Food Anal. Methods (2017) 10:256–262
DOI 10.1007/s12161-016-0576-5

Evaluation and Optimization of Three Different Immunoassays for Rapid Detection Zearalenone in Fodders

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Received: 19 February 2016 / Accepted: 12 June 2016 / Published online: 23 June 2016

Abstract Zearalenone (ZEN) is one of the most common contaminants in fodder with obvious reproduction toxicity and potential carcinogenicity to animals and humans. Thus, simple and sensitive methods are required for the detection of ZEN. In this work, the anti-ZEN monoclonal antibody (mAb) was prepared by hybridoma technique. Then, the mAb-based indirect competitive enzyme-linked immunosorbent assay (ic-ELISA), fluorescence polarization immunoassay (FPIA), and immunochromatographic assay (ICA) were evaluated and optimized for ZEN detection in spiked fodder samples. The ic-ELISA and FPIA showed recovery ranges from 80 to 100 %. The limit of detection (LOD) in ic-ELISA, FPIA and ICA were 0.06, 0.54, and 10 ng/mL, respectively. The detection ranges of IC_{20}~IC_{80} were 2.89 to 115.36 ng/mL in ic-ELISA and 3.0 to 1052 ng/mL in FPIA. Amongst three immunoassays, the ic-ELISA was the most sensitive and stable, nevertheless, most time-consuming with narrower detection range. The FPIA showed good sensitivity, precision, and wide detection range, but it required specific tracer. ICA was a time-saving handy method but exhibited relatively lower precision and quantification compared to other two methods.

Keywords Zearalenone (ZEN) · Monoclonal antibody (mAb) · Indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) · Fluorescence polarization immunoassay (FPIA) · Immunochromatographic assay (ICA)

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Introduction

Zearalenone (ZEN), as one of the commonest contaminants in fodder, has been reported to be reproduction toxic and potential carcinogenic to animals and humans (Streit et al. 2012; Zhu et al. 2012).ZEN is the secondary metabolite produced by some *Fusarium* genera, which mainly contaminates grains including corn, barley, wheat, oats, and sorghum worldwide (Berthiller et al. 2013). ZEN would be produced in food grains, if it is not handled and dried properly (Chun et al. 2009). The ZEN is stable at high temperatures and could not be degraded during food processing (Mirocha et al. 2013).

In order to decrease the ZEN-induced incidences, the USA and Europe have defined the permissible limit of ZEN in food products ranged from 0.02 to 1000 μg/kg (EC/No. 1881/2006). In China, the legal limit is 500 μg/kg in formulated feed and 60 μg/kg in cereals (Zhang et al. 2013). With the focus to prevent the hazards of ZEN contaminated fodders before market entry, various methods have been developed for detecting ZEN, including thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) (Chun et al. 2009; Hadiani et al. 2003; Wang et al. 2014). Each method has different features in terms of cost, time-consuming, and labor. In addition, immunochromatographic assay (ICA) and fluorescence polarization immunoassay (FPIA) have been commonly used for the detection of smaller molecules such as toxin, heavy metal, and antibiotic (Tian et al. 2015; Yang et al. 2015). ICA for antigen detection is based on the colored immunoreactant from

the binding of antibody-gold conjugate and immobilized capture antigen in test line (Huang et al. 2014; Sun et al. 2014; Wang et al. 2014; Yazdanpanah et al. 2012). The ICA is a competitive assay; the intensity of color of test line is the reverse of the concentration of ZEN. FPIA is based on the changing rate of fluorescence polarization value when the antigen present in sample competes with tracer (antigen-fluorescent conjugate) for binding to antibody (Beloglazova and Eremin 2015). Under optimal conditions, the fluorescence polarization value depends on the volume size of the fluorescent molecule (Lippolis et al. 2014; Mi et al. 2013). Small molecules in solution have a smaller fluorescence polarization value because of a fast spin, and the macro molecule vice versa (Lippolis et al. 2014). Thus, in FPIA, the fraction of bound and unbound tracers can be calculated without having to conduct a physical separation of them (Huang et al. 2015; Udenfriend 2014). FPIA has been commercially used for the detection of some small molecular, although, in some cases, they give only qualitative determinations.

The aim of this study is to evaluate and optimized FPIA for ZEN in situ detection in various fodders for using as a screening modality and compare it with a standard technology (ELISA) and a clinical most used technology (ICA).

Materials and Methods

Ethical Approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the Ethics Committee of Northwest A&F University for the use of Laboratory Animals.

Materials

ZEN, egg albumin (OVA), bovine serum albumin (BSA), Dimethyl Formamide (DMF), N,N′-dicyclohexylcarbodiimide (DCC), 4′-(aminomethyl) fluorescein (4AMF), ethylenediamine fluoresceinthiocarbamyl (EDF), N-hydroxysuccinimide (NHS), Freund's complete/incomplete adjuvants, tetramethylbenzidine (TMB), hypoxanthine aminopterin thymidine (HAT), hypoxanthine thymidine (HT), zearalanone, zearalanone, Fluorescein isothiocyanate isomer (FITC), goat anti-mouse IgG antibody conjugated to Horseradish peroxidase were purchased from Sigma-Aldrich (Sigma-Aldrich Co., St. Missouri, USA). 96-well microtiter plates (NUNC, Roskilde, Denmark). RPMI-1640 medium and fetal bovine serum were purchased from Gibco (Invitrogen, New York, USA), Protein G affinity columns were bought from GE heathcare (GE, Pittsburgh, PA). The other reagents and solvents were of analytical grade or higher. Murine myeloma Sp2/0 cells were maintained in our lab.

Experimental Animals

Eight-week-old female BALB/c mice were obtained from the Laboratory Animal Center, Xi'an Jiao Tong University, China, and maintained under strictly controlled conditions.

Preparation of Immunogen and Coating Antigen

In order to increase the immunogenicity, ZEN was conjugated with BSA due to its low molecular mass of 318.15 (Gao et al. 2012). The oxhydryl of ZEN was used to construct a ZEN-carrier protein complex via an active ester reaction. ZEN and DCC were dissolved in 1 mL DMF, respectively, and mixed together slowly, with a drop-wise addition of NHS over constant stirring. Then, the supernatant was obtained and co-incubated with BSA dissolved in PBS for 2 h at room temperature and subsequently incubated overnight at 4 °C. The uncoupled ZEN or BSA were removed after a 3-day dialysis in PBS at 4 °C, and then aliquots of immunogen were stored at −20 °C for further use. Finally, the UV spectra analysis and gel electrophoresis assay were carried out to analyze the association ratio of conjugated immunogen. OVA and ZEN were used to produce the coating antigen as the same method as described above.

Immunization

The mice were subcutaneously injected with ZEN-BSA mixed with Freund's complete adjuvant (Sigma) at multiple points for the first immunization. Four booster injections were given with Freund's incomplete adjuvant (Sigma) at 2-week interval from the first immunization. Sera were collected after the third immunization and screened for anti-ZEN activity by ELISA. One mouse with high specific antibodies was selected as the spleen donor for hybridoma production.

Hybridoma Screening

Mice spleen cells from the immunized mouse were fused with Sp2/0 cell at a 1:1 ratio using PEG-1500. Ten days after cell fusion, the supernatant of hybridoma colonies were recovered, and then followed with a hybridoma screening by ic-ELISA to select specific hybrid strain using ZEN-OVA as coating antigen. The selected wells were subcloned by limiting dilution. Stable antibody-producing clones were expanded and inoculated into the abdominal cavity of BALB/c mice. Ascites fluid was collected 1 week later and the mAb was purified using a protein A affinity column.

mAb Titer Determination

The indirect ELISA was prepared to detect the titer. ZEN-OVA was dissolved in a 0.05 M carbonate buffer solution

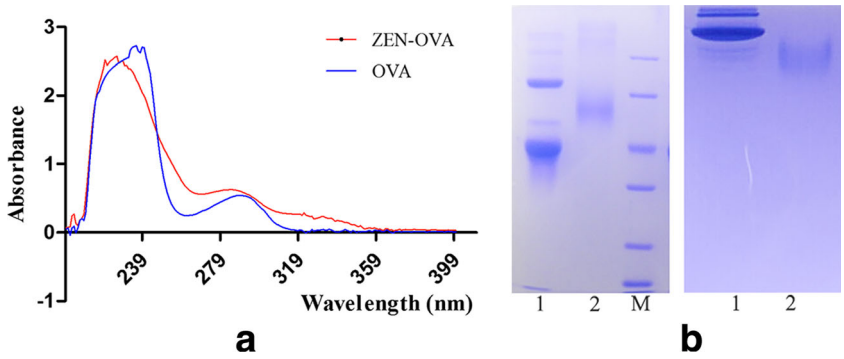

Fig. 1 Identification of ZEN-OVA. **a** Reducing PAGE. Lane 1, OVA; Lane 2, ZEN-OVA. **b** Non-reducing PAGE. Lane M, Marker; Lane 1, OVA; Lane 2, ZEN-OVA). ZEN: Zearalenone; OVA: Albumin

(pH 9.6) and added to 96-microplates for 1 h at 37 °C. After washing three times with PBST, ELISA plates were blocked in 5 % non-fat milk for overnight at 4 °C and washed again. Then, ELISA plate was performed in 5 % non-fat milk using purified mAb for 2 h at 37 °C. After washing, goat anti-mouse antibody IgG (Abcam, Cambridge, UK) (1:20,000) conjugated to Horseradish peroxidase was incubated at 37 °C for 1 h. After three times washing, Tetramethylbenzidine substrate (Sigma-Aldrich, Missouri, USA) solution containing 0.1 mM citrate-phosphate buffer and 1 μL/mL H_2O_2 were added. H_2SO_4 (2 M/L) was added to the ELISA plates for termination. The plates were read directly on microplate reader at 450 nm (ELx800, BioTek, USA).

FPIA for Measuring Anti-ZEN mAb Activity

ZEN-carboxymethyloxime (ZEN-oxime) was prepared as described previously and then conjugated to the amino-group of two fluorescein derivatives (4AMF and EDF) [17]. The conjugate reaction mixture was identified and purified by using thin-layer chromatography (TLC). The main yellow bands for ZEN–EDF at R_f = 0.5 and for ZEN–4AMF at R_f = 0.8 were collected and stored at −20 °C. With the good tracer and a more sensitive FPIA developed, ZEN–AMF with R_f = 0.95 was purified in a second TLC process. The fluorescence intensity of tracer in the gradient dilution were detected and the concentration was selected, under which the fluorescence intensity is five times of borate buffer (0.25 mM, pH 9.0) (BB solution). The antibody working concentration was determined by the binding fraction of optimal tracer and anti-ZEN mAb. ZEN standards were diluted to 0.01, 0.1, 1, 10, 100 and 1000 ng/mL in BB solution. 50 μL ZEN standard, 500 μL tracer and 500 μL optimal diluted antibody and co-incubated for 5–10 min in room temperature and the fluorescence polarization were detected using portable FP instrument Sentry-200 (Diachemix Crop. Grayslake, IL, USA). The inhibition curve and logistic equation were made according to the log concentration of ZEN (lgC) and inhibition ratio (mP/mP_0), where mP was the fluorescence polarization value at each concentration of analyte and mP_0 was the fluorescence polarization value in the absence of analyte. Sensitivity of immunoassay was evaluated by half-maximum inhibition concentration value (IC_{50}) and the detection range was defined as IC_{20}~IC_{80} value.

Cross-reactivity (CR) was conducted with the following equation:

$$CR\ (\%) = [IC_{50}\ (ZEN)/IC_{50}\ (analytes)] \times 100\ \%.$$

Ic-ELISA for Measuring Anti-ZEN mAb Activity

Checkerboard titration method was employed to determine optimal dilutions of coating antigen and purified antibody, as described previously [13]. The above similar procedures of ic-ELISA were performed except that 50 μL dilutions of ZEN standard solution (80, 50, 33.3, 10, 5, 2, and 0 ng/mL) and 50 μL the selected concentration of purified mAb were added to each well successively after emptying skim milk. The inhibition curve and logistic equation were similarly made as per the method described above.

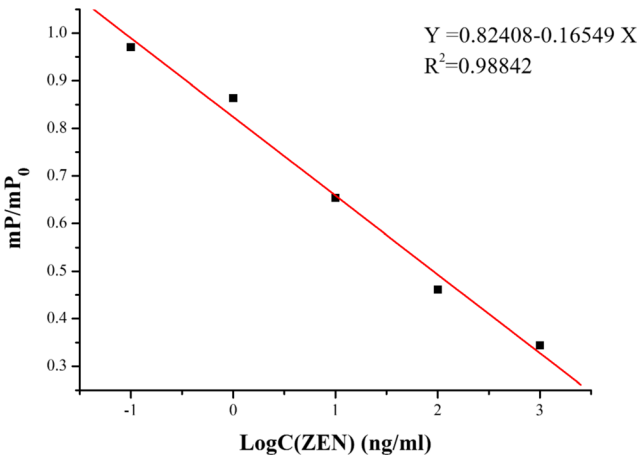

Fig. 2 Standard curves of FPIA for ZEN. X-axis: logarithm concentration of ZEN; Y-axis: represents the inhibition rate (mP/mP_0), B: mP values of the ZEN at the different concentration; mP_0: mP values of ZEN at zero concentration. ZEN: Zearalenone; FPIA: fluorescence polarization immunoassay

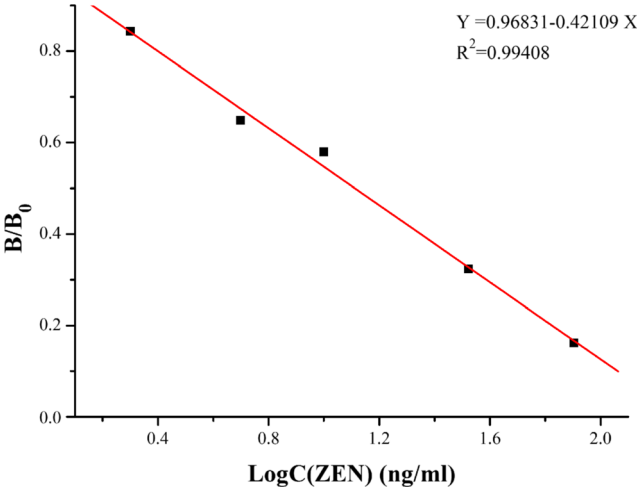

Fig. 3 Standard curves of ic-ELISA for ZEN. X-axis: logarithm concentration of ZEN; Y-axis: represents the inhibition rate (B/B_0), B: OD values of the ZEN at the different concentration; B_0: OD values of ZEN at zero concentration. ZEN: Zearalenone; ic-ELISA: indirect competitive enzyme-linked immunosorbent assay

ICA for Measuring Anti-ZEN mAb Activity

Anti-ZEN mAb-gold conjugate and ICA test strips were prepared as per the method previously described (Sun et al. 2014; Wang et al. 2013). The sensitivity of immunoassay was prepared by spraying ZEN–OVA and goat anti-mouse mAb (1.0 mg/mL) to the nitrocellulose membrane (NC) using BioJet (Biodot, California, USA) as the test line and control line respectively. The mAb-gold conjugate could react with ZEN in sample firstly, and then remaining mAb-gold conjugate would bind with the immobilized ZEN-OVA conjugate at the test line on the membrane. If there was no color present in control line, the test could be considered as invalid.

Sample Preparation

Recovery and repeatability of different immunoassay were assessed in order to evaluate the accuracy and precision of this assay. Six kinds of ZEN-free fodders, such as soybean meal, silage, sorghum, corn, distillers dried grains with soluble (DDGS) and total mixed ration (TMR), were prepared as per the method described in previous reports

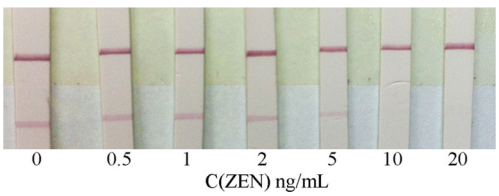

Fig. 4 The limit of detection of ICA for ZEN. Extracts of corn spiked with added ZEN concentrations from left to right: 0, 0.5, 1, 2, 5, 10, and 20 ng/kg. Up line: control line (goat anti-mouse IgG); down line: test line (ZEN-OVA). ZEN: Zearalenone; LOD: The limit of detection

(Chun et al. 2009; Sun et al. 2014). A series of ZEN standard solutions (0, 10, 50, and 100 ng/mL) were added to measure the accuracy and stability of immunoassays. The recoveries of ZEN were calculated based on the ZEN standard curve constructed by ic-ELISA and FPIA.

Results and Discussion

Preparation of Immunogen and Coating Antigen for ZEN

The ZEN-carrier protein complexes were identified by UV spectra and non-reducing PAGE. The absorbance peak and absorbance wave for ZEN-OVA showed obvious difference with that for OVA.ZEN-OVA reached its absorbance peak at around 280 and 320 nm while OVA reached its absorbance peak at 280 nm (Fig. 1). Hence, it suggested that ZEN was successfully conjugated to carrier protein. Reducing and non-reducing PAGE were carried out to confirm the conjugation of carrier-ZEN. The bands and ultra-violet absorption spectra for BSA-ZEN were similar to Fig. 1 (Data not show).

mAb Production

A total of nine hybridomas secreting specific anti-ZEN mAb were obtained by ELISA and three clones namely as 5B4, 2D1, and 1D4 were selected to prepare ascites fluid in BALB/c mouse, of which 5B4 was more stable and sensitive. The subtype of this mAb was identified as IgG1 by using the IsoQuick Kit for Mouse Monoclonal Isotyping (EnviroLogix, Portland, USA) and was chosen for the subsequent investigations.

FPIA

The experimental conditions are significant elements for the immunoassay. Therefore, the optimization is important to enhance the sensitivity and reliability of the method for an approved immunoassay. In this study, tracer and mAb dilutions were analyzed using portable FP instrument Sentry-200 (Diachemix Corp.Grayslake, IL, USA). The fluorescence polarization value of 1:12,800 dilutions of tracer was five times than that of BB solution, which guarantees the sensitivity of the FPIA. Pre-test showed that 1:2000 dilutions of anti-ZEN mAb in BB solution was appropriate for the detection of ZEN. The calibration curve equation for anti-ZEN mAb was $y = 0.82408–0.17374\times$ and exhibited a good linearity ($R^2 = 0.988$, $n = 3$) with the IC_{50} of 56.2 ng/mL (Fig. 2). The IC_{20} to IC_{80} was 3.0 to 1052 ng/mL with LOD was 0.541 ng/mL, which suggested FPIA was not as sensitive as ic-ELISA, but with a wide detection range. The optimized fluorescence polarization immunoassay method developed above was applied to regulate ZEN spiked in ZEN-free corn.

Table 1 Recovery rate of ZEN in different kinds of feed using ic-ELISA and FPIA

Sample (n = 3)	Added (ng/mL)	Ic-ELISA		FPIA	
		Recovery (%)	RSDR (%)	Recovery (%)	RSDR (%)
Soybean meal	10	89.32	5.43	91.43	6.27
	50	88.33	7.58	89.87	5.89
	100	83.85	6.63	90.85	5.79
Silage	10	86.53	6.28	85.93	4.57
	50	86.76	5.24	89.33	6.23
	100	83.96	6.13	92.44	4.55
Sorghum	10	81.67	5.10	85.39	5.29
	50	83.86	4.12	83.25	6.43
	100	82.24	6.28	89.66	4.73
Corn	10	84.43	6.20	92.35	6.47
	50	80.45	5.43	91.83	7.25
	100	84.82	7.58	85.44	4.54
DDGS	10	94.45	6.63	94.39	4.68
	50	94.45	4.21	94.02	4.86
	100	91.60	5.24	89.32	6.32
TMR	10	81.18	5.13	87.49	3.36
	50	84.67	5.12	89.55	4.85
	100	78.45	6.18	87.54	6.27

ZEN was artificially added into the ZEN-free fodders and the ZEN-free fodders have been detected by authentication center of Northwest A&F University

ZEN zearalenone, *ic-ELISA* indirect competitive enzyme-linked immunosorbent assay, *FPIA* fluorescence polarization immunoassay, *DDGS* distillers dried grains with soluble, *TMR* total mixed ration

Ic-ELISA

In this work, the sensitivity of the ic-ELISA was determined in the optimal assay conditions and presented as IC_{50} value. Checkerboard titration showed that 1:40,000 dilution of anti-ZEN mAb could give OD_{450} around 1.0 with ZEN-BSA at 1:10,000 dilutions. Range of detection, defined as from IC_{20} to IC_{80}, was 2.89 to 115.36 ng/mL, and the regression curve equation of the anti-ZEN mAb was $y = -0.41248\times + 0.97696$ and exhibited a good linearity ($R^2 = 0.994$, $n = 10$), which revealed that the IC_{50} value was 14.7 ng/mL and the limit of detection (LOD) was 0.06 ng/mL (Fig. 3). The IC_{50} of 14.87 ng/mL demonstrates that the antibody should be very capable of detecting the ZEN. Then, the specificity of the mAb 5B4 based ic-ELISA was assessed by checking its cross-reactivity with zearalanone and zearalanol. The cross-reactivity was less than 11 % for zearalanone and less than 1 % for zearalanol. This indicates the prepared mAb against ZEN was specific and sensitive.

ICA

ICA has been conventional used as a qualitative assay to detect mycotoxin at a threshold level and the result was evaluated visually. A 0.4 mg/mL of ZEN-OVA conjugate was selected as the immobilization concentration as comparison of the intensity of color among samples and control, this could be

Table 2 Comparison of three immunoassay formats for ZEN detection

Format	Reagent immobilized	Label	Competition between:	What is being detected	Detection range (ng/mL)	LOD (ng/mL)	IC_{50} (ng/mL)	Detection	Time required
Ic-ELISA	ZEN-BSA	HRP	ZEN/ZEN-BSA	Binding of Ab and ZEN-OVA	2.89 to 115.36	0.06	14.7	Absorbance	5 h
FPIA	None	FITC	ZEN/ZEN-FITC	Binding of Ab and ZEN-FITC	3.0 to 1052	0.54	56.2	Polarization of florescence	15 min
ICA	ZEN-BSA	Gold	ZEN/ZEN-BSA	Binding of Ab-gold and ZEN-OVA	–	10	–	Color	5 min

easily distinguished by naked eye. Under the optimal condition, the detection threshold level of ZEN was 10 ng/mL (Fig. 4). This meant if the test strip is positive (no color in test line), the concentration of ZEN in the sample extract was ≥10 ng/mL.

Recovery

In all spiked fodders, the average recovery rates of ic-ELISA and FPIA for ZEN were in the range of 80–100 %, and the coefficient of variation (CV) values were less than 8 % (Table 1). These observations revealed that these assays were repeatable with lower and acceptable variations.

Comparing of Three Kinds of Immunoassay Formats

In order to detect the food contaminants and toxicants rapidly, there is an urgent need to develop in situ detection techniques with high sensitivity and accuracy. The immunological methods such as ELISA, FPIA, and ICA possess different advantages in terms of efficiency, and convenient to be used on the spot detection in the food industry. The development of rapid detection methods could aid the supervision and quality assessments of raw food materials at the early stages of food production. The features of three different immunoassay employed in this study for the detection of ZEN have been shown in Table 2. The ELISA is the most widely used method in mycotoxin detection because of its high sensitivity, inexpensive instrument, and stability. However, it required multiple washing steps, antigen coating, antibody and substrate/antigen incubation, and a step to stop the enzymatic reaction, thus ic-ELISA was the most time-consuming among three formats. Compared with commercial ELISA kit of KBINWON (No. KA05001H), the sensitivity could as low as 2 ng/mL and IC_{20} is 20 ng/mL, while the LOD and IC_{20} of our anti-ZEN monoclonal antibody was 0.06 and 2.89 ng/mL. It demonstrated that our ELISA method for rapid detection of ZEN was more sensitive and was suitable for trace detection. FPIA does not require any additional manipulation as in the ELISA format and LOD as low as 0.1~10 ng/mL along with a wide detection range in previous study for ZEA (Choi et al. 2011). However, the fluorescent tracer may specifically bind to the matrix of the sample and lead to an increased fluorescence polarization value (Mi et al. 2013). The use of new fluorescent dyes, such as long wavelength fluorescent dyes, quantum dot and lanthanide, may be the solutions to improve the precision and stability of FPIA (Beloglazova et al. 2016; Thyrhaug et al. 2013; Tian et al. 2012). In addition, portable FP instrument Sentry-200 could be carried to the trading or production site testing. ICA is always used in the field-based tests, for instance preliminary examinations during outbreaks of animal diseases as well as to deal with large number of environmental samples (Li et al. 2013). Despite the practical use of ICA does not require sequential addition of reagents, it is not suitable in some cases when the quantification of an analyte is concerned. Recent study has reported quantum-dot sub microbeads-based ICA exhibited a good dynamic linear detection for ZEN over the detection range of 0.125 to 10 ng/mL with a IC_{50} of 1.017 ± 0.09 ng/mL (n = 3) (Duan et al. 2015), suggesting that the technological breakthrough based on traditional detection technology and electronic science might be the trend for the development of detection kits with high sensitivity, accurate quantification and suitable for high throughput screening.

Conclusions

Each immunoassay has different characteristic in terms of sensitivity, convenience, cost-effectiveness and high-throughput screening format. FPIA has been developed and evaluated for ZEN detection in six fodders and been compared with ELISA and ICA. Both ic-ELISA and FPIA showed good reproducibility (recoveries in 80–100 %), while FPIA has a wider working range. These results confirmed the suitability of FPIA for the rapid, accurate and precise detection of ZEN in different fodders. The portable instrumentation and simply equipped make FPIA an ideal choice for ZEN in situ testing.

Acknowledgments This study was supported by the key construction program (S2015YFKW0002) of international cooperation base in S&T, Shaanxi Province, the major science and technology projects of Luoyang (150101A), Henan Engineering Laboratory for animal disease diagnosis and food safety detection (2015382), the Ph.D. Programs Foundation of Ministry of Education of China (20130204110023), SCO Regional collaborative innovation project (2016E03012), Xinjiang,International exchanges and cooperation project of Ministry of Agriculture, China, the research was supported by Grant of Russian Foundation for Basic Research (15-53-46015), Russian.

The authors thank Dr. Diraviyam Thirumalai for the language improvement.

Compliance with Ethical Standards All procedures performed in studies involving animals were in accordance with the ethical standards of the Ethics Committee of Northwest A&F University for the use of Laboratory Animals.

Conflict of Interest Xuemei Jiang declares that she has no conflict of interest. Xiumei Li declares that she has no conflict of interest. Zhi Yang declares that he has no conflict of interest. Sergei A. Eremin declares that he has no conflict of interest. Xiaoying Zhang declares that she has no conflict of interest.

Informed Consent Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

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