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ARTICLES

Determination of Zearalenone and Ochratoxin A Mycotoxins in Grain by Fluorescence Polarization Immunoassay

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Abstracts—Rapid procedures are reported for the determination of zearalenone and ochratoxin A, which make possible the analysis of up to 10 grain samples within 20 min by fluorescence polarization immunoassay. The influence of various fluorescein-based tracers on the sensitivity of analysis is examined. The most promising was admitted the use of ethylenediamine thiocarbamoylfluorescein and aminomethylfluorescein. The detection limits of the method for zearalenone and ochratoxin A were 3 and 1.5 ng/mL for a model system and 15 and 10 μg/kg for grain, respectively. The performed analysis of spiked grain samples demonstrated the recovery of mycotoxins at a level of 84–97%.

Keywords: mycotoxins, zearalenone, ochratoxin A, FPIA

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Fluorescence polarization immunoassay (**FPIA**) was first proposed in 1973 and then, after a number of advances made in the scope of instrumentation and branch of application, has become an important tool of bioanalytical chemistry [1]. FPIA is based on the competition between an analyte and a tracer, i.e., an antigen bearing a fluorescent tag, for binding with specific antibodies and the registration of fluorescence polarization of the reaction mixture, which turns to be proportional to the analyte concentration. To its advantages one can attribute rapidity, high specificity and sensitivity, as well as the simplicity of analytical procedures, which ensures a great abundance of the method for the primary screening of various low-molecular contaminants [2].

An important point in the development of a method of fluorescence polarization immunoassay corresponds to the selection of antibodies and the fluorescent tag [3, 4]. Most frequently fluorescein-based tags are used in FPIA. The lifetime of fluorescein and its derivatives in the exited state is about 4 nsec and so is optimal for the registration of fluorescence polarization [5]. The tags based on fluorescein may differ n the following parameters: the length of spacer binding the fluorescein molecule to the antigen one and also its flexibility and location. These factors may affect the sensitivity of FPIA techniques.

Mycotoxins are produced by microscopic mold fungi. Many of them are proved to bear carcinogenic, mutagenic, and hepatotoxic properties and also exert an immunosuppressive effect [6, 7]. These substances are rather stable to environmental effects and do not decompose even upon thermal processing; therefore, the contamination of feed and food stuff with them is potentially harmful to people. In this respect, low minimal permissible concentrations (**MPCs**) are established for the most toxic of them. For instance, according to the EU regulations, MPCs of zearalenone (**ZEN**) varies in the range from 20 to 400 μg/kg (Fig. 1a), and for ochratoxin A (**OTA**), from 0.5 to 80 μg/kg in dependence on the type of food and the degree of grain procession (Fig. 1c) [8].

In Russian Federation according to the governmental sanitary norms, MPC for ZEN varies from 1 to 1000 μg/kg and for ochratoxin, from 1 to 5 μg/kg [9]. Hence, in the development of such procedures, special attention should be paid to the sensitivity achieved.

Most often in the works devoted to the development of FPIA methods for mycotoxins, ethylenediamine thiocarbamoylfluorescein (**EDF**) is used as the tag [10]. In the actual work, we are about to compare EDF with other tags based on fluorescein and examine the influence of the tag on the sensitivity of the determination of zearalenone and ochratoxin A in grain by FPIA.

EXPERIMENTAL

Instruments and reagents. We used zearalenone, ochratoxin A, fluorescein isothiocyanate (**FITC**), ethylene diamine hydrochloride, hexamethylene diamine, piperazine, carboxymethylamine hydrochloride (Sigma, United States), aminomethylfluorescein (**AMF**), and glycinaminofluorescein (**GAF**) (Molecular Probes, United States). As solvents we used methanol, triethylamine, chloroform (Reakhim, Russia), and twice-distilled water. The monoclonal

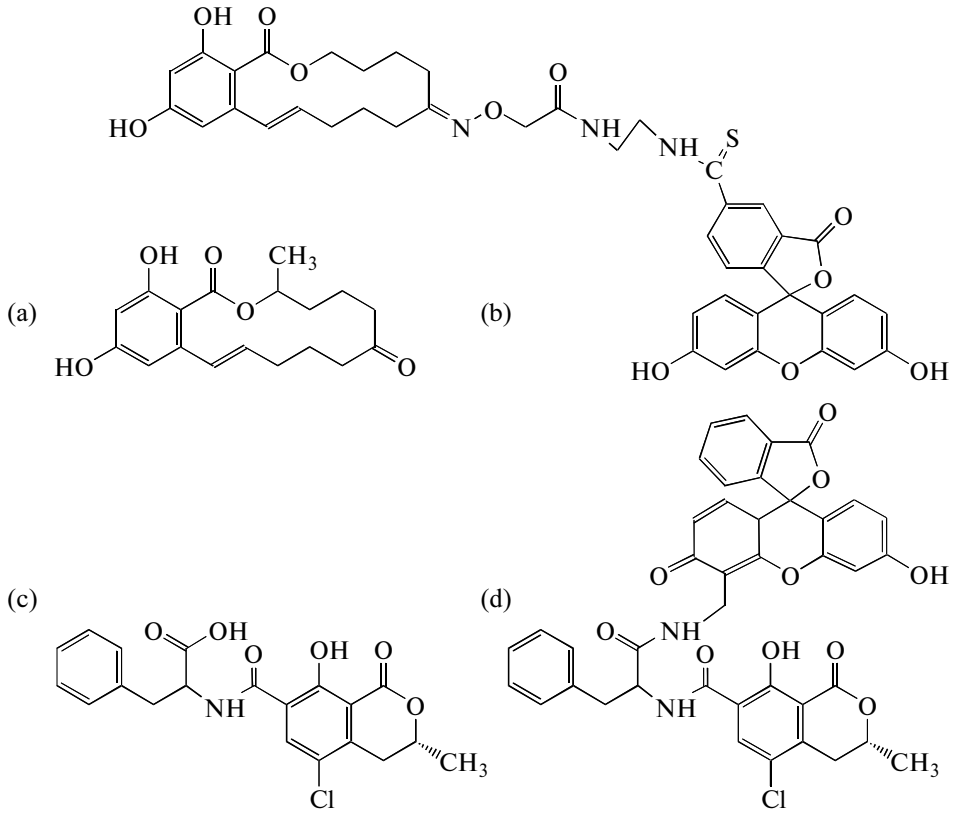

Fig. 1. Structural formulas of (a) zearalenone, (b) tracer ZEN-ATF, (c) ochratoxin A, and (d) tracer OTA-AMF.

antibodies specific against zearalenone were provided by our colleagues from Gyeongsang National University, Korea [11]; the ones against ochratoxin A were provided by scientists from Chung Shan Medical University, Taichung, Taiwan [12].

For experiments we used a 2.5 mM borate buffer solution with pH 8, also containing 0.1% NaN_3. To prepare it, we dissolved 0.1 g of sodium azide (Serva, United States) and 0.95 g of sodium tetraborate decahydrate (Sigma-Aldrich, United States) in 1 L of water.

Fluorescence polarization was registered on a TDx polarization fluorimeter (Abbott, United States) in the Photocheck mode.

In the work we used certified grain of the R-biopharm brand (Germany) and also grain samples earlier tested by HPLC in the FSE All-Russia State Center for Quality and Standardization of Veterinary Medications and Feed. The detection limits for zearalenone and ochratoxin A in HPLC used in there were 0.1 and 0.5 μg/kg, respectively.

For the processing of grain samples, we used a Vibrofix VF1 Electronic vortex (Junke and Kunkel, Kika-Werk) and a Mini Spin centrifuge (Eppendorf).

Synthesis of fluorescent tags. The fluorescent tags were synthesized as described in [13] for the synthesis of EDF from FITC and ethylenediamine dihydrochloride. In the same manner the following tags were prepared: Lys-FITC, piperazinyl fluorescein isocthiocyanate (**PIP**) and hexamethylenediamine fluorescein isothiocyanate (**HMF**) by means of FITC and lysine, piperazine, and hexamethylenediamine, respectively.

Synthesis of tracers. In the preparation of tracers to ZEN, we first synthesized its carboxymethyloxime derivative (**ZEN-CMO**). For that 100 mg of zearalenone and 200 mg of carboxymethylamine hydrochloride were dissolved in 25 mL of pyridine. The reaction mixture was left at ambient temperature for two days until ZEN-CMO formed [14], which was used then for synthesis of tracers to ZEN. The tracers to OTA were prepared from the intact OTA.

We prepared three tracers based on ochratoxin A using ADF, AMF, and Lys-FITC as tags and five tracers based on zearalenone using EDF, GMF, AMF, PIP, and GAF. The synthesis of fluorescein-tagged ZEN and OTA was accomplished by the carbodiimide protocol as described in [15, 16]. For that 4 mg of a micotoxin was dissolved in 0.2 mL of dimethylformamide, which also contained 3mg of *N*-GS and 5 mg of *N,N'*-DCC. A 5-mg portion of a fluorescein label was added to the solution obtained after stirring for two hours at ambient temperature; the reaction mixture was left in a dark place for two days until the tracer formed. Then it was purified by thin-layer chromatography with the

chloroform : methanol 4 : 1 eluent. The purified tracers were dissolved in methanol and stored in a fridge at 4°C. Working solutions of tracers were prepared by the dilution of methanol solutions with a borate buffer solution to the concentrations required.

Method of fluorescence polarization immunoassay. The optimal dilution of antibodies for each tracer was chosen from the curves of titration of antibodies. For that the concentration of antibodies was stepwise reduced twofold, starting from the dilution 200 down to 1024000, after that, we added 500 μL of a tracer working solution to each solution of antibodies in 500 μL of a borate buffer and measured the fluorescence polarization. As the working one, we chose the dilution of antibodies which corresponded to the signal lower by 30%.

To build the relation of fluorescence polarization to the mycotoxin concentration, we prepared series of zearalenone and ochratoxin A standard solutions with concentrations from 1 to 10000 ng/mL in 60% aqueous methanol. Then 50 μL of a standard solution of mycotoxin was transferred into the FPIA cell followed by 500 μL of antibodies and 500 μL of the tracer; the mixture was stirred, and fluorescence polarization was measured immediately.

The detection limit for an analyte in FPIA was established according to the IUPAC recommendations, using the Rodbard's technique [17]. For that we registered the fluorescence polarization of 50 μL of 60% aqueous methanol, 500 μL of the tracer working solution, and 500 μL of the antibody working solution. The experiment was repeated for 20 samples. From the data acquired we found the value of mP_0 as the mean value of fluorescence polarization, and S as the standard deviation of the analytical signal. After that, we determined the value of fluorescence polarization, which corresponded to the detection limit of the method: $mP_{min} = mP_0 - 3S$, so the detection limit for a mycotoxin was established from the calibration graph generated earlier.

To describe and compare the acquired calibration relations, we determined the value of IC_{50}, i.e., the concentration of antigen corresponded to a 50% reduction of the analytical signal. This value is determined by the inflection point in the curve, determined by the approximation of the data by a sigmoid function.

Procedure for pretreatment of grain samples. A 15-mL portion of a mixed extractant composed of methanol and distilled water taken in the ratio 3 : 2 was added to 3.0 g of ground grain. The samples were extracted for 5 min and then centrifuged for 10 min at 10000 rpm. The supernatant prepared was examined for the presence of mycotoxins. Using extracts prepared from pure grain, we prepared series of standard solutions of zearalenone and ochratoxin A with concentrations from 1 to 10000 ng/mL; using them the relations of fluorescence polarization to the mycotoxin concentration with respect to the matrix effect of grain were plotted.

RESULTS AND DISCUSSION

The carboxyl groups in ZEN-CMO and OTA are inactive; for their activation we used *N,N'*-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide in a medium of an aprotic solvent. After the synthesis the tracers were isolated from the reaction mixtures by thin-layer chromatography, and their optimal, i.e., working, concentrations in the borate buffer solution were established. For that we searched for the tracer concentration at which the intensity of fluorescence overpowered the noise by 10.

The binding of the tracers prepared with specific monoclonal antibodies was evaluated. From the obtained curves of relation fluorescence polarization vs. antibody dilution, we selected the optimal dilution for each case, which corresponded to a 70% degree of the tracer bound in an immunocomplex. For all tracers based on zearalenone, the working dilution of monoclonal antibodies specific to ZEN was 1 : 1550. The optimal dilution of monoclonal antibodies to OTA for the tracers OTA–AMF OTA–EDF and OTA–Lys–FITC was 1 : 20000, 1 : 10000, and 1 : 10000, respectively.

To choose an optimal tracer we, generated relations of the relative signal mP/mP_0 to the concentration of each mycotoxin, where mP is fluorescence polarization recorded for a specific concentration of an analyte, and mP_0 is the value for the blank sample (Fig. 2). To compare the sensitivity of analysis methods using tracers with different fluorescent tag for each pair of immunoreagents, factor IC_{50} was calculated as along with the detection limits for mycotoxins; these are presented in Table 1.

The lowest detection limit for zearalenone by FPIA is attained if ZEN-EDF and ZEN-AMF tracers are used (Fig. 2a); moreover, the calibration curves for them virtually coincided. Hexamethylene diamine probably occurs a too long spacer, so fluorescein appears more flexible than with ZEN-EDF and ZEN-AMF; in contrast, aminoglycine and piperazine bind the fluorophore molecule with the antigen too strongly. In the case of ochratoxin, A the optimal choice is also provided by ethylenediamine thiocarbamoylfluorescein and aminomethylfluorescein; therein the use of AMF (Fig. 2b) results in a somewhat higher sensitivity than with EDF. The absence of a competition in using Lys-FITC as a tag may also refer to the significant length and flexibility of the spacer binding the fluorescein with the antigen.

Therefore, using EDF and AMF as fluorescent tags made possible the development of more sensitive methods for ZEA and OTA by FPIA in model systems with detection limits 3 and 1.5 ng/mL, respectively (Fig. 3).

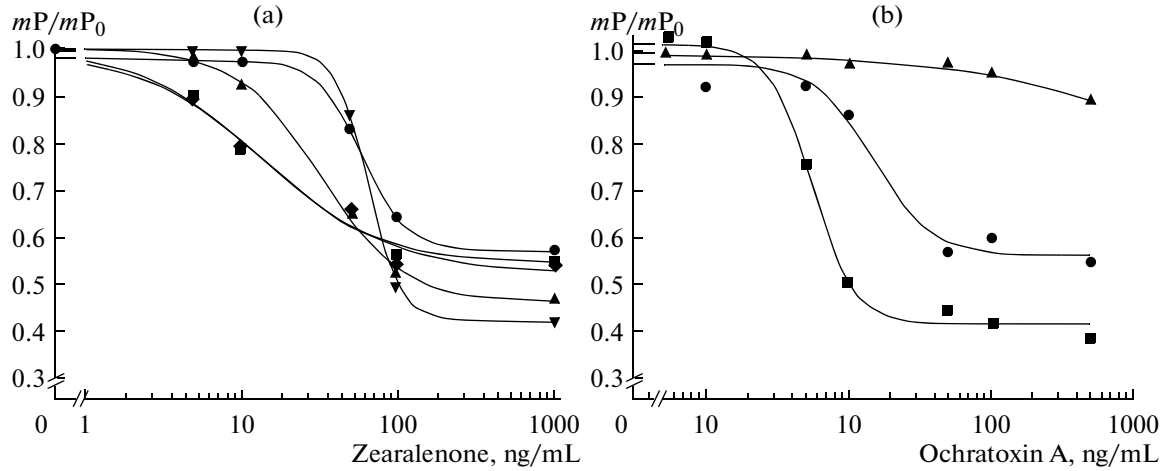

Fig. 2. Analytical signal (mP/mP_0) vs. concentration of analyte for various tracers, for (a) zearalenone: ■ ZEN-EDF, ● ZEN-PIP, ▲ ZEN-GMF, ▼ ZEN-GAF, ◆ ZEN-AMF, and (b) ochratoxin A: ■ OTA-AMF, ● OTA-EDF, ▲ OTA-Lys-FITC.

The optimal pairs of immunoreagents were utilized for the determination of zearalenone and ochratoxin A in grain. As on a common method for the pretreatment of grain samples for analysis, we decided on extraction with a methanol–water mixture in the voluminous ratio 3 : 2. The calibration plots were emulated using a grain pure of mycotoxins. As was found, for the chosen procedure of sample pretreatment for FPIA, the matrix effect of grain virtually does not change the shape of calibration graphs. Apparently this means that the extracts have no specific coloration, and the viscosities of their solutions are practically equal. As it was shown, pretreatment based on the extraction of analytes from the sample with methanol solutions and their subsequent use for FPIA are much more rapid (no more than 15 min) and simpler than the pretreatment of the very same samples required for the chromatography determination of mycotoxins.

The accuracy of the method for mycotoxin quantification was established by the added–found technique (Table 2).

The proposed way of sample pretreatment for grain ensures the high accuracy of mycotoxin analysis, the recovery makes from 84% at low concentrations to 97% in the work in the linear section of the sigmoid relation. All these suggests the high efficiency of the developed procedure for the determination of mycotoxins by FPIA. Since for the analysis of 1 g of ground grain 5 mL of an extraction mixture was used, with respect to dilution the detection limits for zearalenone and ochratoxin A in the developed procedures were 15 and 10 μg/kg in real samples, respectively. By the analysis of 7 samples of raw grain, it was found that two of

Table 1. Determination of mycotoxins with various tracers ($n = 3$)

Analyte	Tracer	IC_{50}, ng/mL	Detection limit, ng/mL
ZEN	ZEN-EDF	15	3
	ZEN-AMF	15	3
	ZEN-HAF	67	50
	ZEN-HMF	36	10
	ZEN-PIP	55	45
OTA	OTA-EDF	14	6
	OTA-AMF	5	1.5
	OTA-Lys-FITC	>500	–

Table 2. Accuracy of mycotoxins determination in spiked grain by added–found method

Mycotoxin	Added, μg/kg	Found, μg/kg	Precision, % ($n = 3$, $P = 0.95$)
ZEN	20	17 ± 1	85
OTA	15	13 ± 1	86
ZEN	100	91 ± 5	91
OTA	100	97 ± 5	97
ZEN	150	140 ± 7	93
OTA	300	280 ± 10	95

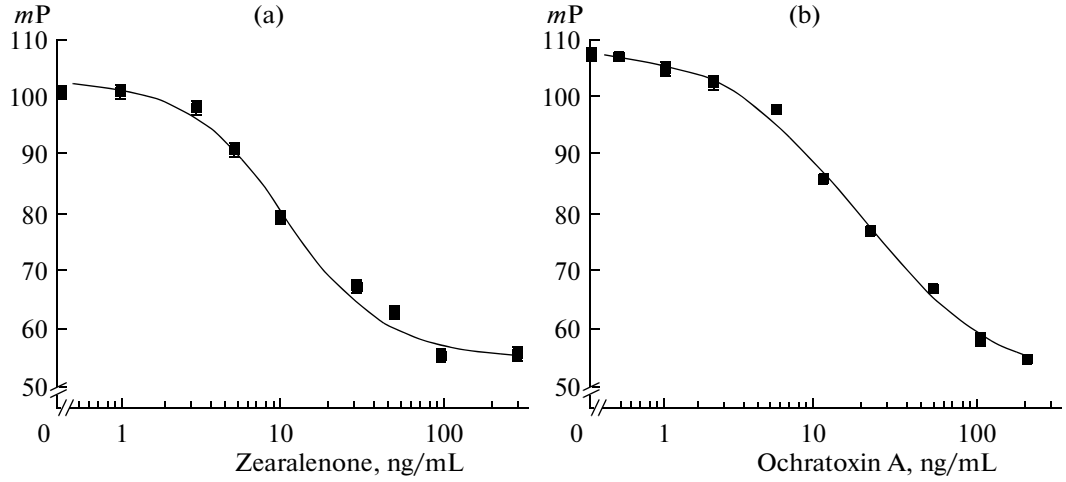

Fig. 3. Calibration graphs for the determination of (a) zearalenone, and (b) ochratoxin A using the optimal tracers ZEN-EDF and OTA-AMF, respectively.

them were contaminated with zearalenone in concentrations below MPC (according to RF regulations 100 μg/kg), one was contaminated at a level of MPC, 100 ± 5 μg/kg, while no presence of ochratoxin A was discovered at all. The data obtained were confirmed by HPLC in the FSE All-Russia State Center for Quality and Standardization of Veterinary Medications and Feed.

* * *

Thus, analytical procedures for the determination of zearalenone and ochratoxin A in grain by fluorescence polarization immunoassay are developed. As established, the optimal fluorescent tags that ensure high sensitivity of analysis are ethylenediamine thiocarbamoylfluorescein and aminomethylfluorescein.

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