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A fluorescent paper biosensor for the rapid and ultrasensitive detection of zearalenone in corn and wheat†

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Zearalenone (ZEN) is a kind of estrogen-like mycotoxin which contaminates primary crops and their products under natural conditions and becomes a serious hazard to human health. In this study, we prepared a sensitive and specific anti-ZEN monoclonal antibody (mAb) belonging to the IgG2b subclass, with a 50%-inhibitory concentration of 0.034 ng mL^{-1}. A lateral flow fluorescence microsphere immunochromatographic test strip (FM-ICTS) for the rapid and ultrasensitive detection of zearalenone in corn and wheat samples was developed based on this mAb. After optimizing experimental parameters, the visual limit of detection (LOD) of the strip assay in both corn and wheat samples was 2.5 ng mL^{-1}, and the cut-off value was 25 ng mL^{-1}. The LOD was calculated to be 0.68 ng mL^{-1} in corn samples and 0.48 ng mL^{-1} in wheat samples. Recovery experiments showed that the test results of the strip were consistent with those of ic-ELISA. As a result, this FM-ICTS assay is reliable, simple and sensitive, and can be used for rapid detection of ZEN in corn and wheat.

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1. Introduction

Mycotoxins are metabolites produced by some fungal species growing in food or feed and result in grain contamination and food poisoning.[1] At present, there are more than 300 mycotoxins known, most of which have carcinogenic, teratogenic and mutagenic effects, posing a great threat to human survival and health.[2,3] In terms of food toxicity caused by mycotoxins, Aspergillus, Penicillium and Fusarium are the most important genera among all the toxic fungi species.[4,5]

Zearalenone (ZEN), also known as F-2 toxin, is a common mycotoxin produced by several Fusarium species, and is commonly found in many cereal grains including corn, wheat, rice, barley, millet and oats.[6–8] ZEN has strong immunotoxicity, reproductive toxicity, genotoxicity and cytotoxicity,[9] and is classified as a class III carcinogen by the International Agency for Research on Cancer (IARC). ZEN's contamination of grains and feed can do harm to animals, especially pigs,[10] causing significant economic losses to livestock farms. In response to the various hazards brought about by ZEN, countries around the world have formulated relevant limit standards. In the European Union, legal limits of ZEN range from 20 to 400 μg kg^{-1} depending on different types of food. In China, the maximum limit of ZEN in wheat, wheat flour, corn, and corn flour is 60 μg kg^{-1}.[11]

At present, the commonly used methods for the detection of zearalenone in grains include high performance liquid chromatography (HPLC), gas chromatography (GC), liquid chromatography-tandem mass spectrometry (LC-MS)[12] and immunoassays. HPLC methods have become the most widespread for zearalenone analysis. These methods have proven to be of high sensitivity and accuracy, but they are not suitable for rapid screening of large quantities due to their disadvantages such as complex pretreatment, expensive equipment and high requirements for operators. Compared with traditional instrumental methods, immunoassays have the advantages of strong specificity, high sensitivity, low cost and high throughput.[13] Many immunoassays have been developed and used for ZEN detection, including enzyme-linked immunosorbent assay (ELISA),[14] fluorescence polarization immunoassay (FPIA),[15] chemiluminescence enzyme immunoassay (CLEIA),[16,17] colloid gold-immunochromatography assay (GICA)[18] and surface plasmon resonance immunoassay.[19] Among them, fluorescence immunochromatography is a fast and sensitive immunochromatography method based on fluorescent microsphere labeling.[20] Compared with colloidal gold, fluorescent microspheres as antibody markers can more effectively amplify the immune response signal and improve the analytical sensitivity.[21] This method has drawn increased attention and has been widely used in medical treatment, environment, food and other fields. By combining fluorescent microspheres with an

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immunochromatographic test strip (FM-ICTS), and then using a strip scan reader to detect the fluorescence intensity ratio, errors caused by visual observation can be avoided, and quantitative analysis of mycotoxins in food can be realized.[22]

Currently, one of the main developmental directions in the field of food safety and quality control is rapid, real-time inspection for harmful substances in foods or raw materials. In pursuit of lower LODs and shorter detection time, a sensitive and rapid FM-ICTS assay for the detection of ZEN in corn and wheat was developed in this study (Table S1†).

2. Materials and methods

2.1. Chemicals and materials

Zearalenone, zearalanone, α-zearalenol, β-zearalenol, α-zearalanol, β-zearalanol, ochratoxin A (OTA), deoxynivalenol (DON), T-2 toxin and aflatoxin B1 were acquired from Pribolab Pte. Ltd (Qingdao, China). Freund's complete adjuvant, Freund's incomplete adjuvant, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS) and 2-(4-morpholino)-ethanesulfonic acid (MES) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Keyhole-limpet haemocyanin (KLH), bovine serum albumin (BSA), *O*-(carboxymethyl) hydroxylamine hemihydrochloride (CMO), horseradish peroxidase and 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from Aladdin Chemical Co., Ltd (Shanghai, China). Reagents for cell experiments such as RPMI-1640 cell culture medium, polyethylene glycol (PEG) and dimethyl sulfoxide (DMSO) were supplied by Gibco BRL (Paisley, UK). All reagents used in the experiment were of analytical grade, and all buffer solutions were prepared with ultrapure water (Waters Maldi Synapt Q-Tof MS, Waters, Shanghai, China).

Fluorescent microspheres (product number MD021, 210 nm) were obtained from Invitrogen (Carlsbad, CA, USA). The nitrocellulose membrane (NC), sample pad (CB-SB08), absorbent pad and polyvinyl chloride pad (PVC) were purchased from Goldbio Tech Co., Ltd (Shanghai, China). Female BALB/c mice (aged 6–8 weeks) were provided by Vital River Laboratory Animal Technology Co. Ltd (Beijing, China) and the relevant animal experiments were approved by the Animal Ethics Committee of Jiangnan University.

2.2. Preparation of haptens and antigens

Zearalenone has no groups that can be directly coupled to proteins. It can be oximated and derivatized by CMO to introduce carboxyl groups[23] (Fig. 1). Briefly, 1 mg of ZEN and 2 mg of CMO were dissolved in 500 μL of anhydrous pyridine and magnetically stirred at 70 °C for 3 hours in the dark. After the reaction was over, the reaction liquid was dried with nitrogen, dissolved in 3 mL of chloroform and extracted with an equal volume of ultrapure water. The lower chloroform phase was collected and dried with nitrogen to obtain the derivative ZEN-CMO.

Synthesis of the immunogen was performed according to a previous study.[24] 1 mg of ZEN-CMO, 2.4 mg of EDC and 1.2 mg of NHS were dissolved in 400 μL of DMF solution and then the solution was magnetically stirred for 2 h at room temperature in the dark. The reaction solution was added slowly to a KLH solution (6.3 mg of KLH dissolved in 2 mL of 0.1 M carbonate–bicarbonate buffer; pH 9.6), and then the reaction was stirred overnight under dark conditions in an ice bath. The conjugates were dialyzed with 0.01 M PBS for three days to obtain the immunogen. In the synthesis of the coating antigen, KLH was replaced with BSA, and other conditions remained the same.

2.3. Monoclonal antibody production and characterization

Six female BALB/c mice (aged 6–8 weeks) were immunized with the ZEN-CMO-KLH at 3 week intervals. The first immunization dose was 80 μg per mouse and the subsequent injection dose was 40 μg per mouse.[25] From the third immunization, serums were collected from the tail vein of the mice and evaluated by ic-ELISA.[26] After the fifth immunization, the mouse with the highest titer and specificity to zearalenone was selected for cell fusion.

Cell fusion was performed as described previously.[27] The spleen cells of positive mice were fused with SP2/0 cells by PEG. After three rounds of screening and subcloning, the hybridoma cells with high titer and good inhibition were expanded and injected into the abdominal cavity of mice to produce ascites. One week later, ascites were collected and purified using a caprylic-acid–ammonium sulfate precipitate to obtain monoclonal antibodies. The obtained antibodies were dialyzed in PBS for 3 days and then stored at −20 °C.

We used a mouse mAb isotyping kit for identification of antibody subtypes. By observing the specific binding of mouse mAb to HRP-labeled antibodies of anti-mouse subtypes (IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM), the antibody subtype corresponding to the positive well with the highest OD value was the subtype of anti-ZEN mAb. We evaluated the sensitivity of the mAb using an ic-ELISA. The work point (optimal coating antigen and antibody concentration) was determined by ic-ELISA, and a series of ZEN standard solutions (0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1 ng mL^{-1}) were used to establish

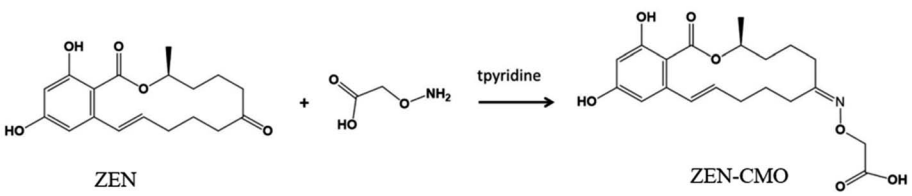


Fig. 1 Hapten synthesis of ZEN.

a standard curve under this work point. The half-maximal inhibitory concentration (IC_{50}) and the limit of detection (LOD), which were used to characterize the degree of mAb sensitivity, were calculated according to the standard curve. The cross-reactivity (CR) was used to identify the specificity of the mAb, and the calculation formula was as follows[28]

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

2.4. Fluorescent microsphere–mAb conjugates

Fluorescent microsphere–mAb conjugates were prepared using the EDC mediated activated ester method.[29] First, 100 μL of fluorescent microspheres was mixed with 400 μL of 0.05 M borate saline buffer (BB) (pH 8.0) by vortexing for one minute. 300 μg of EDC was dissolved in the FM solution for 15 min at room temperature to activate the carboxyl groups on the surface of the microspheres. After the centrifugation (13 000 rcf, 10 °C, 10 min) of the solution, the supernatant was discarded. The lower precipitate was dissolved in 0.05 M BB (pH 8.0) and sonicated at 100 W for 1 min, and then 100 μL of mAb (diluted to 0.5 mg mL^{-1} with 0.02 M BB) was added for 2 h at room temperature. After that, 50 μL of 10% BSA was added and incubated for 2 h at room temperature to terminate the reaction. The mixture was centrifuged (8500 rcf, 4 °C, 1 h) to remove the supernatant, and the precipitate was reconstituted with 0.05 M BB (pH 8.0). After sonicating the reconstituted solution for 1 min, FM–mAb conjugates were obtained and stored at 4 °C in the dark.

2.5. Assembly of the test strip components

The fluorescent immunoassay strip consists of four components including a nitrocellulose (NC) membrane, a sample pad, an absorption pad and a polyvinylchloride (PVC) backing card. The ZEN-CMO-BSA and goat anti-mouse IgG antibodies were coated on the NC membrane, respectively, as the test (T) line and the control (C) line, and then the NC membrane was dried in an oven at 37 °C for 12 h. As previously reported,[30] the NC membrane was attached to the middle of the PVC backing card, and the absorption pad and the sample pad were assembled at the upper and lower ends of the PVC backing card, overlapping 2 mm with the NC membrane. Finally, the assembled strips were cut into 3 mm wide strips and stored in an airtight and dry place for later use.[31]

2.6. Sample pretreatment

Corn and wheat were purchased from the local market as test samples and confirmed to be negative for ZEN by LC-MS (Fig. S1†). Sample pretreatment was performed as described in previous studies.[32] First, samples of corn and wheat were pulverized and sieved to obtain the solid powder. Then, 5 g of sample powder and 25 mL of aqueous solution containing 80% methanol were mixed evenly in a 50 mL centrifuge tube by vortexing for 5 min. After centrifuging the mixture at 6000 rcf for 10 min, the supernatant was diluted 5-fold with 0.01 M PBS + 3% Rhodasurf® On-870 (an ethoxylated oleyl alcohol) and used for ic-ELISA and FM-ICTS detection.

2.7. Quantitative detection using the FM-ICTS

The FM-ICTS is established based on the competitive combination between ZEN and ZEN-CMO-BSA with an anti-ZEN mAb labeled with FMs.[33] Briefly, 10 μL of FM–mAb, 40 μL of resuspension buffer and 15 μL of sample extraction solution were added to the sample well and mixed for 3 min. Next, the test strip was inserted into the microwell plate and allowed to stand for 15 min. For negative samples, the FM–mAb conjugate binds to ZEN-CMO-BSA on the T line to form a strongly fluorescent line. In contrast, the FM–mAb conjugate preferentially combines with ZEN in the positive samples, resulting in the decrease of fluorescence intensity on the T line. The amount of the FM–mAb conjugate bound to the C line has nothing to do with the concentration of ZEN in the sample. The fluorescence intensity of T and C lines can be read through the strip reader to provide information for quantitative analysis.

3. Results and discussion

3.1. Hapten and antigen characterization

The hapten of ZEN was synthesized according to the above method and characterized by LC-MS. The target compound ZEN-CMO (MW 391) has a chiral carbon structure and two isomers, D-ZEN-CMO and L-ZEN-CMO. Fig. S2(a)† shows that

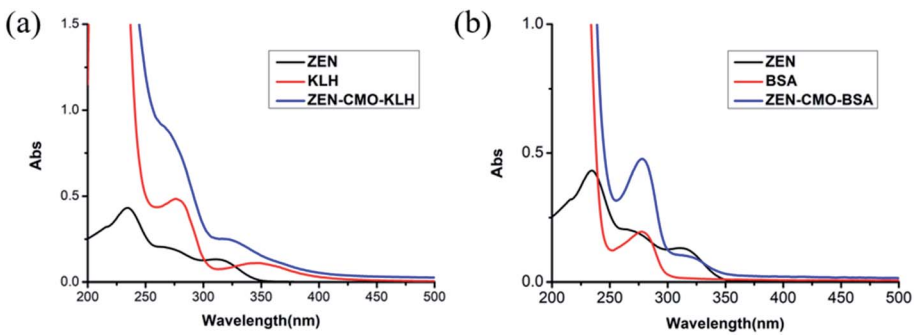


Fig. 2 The UV-Vis spectra of ZEN, proteins and conjugates. (a) Confirmation of the immunogen (ZEN-CMO-KLH); (b) confirmation of the coating antigen (ZEN-CMO-BSA).

the main products had obvious chromatographic peaks at 5.43 min. Fig. S2(b)† shows that fragments with a molecular weight of 390 Da were detected by mass chromatography, consistent with the size of the ZEN-CMO. Therefore, the synthesis reaction of ZEN-CMO was successful and the purity was very high.

The complete antigen of ZEN was identified using a UV-Vis spectrophotometer. As shown in Fig. 2(a), the ZEN molecule had an absorption peak at 240 nm and 310 nm each, while KLH had two characteristic peaks at 280 nm and 340 nm. ZEN-CMO-KLH had absorption peaks in the range of 240–280 nm and 310–340 nm, covering the peaks of ZEN and KLH. As shown in Fig. 2(b), BSA only had a characteristic absorption peak at 280 nm. Meanwhile, ZEN-CMO-BSA had ultraviolet absorption at 280 nm and 310 nm, which were consistent with the absorption peak of ZEN and BSA. These results suggested that ZEN-CMO was successfully conjugated with carrier proteins to synthesize the immunogen and coating antigen.

3.2. Characterization of a mAb against ZEN

After screening by ic-ELISA, mAb 2F6 with an optimal specificity and sensitivity was selected for subsequent experiments. Fig. 3(a) shows that the subtype of mAb 2F6 was identified as IgG2b. To calculate the IC_{50} and LOD of the mAb, a standard curve was constructed by plotting the relationship between the optical density (OD) and the concentration of ZEN. As shown in Fig. 3(b), the standard curve equation was $y = 0.174 + 1.760/(1 + [x/0.034]^{1.544})$, with a correlation coefficient (R^2) of 0.998. The IC_{50} and LOD values of mAb 2F6 were 0.034 and 0.0068 ng mL^{-1}, respectively, and the linear range of detection (IC_{20}–IC_{80}) was 0.014–0.083 ng mL^{-1}. Table 1 presents the cross-reactivity of mAb 2F6. It was not hard to see that mAb 2F6 had a strong cross-reactivity with the metabolites of ZEN and a poor cross-reactivity with other common mycotoxins such as DON, OTA, aflatoxin B1 and T2 toxin, indicating that this mAb has a good broad spectrum and strong specificity.

3.3. Optimization of the FM-ICTS assay

The test principle of the FM-ICTS requires that FMs should maintain good fluidity on the strip.[34] Because the NC membrane is hydrophobic, supplementing surfactants to the resuspension buffer can increase its hydrophilic characteristics and improve the sample fluidity. Different surfactants lead to different flow rates on the NC membrane, which greatly affects the detection sensitivity of the FM-ICTS assay.

In this study, we evaluated four surfactants including polyvinylpyrrolidone (PVP), PEG, BSA, and Rhodasurf® On-870 by using the test strip to test 0 and 1 ng mL^{-1} ZEN in PBS. As shown in Fig. 4(a), when testing ZEN negative samples, the surfactant PVP led to the most bright color intensity of the T line. Except in the PBS matrix, corn samples spiked with 0 and 10 ng mL^{-1} ZEN were also analyzed using the FM-ICTS assay. Fig. 4(b) shows that PVP still produced the best color intensity on the T line of the negative samples and provided the greatest sensitivity when detecting positive samples. Therefore, PVP was chosen as the optimal surfactant for the next set of experiments.

3.4. Sensitivity of the strip assay

Under optimized conditions, we evaluated the sensitivity of the strip assay based on the visual limit of detection (vLOD) and the cut-off value. For the naked eye, the vLOD was defined as the lowest concentration leading to a weaker color intensity on the T line than a ZEN negative sample, and the cut-off value was defined as the threshold concentration that produced a colorless T line.

In this experiment, a series of ZEN standards (0, 0.1, 0.25, 0.5, 1, 2.5, 5 and 10 ng mL^{-1}) added to 0.01 M PBS were tested by the test strip, and the test results were available within 15 min (Fig. 5(a)). As the concentration of ZEN increased, the fluorescence intensity of the T line gradually decreased. When the ZEN concentration in PBS was 0.5 ng mL^{-1}, the color intensity of the T line became obviously weaker. When the ZEN concentration reached 5 ng mL^{-1}, the T line completely disappeared. Thus, the vLOD of this method was 0.5 ng mL^{-1} and the cut-off value was 5 ng mL^{-1}. For quantitative detection, a strip scan reader was used to measure the fluorescence intensity of the T and C lines. Based on the relationship between ZEN concentrations and the T/C value, a standard curve was set up (Fig. 5(b)). The T/C value of the unknown sample was obtained with the help of a strip scan reader and then put into the curve equation to calculate the ZEN concentration in the sample.

To test the performance of the FM-ICTS in actual samples, corn and wheat samples were selected owing to the matrix effect. Eight concentrations of ZEN standards were added into

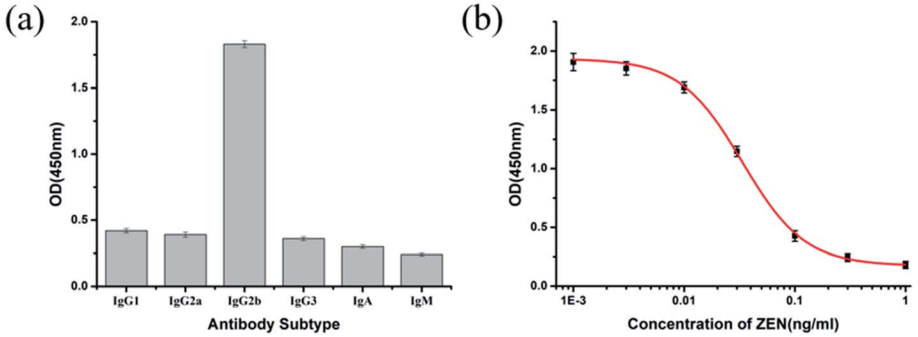
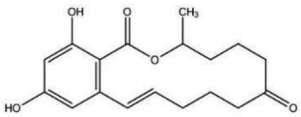
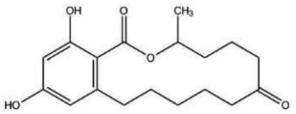
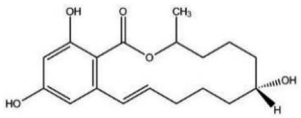
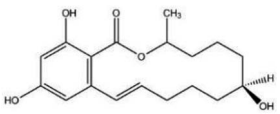
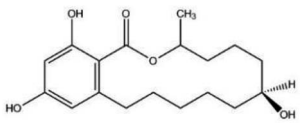
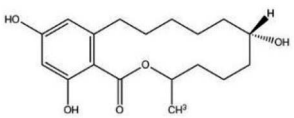
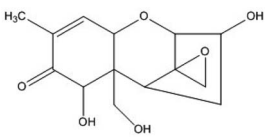
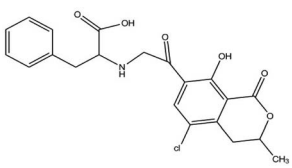
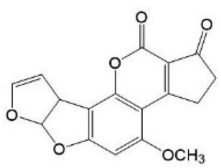
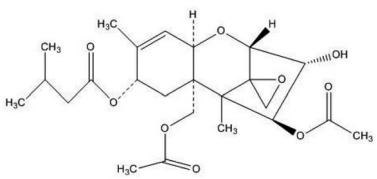


Fig. 3 Characterization of the anti-ZEN mAb. (a) Isotype determination; (b) standard curve for ZEN detection.

Table 1 The cross-reactivity of the anti-ZEN mAb

Analytes	Structure	IC_{50} (ng mL^{-1})	CR (%)
Zearalenone		0.034	100.0
Zearalanone		0.039	87.2
α-Zearalenol		0.045	75.6
β-Zearalenol		0.053	64.2
α-Zearalanol		0.041	82.9
β-Zearalanol		0.062	54.8
Deoxynivalenol		>10	<0.01
Ochratoxin A		>10	<0.01
Aflatoxin B1		>10	<0.01
T-2 toxin		>10	<0.01

negative samples, including 0, 0.5, 1, 2.5, 5, 10, 25, and 50 ng mL^{-1}. For the corn sample (Fig. 5(c)), it can be observed that a significant visual difference was generated between T and C lines when the ZEN concentration in the sample was 2.5 ng mL^{-1}, and the T line was absent when the concentration was 25 ng mL^{-1}, suggesting that the vLOD of the FM-ICTS assay was 2.5 ng mL^{-1} (corresponding to 25 μg kg^{-1} in corn samples according to the extraction procedure) and the cut-off value 25 ng mL^{-1} (125 μg kg^{-1} in corn samples). From the curve shown in Fig. 5(d), the equation for the calibration curve was $y = 0.003$

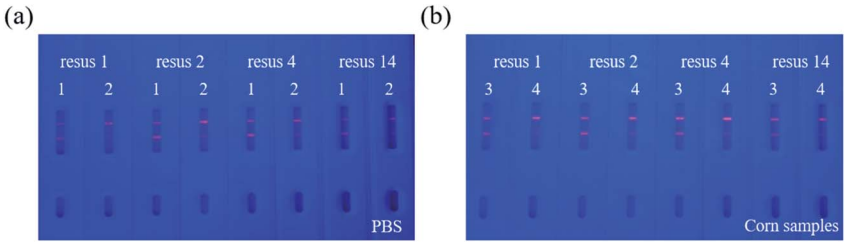


Fig. 4 Optimization of surfactants. Resus 1, 2, 4 and 14 represent PEG, PVP, BSA and On-870, respectively. (a) 1 and 2 represent ZEN standard concentrations of 0 and 1 ng mL^{-1}, respectively, prepared in PBS; (b) 3 and 4 represent ZEN standard concentrations of 0 and 10 ng mL^{-1}, respectively, prepared in corn samples.

$+ 1.445/(1 + [x/2.641]^{1.606})$, and the LOD was calculated to be 0.68 ng mL^{-1}. For the wheat sample (Fig. 5(e) and (f)), the equation for the calibration curve was $y = 0.004 + 0.892/(1 + [x/2.908]^{1.630})$, with a vLOD of 2.5 ng mL^{-1}, a cut-off value of 25 ng mL^{-1}, and a LOD of 0.48 ng mL^{-1}. These values were far below the limit standards for most countries around the world.

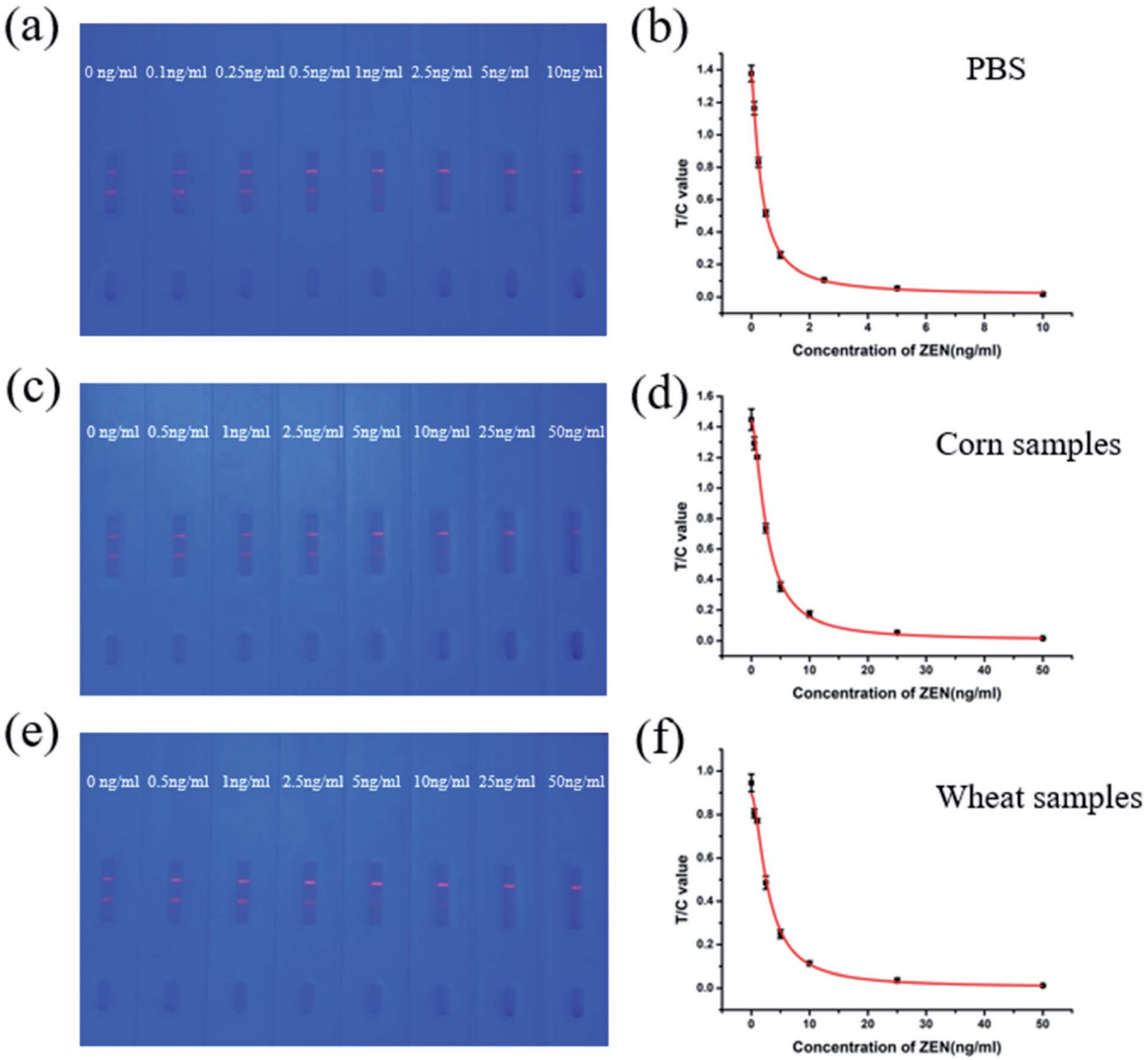


Fig. 5 Photo images of ZEN detection by a fluorescent test strip in PBS (a), corn samples (c) and wheat samples (e). Calibration curve of FM-ICTS analysis for ZEN in PBS (b), corn samples (d) and wheat samples (f). The *X*-axis represents the ZEN concentration, and the *Y*-axis represents the T/C value.

Table 2 Recovery of ZEN in corn and wheat samples by ic-ELISA and FM-ICTS assay

		ic-ELISA ($n = 5$)		Strip ($n = 5$)	
Sample	Spiked level (μg kg^{-1})	Recovery (%) ± SD	CV (%)	Recovery (%) ± SD	CV (%)
Corn	20	96.8%	4.2	93.7%	1.6
	40	94.8%	5.1	98.7%	6.4
	60	103.6%	4.0	101.3%	2.2
Wheat	20	92.2%	1.8	86.3%	2.5
	40	87.9%	4.5	95.1%	3.6
	60	94.5%	2.7	90.4%	6.1

Table 3 Comparation of LC-MS/MS and FM-ICTS for ZEN in corn and wheat samples

Sample	LC-MS/MS (μg kg^{-1})	Visual	Strip scan reader
Corn-1	95.76 ± 7.34	±[a]	107.73 ± 8.46
Corn-2	182.94 ± 11.76	+[b]	—[c]
Corn-3	334.83 ± 18.47	+	—
Corn-4	411.75 ± 16.28	+	—
Corn-5	550.46 ± 27.56	+	—
Wheat-1	88.70 ± 9.28	±	78.45 ± 5.52
Wheat-2	227.39 ± 13.71	+	—
Wheat-3	382.06 ± 20.95	+	—
Wheat-4	527.23 ± 23.66	+	—
Wheat-5	661.35 ± 32.93	+	—

[a] Weak positive: the concentration of ZEN was in the range of 12.5–125 μg kg^{-1}. [b] Positive: the concentration of ZEN exceeded 125 μg kg^{-1}. [c] Result cannot be calculated.

Moreover, the FM-ICTS assay required shorter detection time than other methods, which can meet the need for rapid detection of large quantities of corn and wheat in the field.

3.5. Sample analysis

To ensure the reliability of the FM-ICTS assay in practical application, recovery experiments of ZEN in corn and wheat samples were performed. As observed in Table 2, the average recovery rates ($n = 5$) ranged from 94.8% to 103.6% and 87.9% to 94.5% for ZEN using an ic-ELISA in corn and wheat samples, compared with 93.7% to 101.3% and 86.3% to 95.1% using FM-ICTS assay, suggesting that results of FM-ICTS assay had a good correlation with those of ic-ELISA. To further confirm the practicability of this FM-ICTS, ten different positive samples of corn and wheat were subjected to both an LC-MS/MS method and the FM-ICTS. As shown in Table 3, two samples (corn-1 and wheat-1) could be quantified effectively using the strip reader, with recovery rates of 112.5% and 88.4%. Only one C line could be observed for samples above the cut-off value. These results demonstrated that the strip assay we established was accurate, sensitive and reliable.

4. Conclusion

In this paper, an FM-ICTS for the rapid and ultrasensitive detection of ZEN in corn and wheat was developed, based on the preparation of anti-ZEN mAb 2F6, using FMs as reporters and PVP as the surfactant. This method provided not only qualitative results for rapid assessment but also quantitative results for the accurate determination of ZEN in corn and wheat samples. Overall, our FM-ICTS assay has proven to be an effective tool for onsite detection and rapid screening of ZEN in corn and wheat products.

Author contributions

Chuanlai Xu participated in the design of experiments. Yunjie Sun and Liqiang Liu performed the experiments. Aihong Wu, Shanshan Song, and Hua Kuang participated in data analysis. Hua Kuang contributed analysis tools. All authors drafted, read and approved the manuscript prior to submission.

Conflicts of interest

No potential conflict of interest was reported by the authors.

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