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Fluorescence Polarization Immunoassay Based on a New Monoclonal Antibody for the Detection of the Zearalenone Class of Mycotoxins in Maize

Xiya Zhang,[†,‡,§] Sergei A. Eremin,[∥] Kai Wen,[†,‡,§] Xuezhi Yu,[†,‡,§] Chenglong Li,[†,‡,§] Yuebin Ke,[⊥] Haiyang Jiang,[†,‡,§] Jianzhong Shen,[†,‡,§] and Zhanhui Wang*[,†,‡,§]

[†]Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Veterinary Medicine, China Agricultural University, Beijing 100193, People's Republic of China

[‡]Beijing Key Laboratory of Detection Technology for Animal-Derived Food Safety, Beijing 100193, People's Republic of China

[§]Beijing Laboratory for Food Quality and Safety, Beijing 100193, People's Republic of China

[∥]Department of Chemical Enzymology, Faculty of Chemistry, M. V. Lomonosov Moscow State University, Moscow 119991, Russia

[⊥]Department of Genetic Toxicology, Shenzhen Center for Disease Control and Prevention, Shenzhen, Guangdong 518020, People's Republic of China

Supporting Information

ABSTRACT: To develop a sensitive fluorescence polarization immunoassay (FPIA) for screening the zearalenone class of mycotoxins in maize, two new monoclonal antibodies with uniform affinity to the zearalenone class and four fluorescein-labeled tracers were prepared. After careful selection of appropriate tracer−antibody pairs in terms of sensitivity and specificity, a FPIA that could simultaneously detect the zearalenone class with similar sensitivity was developed. Under optimum conditions, the half maximal inhibitory concentrations of the FPIA in buffer were 1.89, 1.97, 2.43, 1.99, 2.27, and 2.44 μg/L for zearalenone, α-zearalenol, β-zearalenol, α-zearalanol, β-zearalanol, and zearalanone, respectively. The limit of detection of FPIA for the zearalenone class was around 12 μg/kg in maize, and the recoveries ranged from 84.6 to 113.8%, with coefficients of variation below 15.3% in spiked samples. Finally, the FPIA was applied for screening naturally contaminated maize samples, and the results indicated a good correlation with that of high-performance liquid chromatography−tandem mass spectrometry.

KEYWORDS: *zearalenone, monoclonal antibody, fluorescence polarization immunoassay, maize, detection*

INTRODUCTION

Humans and animals are continuously exposed to a variety of mycotoxins that naturally occur in various food products, such as maize.[1] Zearalenone (**1**) is an estrogenic mycotoxin that is an important secondary metabolite produced by several species of the genus *Fusarium*, such as *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium cerealis*, *Fusarium equiseti*, *Fusarium crookwellense*, and *Fusarium semitectum*.[2] It is one of the most prevalent mycotoxins worldwide in maize, barley, wheat, and other cereal grains.[2−5] It has been classified as a group III carcinogen by the International Agency for Research on Cancer because it has been associated with early puberty, hyperplastic and neoplastic endometria, and human cervical cancer, and it exhibits a genotoxic potential *in vitro* and *in vivo* through induction of micronuclei, chromosome aberrations, DNA fragmentation, and cell cycle arrest.[6−9] As a result of the high toxicity of compound **1** in both animals and humans, the European Union (EU) has set maximum residue levels (MRLs) of 100 μg/kg for cereals and 350 μg/kg for unprocessed maize, while the legal limit of 60 μg/kg has been set for cereal and cereal-based products in China.[3,10] Actually, two other structurally similar mycotoxins α- and β-zearalenol (**2** and **3**) usually co-occur with compound **1** in cereals infected with *Fusarium* (see Figure 1).[3,11−13] In addition, it has been reported that compound **1** could be metabolized to compounds **2** and **3** and α- and β-zearalanol (**4** and **5**) in some animal species.[12,14] These mycotoxins and metabolites, i.e., compounds **2**−**5** and zearalanone (**6**) (see Figure 1), have also been reported to be harmful because of their estrogenic activity.[12,15,16] Thus, monitoring the presence of the zearalenone class of resorcylic acid lactones in maize is necessary to reduce the risk of zearalenones to humans and livestock. Considering the speed, cost, and efficiency, a detection method that can simultaneously detect the zearalenone class, instead of each individual analogue, would be preferable.

Antibody-based methods, i.e., immunoassays, are relatively inexpensive, rapid, and sensitive screening methods that are used to detect chemical contaminants in large-scale monitoring programs.[17] There have been some reports of the immunoassays that can be used for the detection of the zearalenone class, mainly enzyme-linked immunosorbent assays (ELISAs)[3,18,19] and lateral-flow immunoassays (LFAs).[20,21] ELISA, although very sensitive, requires multiple incubations, washing steps, and extensive pipetting and, thus, is time-consuming.[22] LFAs are usually used for qualitative analysis and low

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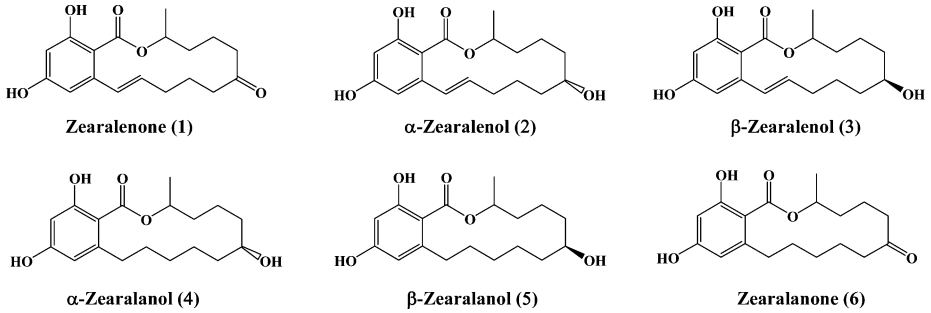

Figure 1. Chemical structures of the zearalenone structural analogues.

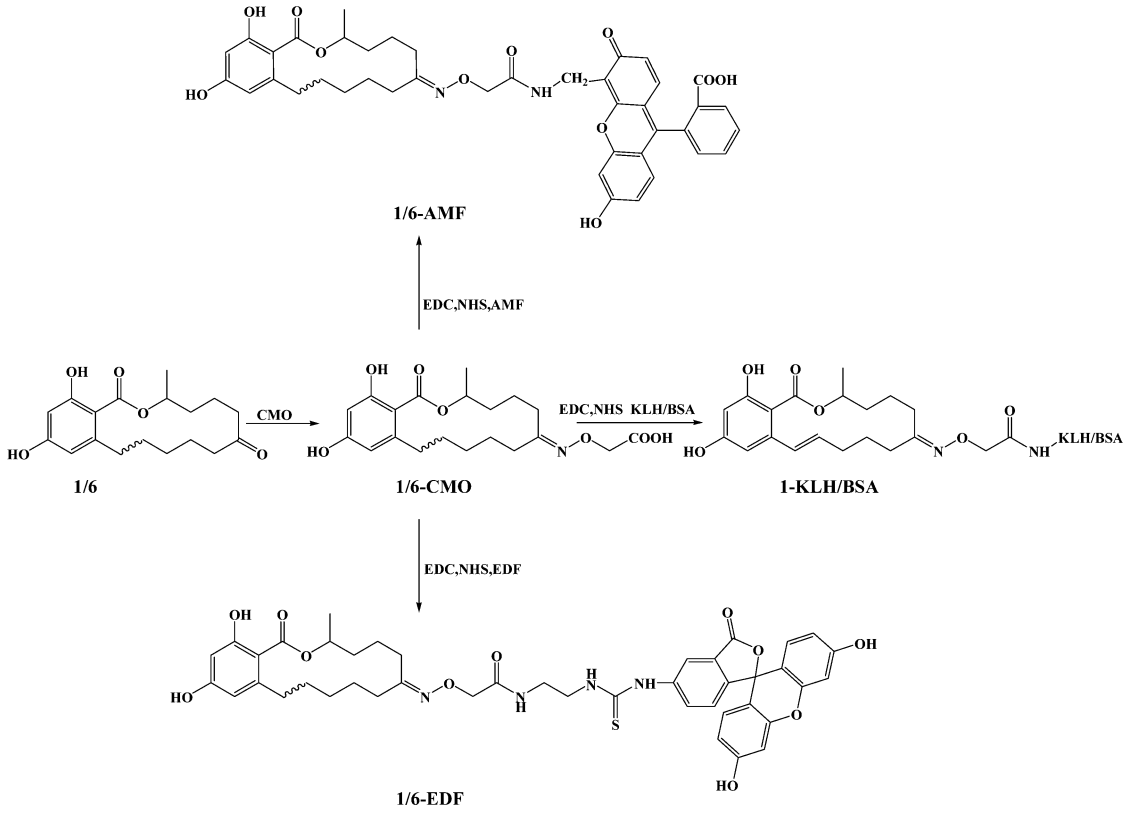

Figure 2. Synthetic of the compound **1** conjugates and **1**/**6** fluorescence tracers.

throughput. In comparison to ELISA and LFA, the fluorescence polarization immunoassay (FPIA) takes only a few minutes before measuring, needs no separations and washing steps,[23] and also can give the quantitative results. These advantages make this method suitable for monitoring large-scale samples. For example, commercial FPIA kits have been developed for aflatoxins, deoxynivalenol, and fumonisins in cereals. Recently, some FPIA methods for the detection of compound **1** in corn have been reported.[22,24−26] However, these methods were less accurate for detection of those zearalenone structural analogues as a result of the diverse cross-reactivity (CR) values of $35.3 \leq CR \leq 522.2\%$[25] or $20 \leq CR \leq 195\%$[22] derived from large variations in affinities of antibodies to each zearalenone analogue. Thus, establishing a FPIA with high sensitivity and accuracy for simultaneous detection of the zearalenone class is necessary.

Antibodies and tracers are two main factors that could directly improve the sensitivity and accuracy of an FPIA for the zearalenone class detection. All reported antibodies against zearalenones do not meet the requirement of detection of the zearalenone class with high accuracy because of diverse CR values. For example, a monoclonal antibody (mAb) named 2D3 was prepared with an half maximal inhibitory concentration (IC_{50}) value of 0.02 μg/L for compound **1**, but this exhibited a low CR with compound **2** (4.4%).[27] Thus, an antibody that could be employed to develop a multi-analyte FPIA should provide not only high but also uniform affinity to all of the targets of interest. In addition, the fluorescein thiocarbamyl hexamethylenediamine-labeled zearalenone (**1**-HMDF) as a tracer was designed to establish a FPIA for screening compound **1** in corn with the limit of detection (LOD) value of 137 μg/kg.[24] The sensitivity of the FPIA was improved 2-fold (LOD of 77 μg/kg) when 4′-(aminomethyl)fluorescein-labeled zearalenone (**1**-AMF) was selected as a tracer using the same antibody in a follow-up study.[25] In other words, the sensitivity of FPIA is significantly influenced by the structure of the tracer. Therefore, we have to search for a better tracer.

The aim of the present study was to produce new mAbs with uniform affinity toward the zearalenone structural analogues and tracers to develop a FPIA for the simultaneous detection of

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Table 1. Comparison of IC_{50} in a Reported icELISA Format and That of This Study[a]

mAb name	performance index	1	2	3	4	5	6
3D4	IC_{50} (μg/L)	0.041	0.038	0.072	0.04	0.056	0.063
	CR (%)	100	107.9	57.2	103.5	73.9	66
11C6	IC_{50} (μg/L)	0.078	0.062	0.677	0.063	0.617	0.044
	CR (%)	100	125.8	11.5	123.6	12.5	177.7
7B2[3]	IC_{50} (μg/L)	0.18	0.39	0.46	0.3	0.73	0.3
	CR (%)	100	46.7	39.2	60.5	24.7	59.5
2D8[19]	IC_{50} (μg/L)	0.8	1.16	*b*	1.9	*b*	3.6
	CR (%)	100	69	<1	42	<1	22
2D3[27]	IC_{50} (μg/L)	0.02	0.455	0.023	*b*	0.435	*b*
	CR (%)	100	4.4	88.2	*b*	4.6	*b*
4A3-F9[36]	IC_{50} (μg/L)	1.115	44.618	39.827	28.933	65.296	2.099
	CR (%)	100	2.5	2.8	3.8	1.7	53.1
KK-ZEN[37]	IC_{50} (μg/L)	131.3	121.3	109.9	115.1	100.3	*b*
	CR (%)	100	108.1	119.3	114.1	130.3	*b*
1H9B4[39]	IC_{50} (μg/L)	5.4	5.5	433.3	3	8	3.9
	CR (%)	100	98.2	1.2	180	67.5	138.5

[a]All were calculated using the CR of compound **1** as 100%. [b]Not detected.

the zearalenone class with high sensitivity and accuracy. The feasibility of the developed method was confirmed by high-performance liquid chromatography−tandem mass spectrometry (HPLC−MS/MS), and the results showed that the FPIA could be used as a screening method for the simultaneous detection of the zearalenone class in maize.

MATERIALS AND METHODS

Reagents and Materials. Zearalenone (**1**), α-zearalanol (**2**), β-zearalanol (**3**), α-zearalenol (**4**), β-zearalenol (**5**), zearalanone (**6**), aflatoxin B_1, ochratoxin A, fumonisins B_1, deoxynivalenol, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), 3,3′,5,5′-tetramethylbenzidine (TMB), Freund's complete/incomplete adjuvant, polyethylene glycol 1500 (PEG 1500), and hypoxanthine aminopterin thymidine (HAT) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum was provided by Gibco BRL (Paisley, Scotland). Dulbecco's modified Eagle's medium (DMEM) was obtained from Shanghai Lifei Biotechnology Co., Ltd. (Shanghai, China). Ethylenediamine fluoresceinthiocarbamyl (EDF) was previously synthesized by our group.[28] 4′-(aminomethyl)fluorescein (AMF) was obtained from Molecular Probes (Eugene, OR). Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) (H + L) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). *O*-Carboxymethyl oxime (CMO), *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl), pyridine, dimethyformamide, and *N*-hydroxysuccinimide (NHS) were obtained from Aladdin Chemistry Co., Ltd. (Shanghai, China). Borate buffer (0.05 M, pH 8.0) was selected as a working buffer for the FPIA experiment. The fluorescein-labeled tracers of **1**-EDF/AMF were previously synthesized (Figure 1B).[24]

Non-binding surface, non-sterile, and 96-well black polystyrene microplates were purchased from Corning Life Sciences (Corning, NY). Thin-layer chromatography (TLC) aluminum sheets (M5554 silica gel 60 F254) were obtained from Merck (Darmstadt, Germany).

Five female BALB/c mice, which were supplied by Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), were raised in specific pathogen-free conditions, and those mice were disposed in compliance with the Animal Care Center of China Agricultural University (2012-SYXK-0037) and Chinese laws and guidelines (GKFCZ2001545).

Apparatus. Optical density (OD_{450}) signals, fluorescence intensity (FI), and fluorescence polarization (FP) values were measured by a Spectramax M5 microplate reader (Sunnyvale, CA). Deionized water for the FPIA detection system was provided by a Milli-Q synthesis system (Bedford, MA).

Production of mAbs against the Zearalenone Class. The hapten **1**-CMO (see Figure 2) was synthesized according to a previous study.[29] Briefly, 16 μmol of compound **1** was dissolved in 1 mL of pyridine and reacted with 80 μmol of CMO at 90 °C for 8 h. Those reaction solutions were dried by a vacuum pump and then redissolved in 5 mL of distilled water (adjusted to pH 8 with 1 M sodium bicarbonate buffer). Unchanged compound **1** was extracted from the sodium bicarbonate buffer by 10 mL of ethyl acetate. Subsequently, the aqueous phase was adjusted to pH 3 by 0.1 M HCl, and the hapten **1**-CMO was extracted from the water phase with 5 mL of ethyl acetate 3 times. The extraction was dried to obtain the hapten **1**-CMO. The hapten **1**-CMO was coupled to the carrier protein (KLH and BSA) using the active ester method, as previously described.[30]

The female BALB/c mouse was injected subcutaneously with 100 μg of **1**-KLH (100 μL) emulsified with 100 μL of adjuvant 3 times.[31] The mouse that showed competitive inhibition of antibodies binding to **1**-BSA by the free zearalenone structural analogues was sacrificed for fusion. The culture supernatants of the hybridoma were screened with an indirect competitive enzyme-linked immunosorbent assay (icELISA), as previously described.[32] The positive hybridomas were used to produce the ascites fluids.

Preparation of the Fluorescein-Labeled Tracers. Compound **6** was selected for coupling to EDF and AMF (see Figure 2). Because compound **6** has no reactive groups to enable coupling reactions, the first step was converting it into **6**-CMO, prepared in the same way as for **1**-CMO. Then, **6**-CMO was conjugated to amino-fluorescein derivatives (EDF/AMF) with a six-carbon bridge using the active ester method.[24,25] Briefly, **6**-CMO (2 μmol), EDC (6 μmol), and NHS (6 μmol) were dissolved in 0.2 mL of DMF and reacted at room temperature for 12 h. The precipitates were removed by centrifugation. EDF or AMF (3 μmol) was added to the clear solutions and reacted for another 12 h at room temperature. TLC with trichloromethane/methanol (4:1, v/v) as a mobile phase was used to purify the fluorescein-labeled tracers of **6**-EDF/AMF, as described previously.[23] The major yellow band (R_f of around 0.5) was scraped off, soaked into 100 μL of methanol for 5 min, and centrifuged at 3000*g* for 5 min. The clear solutions were the tracers of **6**-EDF/AMF and further diluted by 0.05 M borate buffer (pH 8.0) to obtain the working concentration or stored at −20 °C in the dark until use.

Protocol of the FPIA. The procedure of FPIA was as follows: 70 μL/well of standards, 70 μL/well of fluorescein-labeled tracers, and 70 μL/well of the working solution of optimally diluted mAb were sequentially added to a microplate. Following a shaking step in the Spectramax M5 microplate reader, FP values were measured at λ_{ex} of 485 nm and λ_{em} of 530 nm, with an emission cutoff of 515 nm. The standard curve was calculated as the reference.[33]

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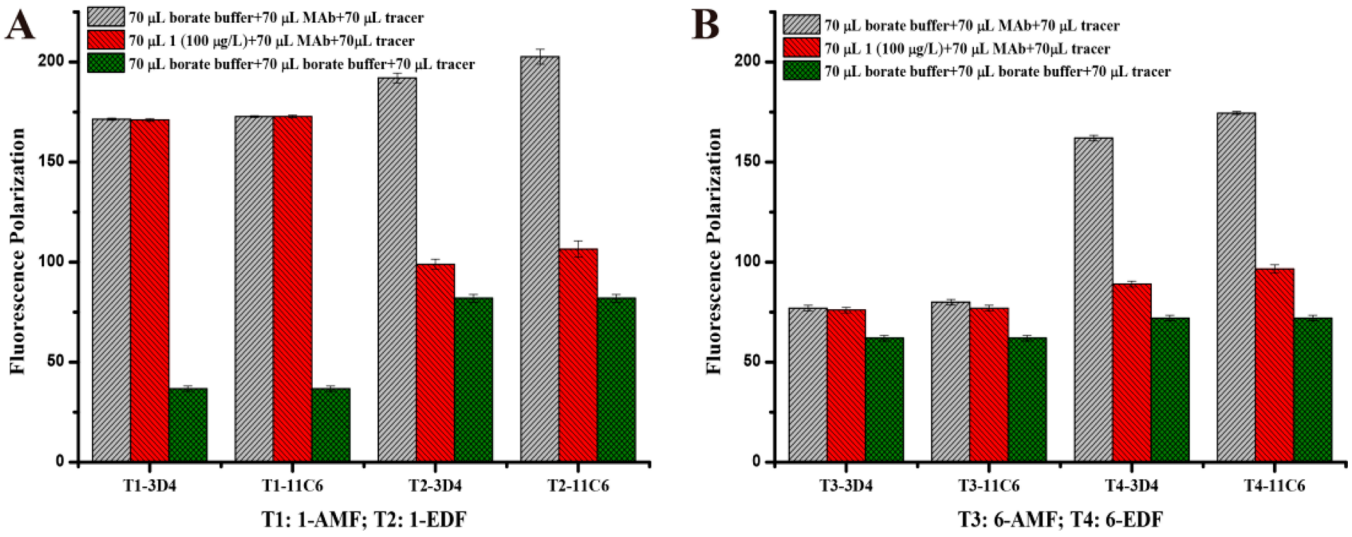

Figure 3. Results of the four fluorescence tracers (1000-fold diluted) binding with mAbs 3D4 (5000-fold diluted) and 11C6 (8000-fold diluted) (n = 3).

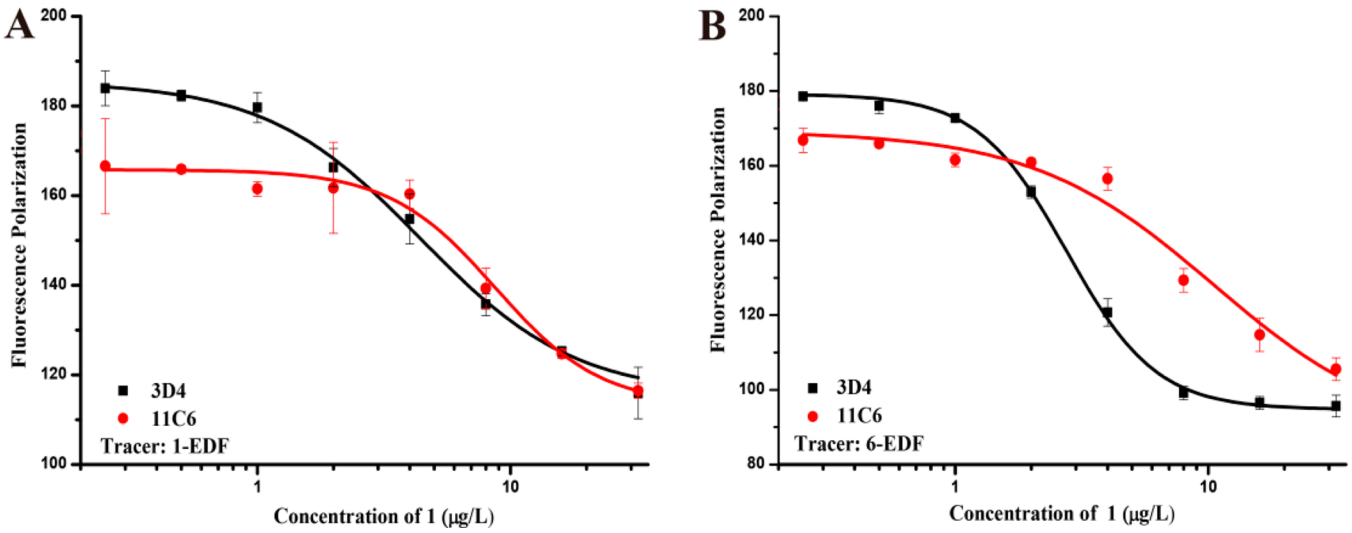

Figure 4. Calibration curves based on mAbs 3D4 (5000-fold diluted) and 11C6 (12 000-fold diluted) for (A) **1**-EDF and (B) **6**-EDF (1000-fold diluted) (n = 3).

To determine the specificity of the developed FPIA, CR values with other mycotoxins, including compounds **1**–**6**, aflatoxin B_1, ochratoxin A, fumonisins B_1, and deoxynivalenol, were calculated with the following equation:[31]

$$CR = (IC_{50} \text{ of zearalenone}/IC_{50} \text{ of competitor}) \times 100\%$$

Preparation of the Maize Samples. Zearalenone-free and naturally contaminant maize flour samples were kindly supplied by Prof. De Saeger (Ghent University). A total of 5 g of the maize flour was weighed into 50 mL polypropylene centrifuge tubes, and 15 mL of methanol/borate buffer (7/3, v/v) was added to each sample for extraction. The mixture was vortexed for 5 min and centrifuged at 3000g for 5 min. The obtained supernatant was diluted by 0.05 M borate buffer (pH 8.0) for analysis without any further processing. A total of 20 blank samples were measured by the development of FPIA as a control. The LOD was calculated using the following formula:

$$LOD = (\bar{X} + 3SD)$$

$\bar{X}$ was the average detection concentration of the 20 blank samples. The working range corresponds to the concentration of the standard varying from IC_{20} to IC_{80} of the calibration curves. Naturally contaminated maize samples confirmed by HPLC–MS/MS[3,34] were prepared as the same procedure and submitted to the FPIA.

RESULTS AND DISCUSSION

mAb Production. Zearalenone structural analogues are small molecules [molecular weight (MW) of <1000] that should be conjugated to a carrier protein to elicit an immune response in the host animal. The preparation of an effective immunogen, i.e., immune conjugate, is the critical step in the process of antibody production.[17] In the reported studies, there are three strategies that are employed to conjugate compound **1** to carrier proteins; i.e., formaldehyde,[35] 1,4-butanediol diglycidyl ether,[36] and **1**-CMO.[18,19,37,38] These results showed that the antibody derived from the formaldehyde method provided lower affinity,[35] while, the antibody derived from 1,4-butanediol diglycidyl ether as the linker agent was highly specific to compounds **1** and **6** and could not recognize other zearalenone structural analogues.[36] The strategy of using **1**-CMO as a hapten was promising to produce broadly specific antibodies,[18,19,37,38] although these reported antibodies showed

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Table 2. Comparison of IC_{50} and CR Values in Reported FPIAs and That of This Study[a]

mAb tracer	performance index	1	2	3	4	5	6
mAb 3D4/**6**-EDF	IC_{50} (μg/L)	1.89	1.97	2.43	1.99	2.27	2.44
	CR (%)	100	95.9	77.8	95	83.3	77.5
mAb 3D4/**1**-EDF	IC_{50} (μg/L)	3.65	4.26	5.12	3.86	3.94	5.62
	CR (%)	100	85.7	71.3	94.5	92.6	64.9
mAb 11C6/**6**-EDF	IC_{50} (μg/L)	10.52	8.96	82.3	9.25	89.3	8.87
	CR (%)	100	117.4	12.8	113.7	11.8	118.6
mAb 11C6/**1**-EDF	IC_{50} (μg/L)	8.71	7.89	61.35	7.64	63.5	6.32
	CR (%)	100	110.4	14.2	114	13.7	137.8
mAb 5B4/**1**-FITC[26]	IC_{50} (μg/L)	56.2	*b*	*b*	*b*	*b*	*b*
	CR (%)	100	*b*	*b*	*b*	*b*	*b*
mAb (Sigma)/**1**-AMF[25]	IC_{50} (μg/L)	517	594	673	217	1463	99
	CR (%)	100	87	76.8	238.2	35.3	522.2
mAb (Sigma)/**1**-HMDF[24]	IC_{50} (μg/L)	500	*b*	*b*	*b*	*b*	*b*
	CR (%)	100	*b*	*b*	*b*	*b*	*b*
mAb 70/**1**-AMF[22]	IC_{50} (μg/L)	*b*	*b*	*b*	*b*	*b*	*b*
	CR (%)	100	102	71	139	20	195

[a]All were calculated using the CR of compound **1** as 100%. [b]Not detected.

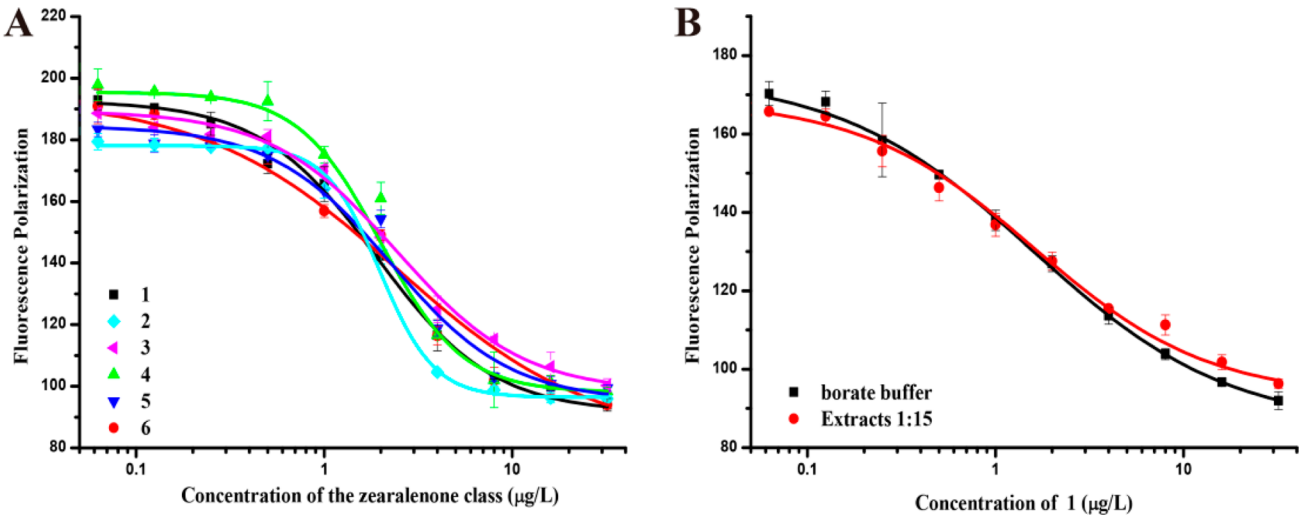

Figure 5. (A) Calibration curves of the zearalenone class based on **6**-EDF (2000-fold diluted) and mAbs 3D4 (7000-fold diluted) and (B) calibration curves for compound **1** in borate buffer and flour maize extract diluted 15-fold by the FPIA (n = 3).

Table 3. Recoveries and CV for the Zearalenone Class in Maize in the FPIA[a]

spiked (μg/kg)	test (μg/kg)	recovery (%)	CV (%)
50	48.45 ± 6.56	96.9	13.1
100	91.2 ± 13.62	91.2	13.6
200	204.9 ± 30.62	102.4	15.3

[a]Flour maize samples were spiked with indicated amounts of compounds **1**−**6** (weight ratios of 1:1:1:1:1:1) (n = 3).

significantly varied affinity to compounds **1**−**6**. Thus, **1**-CMO was selected for this study.

At 7 days after the third immunization, the blood from the tail vein was gathered and characterized by the icELISA. Mouse number 5 was sacrificed for cell fusion as a result of its uniform affinity to all zearalenone structural analogues (the inhibition ratios were around 25%). Two cell lines, named 3D4 and 11C6, were obtained. The IC_{50} and CR values of mAbs 3D4 and 11C6 and other antibodies from recent references are shown in Table 1. The results showed that mAbs 3D4 and 11C6 displayed high affinity for the class zearalenone, but mAb 11C6 showed low CR values with compounds **3** (11.5%) and **5** (12.5%). mAb 3D4 had broad selectivity for the class zearalenone with uniform affinity ($0.038 \leq IC_{50} \leq 0.072$ μg/L) compared to others.[3,19,27,36,39] Although the reported antibody named KK-ZEN could recognize five zearalenone structural analogues (**1**−**5**), the IC_{50} values of the antibody ranged from 100.3 to 131.3 μg/L, which were not sufficiently sensitive for developing a FPIA.[37] Thus, mAbs 3D4 and 11C6 were used in the subsequent studies.

Fluorescein-Labeled Tracer Synthesis and Selection. The structure of the tracers had a dramatic influence on the FPIA characteristics. In certain cases, the sensitivity of the FPIA could be significantly improved by a careful choice of the appropriate tracer.[23−25,40] Several tracers have been reported for the detection of compound **1** in a FPIA format in previous studies.[22,24−26] It has been reported that the tracer (**1**-HMDF) containing a six-carbon bridge resulted in the most sensitive FPIA for compound **1** in corn (LOD of 137 μg/kg).[24] In subsequent studies, one new tracer (**1**-AMF) was prepared by the same group, and this resulted in a 2-fold improved sensitivity (LOD of 77 μg/kg) using the same antibody,[25]

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Table 4. Naturally Contaminated Maize Flour Samples[a]

	HPLC–MS/MS (μg/kg)										FPIA (μg/kg)	
number	deoxynivalenol	ochratoxin A	fumonisins B_1	aflatoxin B_1	**1**	**2**	**3**	**4**	**5**	**6**	measured	CV (%)
1	134	*b*	569	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
2	281	*b*	*b*	*b*	70	*b*	*b*	*b*	*b*	*b*	63 ± 6	9.5
3	522	*b*	*b*	*b*	23	2	4	*b*	*b*	*b*	28 ± 3	10.7
4	300	*b*	67	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
5	679	*b*	328	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
6	316	*b*	786	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
7	78	30	1184	18	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
8	*b*	*b*	379	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
9	66	*b*	7262	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
10	2197	*b*	297	*b*	89	*b*	*b*	*b*	*b*	*b*	76 ± 10	13.2
11	213	*b*	291	*b*	130	*b*	*b*	*b*	*b*	*b*	110.5 ± 16	14.5
12	705	*b*	742	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
13	213	*b*	236	*b*	4	*b*	*b*	*b*	*b*	*b*	*b*	*b*
14	535	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
15	990	*b*	1686	*b*	5	*b*	*b*	*b*	*b*	*b*	*b*	*b*

[a]Data are represented as an average ± standard deviation of three replicates. [b]Not detected.

implying the important role of the tracer on the sensitivity of the FPIA. This study prompted us to prepare new tracers suitable for developing a highly sensitive FPIA for screening the class zearalenone. In this study, we prepared two new tracers by conjugated **6**-CMO to different fluoresceins (AMF/EDF), as shown in Figure 2. The new tracers were confirmed by mass spectrometry after purification by preparative TLC. The molecular ion peaks (m/z) of **6**-EDF/AMF were 754.6 $[m\text{-}NH_4]^+$ and 842.2 $[m\text{-}NH_4]^+$, respectively, indicating that the tracers of **6**-EDF/AMF were synthesized successfully.

In combination with two previously prepared tracers of **1**-EDF/AMF,[24] in total, four tracers were preliminarily characterized for their binding ability to mAbs 3D4 and 11C6 (see Figure 3). The detection windows [δFP ($FP_{bind} - FP_{free}$)] and inhibition ratios were the main parameters for the selection of the best antibody–tracer pair. It can be observed in Figure 3 that 6-AMF only induced a small change of FP values when binding with both antibodies, indicating that the tracer could not be specifically recognized by mAbs. Although **1**-AMF could specifically bind to the two mAbs and give an adequate increase in FP signals (δFP of around 130), obvious inhibitions were not observed when free compound **1** at 100 μg/L was present in the mixture, meaning that the **1**-AMF was not suitable for developing a sensitive FPIA. Satisfactory binding (δFP of 90–121 mP) and inhibitions (around 20%) by free compound **1** at 100 μg/L were observed when **1**/**6**-EDF were mixed with the two mAbs, guaranteeing establishment of a highly sensitive FPIA.[41] Thus, **1**/**6**-EDF were selected for the following studies.

To further evaluate the best tracer and antibody pair for developing a FPIA with broad specificity, **1**/**6**-EDF and mAbs 3D4 and 11C6 were used to construct FPIA standard curves for compound **1** (see Figure 4). Among these combinations of tracers and mAbs, the IC_{50} value of FPIA based on **6**-EDF and mAb 3D4 was 2.76 μg/L, which was obviously lower than those of FPIAs based on other pairs, ranging from 4.41 to 10.52 μg/L. After optimization of the working dilution of mAb 3D4 (1/7000) and **1**/**6**-EDF (1/2000 and 1/4000, respectively) and mAb 11C6 (1/12 000) and **1**/**6**-EDF (1/1000), the IC_{50} values and CR values for the zearalenone structural analogues are calculated and shown in Table 2. The results indicated that mAb 3D4 in FPIA for screening the zearalenone class had more uniform CR values ($64.9 \le CR \le 100\%$) than mAb 11C6 in FPIA ($11.8 \le CR \le 100\%$) when paring **1**/**6**-EDF, which agreed with CR values of these mAbs in icELISA. Moreover, the sensitivity (expressed by IC_{50} values in the case) of the FPIA based on mAb 3D4 was improved almost 2-fold when **6**-EDF was selected as a tracer rather than **1**-EDF. Thus, the combination of **6**-EDF and mAb 3D4 was finally selected to develop a FPIA for the detection of the zearalenone class, and the standard curves of FPIA were then constructed, as shown in Figure 5A. It was observed that the IC_{50} values of standard curves were 1.89 μg/L for compound **1**, 1.97 μg/L for compound **2**, 2.43 μg/L for compound **3**, 1.99 μg/L for compound **4**, 2.27 μg/L for compound **5**, and 2.44 μg/L for compound **6**. The CR values of the developed FPIA for the above zearalenone structural analogues were 100, 95.9, 77.8, 95, 83.3, and 77.5%, respectively, and below 0.1% with other mycotoxins, such as aflatoxin B_1, ochratoxin A, fumonisins B_1, and deoxynivalenol. The uniform CR values of the developed FPIA could ensure the accurate detection of the zearalenone class in maize. We summarized the IC_{50} values and CR values recently reported for FPIA methods in the literature, as shown in Table 2. It can be concluded that the FPIA that we developed was more sensitive ($1.89 \le IC_{50} \le 2.44$ μg/L) and accurate ($77.5 \le CR \le 100\%$) than those in the literature.[22,24−26] We thought that the broad-specific mAb 3D4 ($0.038 \le IC_{50} \le 0.072$ μg/L in an ELISA format) and new tracer **6**-EDF both contributed to the high sensitivity and potential accuracy of a FPIA for the detection of the zearalenone class.

Development of FPIA in the Maize Matrix. In general, the zearalenone structural analogues could be extracted from maize samples with a mixture of methanol/water.[3,24,25] According to a previous study, 70% methanol was chosen for the recovery study.[25] As a homogeneous immunoassay, FPIA was more susceptible to the matrix interference than other heterogeneous immunoassays, such as LFA and ELISA.[23,25] Sample dilution is a commonly employed method to eliminate the matrix effect. Therefore, investigation of the matrix effect is a critical point in quantitative analysis of FPIA. It can be observed in Figure 5B that the matrix effect could be removed after 15 times dilution of the extract by 0.05 M borate buffer (pH 8.0). The LOD calculated by taking into account dilution as a result of extraction and sample pretreatment was around 12

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μg/kg, which was lower than that of the reported FPIA in the references.[22,24−26] Also, the working range was 14.6−340.5 μg/kg. According to the uniform affinity of this FPIA (77.5 ≤ CR ≤ 100%), a mixed standard (the six zearalenone structural analogue weight ratios were 1:1:1:1:1:1) was used for the recovery study. The recoveries ranged from 84.6 to 113.8%, with a coefficient of variation (CV) below 15.3% acceptable for the detection of the zearalenone class in maize samples (Table 3). Then, the developed FPIA was applied to 15 naturally contaminated maize flour samples that were already confirmed by the HPLC−MS/MS method.[3,34] As shown in Table 4, a satisfactory correlation was obtained between the results of FPIA and HPLC−MS/MS. Several FPIA methods have been used for screening the zearalenone class; however, the methods may have a slightly false positive result of naturally contaminated samples containing those zearalenone structural analogues because of the relatively high CR values of compound **6** (522.2%[25] or 195%[22]) and compound **4** (238.2%[25] or 139%[22]). In comparison to those FPIA methods,[22,25] the FPIA that we developed had more accuracy for the detection of the zearalenone class in maize because of the uniform CR values (77.5 ≤ CR ≤ 100%).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b05614.

IC_{50} values using **1**/**6**-EDF with mAbs 3D4 and 11C6 (Table S1), results of the immunization (Figure S1), calibration curves of the icELISAs (Figure S2), and mass spectrometry of **6**-EDF/AMF (Figure S3) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +86-10-6273-4565. Fax: +86-10-6273-1032. E-mail: wangzhanhui@cau.edu.cn.

ORCID

Xiya Zhang: 0000-0002-0996-9710

Zhanhui Wang: 0000-0002-0167-9559

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Notes

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