ng/g_{dm}]	[ng/g]	[ng/g_{dm}]	[ng/g]	[ng/g_{dm}]
Oat rings	3.75 ± 0.26	4.05 ± 0.28	<0.15 ± 0.02	< 0.16 ± 0.02	<0.075 ± 0.005	<0.081 ± 0.006
Corn flakes	0.30 ± 0.02	0.37 ± 0.02	<0.15 ± 0.02	< 0.19 ± 0.02	<0.075 ± 0.005	<0.092 ± 0.006
Walnuts I	0.30 ± 0.02	0.32 ± 0.02	<0.15 ± 0.02	< 0.16 ± 0.02	<0.074 ± 0.005	<0.078 ± 0.006
Walnuts II	0.30 ± 0.02	0.32 ± 0.02	<0.15 ± 0.02	< 0.16 ± 0.02	<0.074 ± 0.005	<0.078 ± 0.006
Snickers cream	<0.14 ± 0.02	<0.15 ± 0.02	<0.14 ± 0.02	< 0.16 ± 0.02	<0.068 ± 0.005	<0.073 ± 0.006
Buckwheat cereals	0.30 ± 0.02	0.33 ± 0.02	<0.11 ± 0.02	< 0.12 ± 0.02	<0.055 ± 0.005	<0.062 ± 0.005
Cocoa balls	0.29 ± 0.02	0.30 ± 0.02	<0.15 ± 0.02	< 0.16 ± 0.02	<0.074 ± 0.005	<0.076 ± 0.006
Pearl barley	<0.15 ± 0.02	<0.17 ± 0.02	<0.15 ± 0.02	< 0.17 ± 0.02	<0.075 ± 0.005	<0.083 ± 0.006
Rice	<0.15 ± 0.02	<0.17 ± 0.02	<0.15 ± 0.02	< 0.17 ± 0.02	<0.074 ± 0.005	<0.085 ± 0.006
Wheat flour	<0.15 ± 0.02	<0.18 ± 0.02	<0.15 ± 0.02	< 0.18 ± 0.02	<0.073 ± 0.005	<0.088 ± 0.006
Almonds	<0.15 ± 0.02	<0.16 ± 0.02	<0.15 ± 0.02	< 0.16 ± 0.02	<0.074 ± 0.005	<0.077 ± 0.006
Hazel nuts	<0.15 ± 0.02	<0.16 ± 0.02	<0.15 ± 0.02	< 0.16 ± 0.02	<0.074 ± 0.005	<0.077 ± 0.006
Liver	<0.13 ± 0.02	<0.39 ± 0.02	<0.13 ± 0.02	< 0.39 ± 0.02	<0.064 ± 0.005	<0.190 ± 0.013

of aflatoxins by the ELISA method to detect one metabolite accumulation of which is accompanied by the mildew development (YONG 1995; GOURAMA & BULLERMAN 1995). Such a metabolite can be for example ergosterol (GOURAMA & BULLERMAN 1995) and its accumulation results in aflatoxin formation. It follows from our studies that the ELISA method is very convenient and most sensitive among many available methods. This method makes it possible to carry out the fast analysis of a considerable number of samples without any laborious stage of sample preparation.

CONCLUSIONS

The immunoenzymatic method (ELISA) can be successfully used for the determination of pollutans contaminatants such as aflatoxin in food. The method makes it possible to determine aflatoxin M_1 at a level of 2.5 ng/kg, aflatoxin B_1 – 12.5 ng/kg and total aflatoxin – 25 ng/kg. Aflatoxins were found in some samples at a very low level.

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Souhrn

LESZCZYŃSKA J., MASŁOWSKA J., OWCZAREK A., KUCHARSKA U. (2001): **Stanovení aflatoxinů v potravinářských produktech metodou ELISA**. Czech J. Food Sci., **19**: 8–12.

Metodou ELISA byl v mléce, mléčných výrobcích a ve vzorcích cereálních výrobků stanoven celkový obsah aflatoxinů, obsah aflatoxinu B_1 a aflatoxinu M_1. Bylo zjištěno, že některé vzorky byly aflatoxiny kontaminovány. Byly stanoveny selektivita a citlivost metody ELISA pro stanovení aflatoxinů v potravinách.

Klíèová slova: aflatoxiny; ELISA; imunoenzymatická metoda

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