



Rapid detection of zearalenone and its metabolite in corn flour with the immunochromatographic test strip

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ABSTRACT

Zearalenone (ZEN) is a mycotoxin produced by fungal species which are widespread in nature and commonly contaminate many cereal grains, such as wheat, maize, corn, barley, and other cereal grains. An immunochromatographic (ICA) test strip was developed for the rapid simultaneous detection of ZEN and its metabolite in corn flour samples. For the ICA test, antigen (ZEN-BSA) and goat anti-mouse IgG were, respectively, drawn on nitrocellulose membrane as control line and test line. Under the optimized condition, the cut-off limits of test strips for ZEN, α -ZAL, and β -ZAL were found to be 0.5 ng/mL in 0.01 M PBS and 50 ng/mL in corn flour samples, meanwhile α -ZOL, β -ZOL, and ZEA were found to be 1 ng/mL in 0.01 M PBS and 75 ng/mL in corn flour samples. Our data indicate that the ICA is sensitive, rapid, cost-effective, and specific on-site screening for ZEN and its metabolite.

ARTICLE HISTORY

Received 4 October 2017
Accepted 5 October 2017

KEYWORDS

Immunochromatographic assay; monoclonal antibody; mycotoxin; strip test; zearalenone

Introduction

Mycotoxins are toxic metabolites produced under particular environmental conditions by fungi and contaminate a large variety of grains and feedstuffs worldwide (Anater et al., 2016; Serrano, Font, Maes, & Ferrer, 2016). Infection occurs through ingestion, inhalation, or skin contact with fungi. Cereal grains can be infected by mycotoxigenic fungi at pre-harvest or post-harvest. These grains can be consumed by humans leading to contamination of plant origin food (cereal grain) (Glenn, 2007). It is estimated that approximately 25% of the world's crop production is contaminated with mycotoxins, which can result in the reduction of livestock production, pose a great potential health risk to consumers, and will lead to tremendous economic losses (Chen et al., 2016; Sirot, Fremy, & Leblanc, 2013).

Zearalenone (Figure 1, ZEN), a non-steroidal β -resorcylic acid lactone with the systematic name of 3, 4, 5, 6, 9, 10-hexahydro-14, 16-dihydroxy-3-methyl-[S-(E)]-1H-2-benzoxacyl-otetradecin-1, 7(8H)-dione, mainly produced by several *Fusarium* species, such as *Fusarium graminearum* (*Gibberella zeae*), *Fusarium culmorum*, *Fusarium cerealis*, *Fusarium equiseti*, *Fusarium crookwellense*, and *Fusarium semitectum*, which are widespread in

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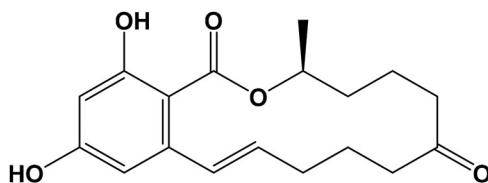


Figure 1. Chemical structure of zearalenone.

nature and commonly contaminate many cereal grains, such as wheat, maize, corn, barley, and other cereal grains (Cetin & Bullerman, 2006). ZEN shows only a low acute toxicity and there is limited evidence for its carcinogenicity and was proposed to be better fit the classification of a non-steroidal oestrogen or a myco-oestrogen. Because of the potential health risk, the provisional maximum tolerable daily intake (PMTDI) was 0.5 µg/kg body weight per day for ZEN. To reduce the intake ZEN maximum limits (MLs) for ZEN, it has been regulated in many countries and global commissions ranging from 20 to 3000 µg/kg in various food and feed stuffs (Briones-Reyes, Gomez-Martinez, & Cueva-Rolon, 2007; Chang et al., 2017; Liu et al., 2012; Meneely et al., 2010).

Due to the potential impact on human health and economical aspects, the development of a simple, rapid, and low-cost analytical methods of ZEN and its metabolite was urgently in need. The most common analytical techniques include thin-layer chromatography (TLC) (Swanson, Corley, White, & Buck, 1984), high-performance liquid chromatography (Chen, Luan, Wang, Wang, & Shao, 2017; Ok, Choi, Kim, & Chun, 2014; Sadeghi et al., 2016), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (De Santis et al., 2017; Kim et al., 2017). TLC is now rarely used because of its low sensitivity and specificity (Khaemba et al., 2016; Suryoprabowo, Liu, Peng, Kuang, & Xu, 2015). HPLC and LC-MS/MS are the methods that are most frequently used due to high sensitivity and accuracy, but these methods have disadvantages as they are complex, time-consuming, and labour intensive (Isanga et al., 2017; Ding, Liu, Song, Kuang, & Xu, 2017; Kong et al., 2017; Liu, Suryoprabowo, Zheng, Song, & Kuang, 2017b).

A variety of immunoassay methods used for ZEN detection have recently been described, including enzyme-linked immunosorbent assay (ELISA) (Bolechova et al., 2015; Du et al., 2014; Huang et al., 2014; Pei et al., 2013). The ELISA has high specificity, is applicable for high-throughput applications, requires simple sample preparation steps, and is cost-effective (Kong, Liu, Song, Kuang, & Xu, 2016; Peng, Liu, Kuang, Cui, & Xu, 2017; Suryoprabowo et al., 2015). However, the lateral-flow immunochromatographic assay (ICA) is increasingly popular because it is simple, rapid, specific, and sensitive. The ICA method has advantage that all the reagents are included in the strip and the results can be obtained within 5–10 min (Liu et al., 2014; Liu, Suryoprabowo, Zheng, Song, & Kuang, 2017a; Mukunzi, Isanga, Suryoprabowo, Liu, & Kuang, 2017).

Development of ZEN residues in wheat and maize (Huang et al., 2012), agro-food (Li et al., 2013; Majdinasab et al., 2015), and three mycotoxins in corn, rice, and peanut (Chen et al., 2016) have been detected using the ICA assay. However, no studies have used the ICA method to determine ZEN and its metabolites, such as α -zearalanol (α -ZAL),

β -zearalanol (β -ZAL), α -zearalenol (α -ZOL), β -zearalenol (β -ZOL), and zearalanone (ZEA) in corn flour.

Material and methods

Chemicals and reagents

ZEN, α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZEA standards were obtained from Sigma (St. Louis, MO, USA). Goat anti-mouse immunoglobulin (IgG) antibody was purchased from Jackson ImmunoResearch Laboratories (PA, USA). QuickAntibody-Mouse 5W was obtained from Biodragon-Immunotech Co., Ltd. (Beijing, China). Gelatin was obtained from Beijing Biodee Biotechnology Co., Ltd. (Beijing, China). Tetramethylbenzidine (TMB) and horseradish peroxidase (HRP) were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). All reagents used for cell fusion were from Sunshine Biotechnology Co., Ltd. (Nanjing, China). Bovine serum albumin (BSA) and keyhole-limpet haemocyanin (KLH) were obtained from Solarbio Science & Technology, Co, Ltd. (Beijing, China). All other reagents and chemicals were obtained from the National Pharmaceutical Group Chemical Reagent Co, Ltd. (Shanghai, China).

Nitrocellulose (NC) high-flow plus membranes (Pura-bind RP) were obtained from Whatman-Xinhua Filter Paper Co., Ltd. (Hangzhou, China). Glass fibre membrane (CB-SB08) used for the sample pad, polyvinylchloride (PVC) backing material and absorbance pad (SX18) were supplied by Goldbio Tech Co., Ltd. (Shanghai, China). A BioDot TSR 3000 Membrane Strip Reader (Gene Company Limited, Shanghai Branch, Shanghai (China)) was used to test the colour intensities of colloidal gold in the test zone. The complete Freund's adjuvant (FCA), incomplete Freund's adjuvant (FIA), and enzyme immunoassay-grade HRP-labeled goat anti-mouse immunoglobulin were obtained from Sigma (St. Louis, MO, USA). BSA and specific monoclonal antibodies (anti-ZEN monoclonal antibody (mAb), No. #3) were generated in our laboratory.

All buffer solutions were prepared using ultrapure water (Milli-Q purification system, Millipore Co., Bedford, MA, USA). The strip cutting instrument was CM 4000 (Gene, Shanghai, China). The Airjet Quanti 3000TM and Biojet Quanti 3000TM (XinqidianGene-Technology Co., Ltd., Beijing, China) were used as dispensers.

Preparation and characterization of monoclonal anti-ZEA antibody

ZEA was initially derived using carboxymethoxylamine (CMO) (Figure 2) with protein and then the ZEA-CMO-BSA or ZEA-CMO-KLH was synthesized by the active ester

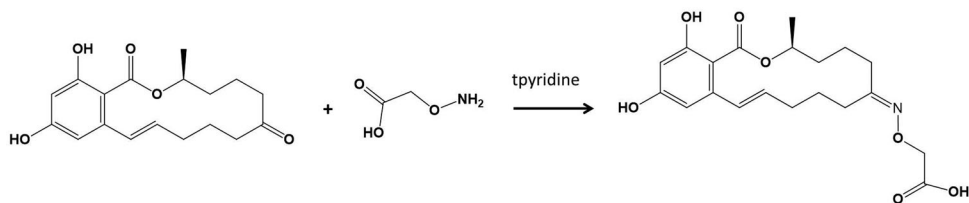


Figure 2. Oximation of zearalenone.

method (Kong, Liu, Song, Suryoprabowo, et al., 2016; Suryoprabowo et al., 2015). Briefly, a mixture of ZEN-CMO, carboxyl-reactive carbodiimide cross linker (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) (EDC), and N-hydroxysuccinimide (NHS) was added to *N,N*-dimethyl formamide (DMF) and incubated for 24 h (solution 1). KLH or BSA was mixed with 0.05 mol/L NaHCO_3 (solution 2). Solution 1 was slowly added to solution 2 with stirring and the mixture was then stirred continuously for 8 h at room temperature. The conjugates were dialysed against distilled water for 3 days and subsequently with 0.01 M PBS for a further 3 days. Ultraviolet absorption was used to check the conjugation of protein and ZEN (Figure 3).

We used FCA in the first immunization and FIA was used in the subsequent booster injections. The mice were immunized with ZEN-KLH every three weeks with 100 μg (first immunization) and 50 μg (subsequent injections; two to five times). We analysed the antibodies in blood samples of mice using ELISA. The mouse with the highest titre was sacrificed and its splenocytes were fused with Sp 2/0 murine myeloma cells. The resulting hybridomas were screened using indirect ELISA. The selected hybridoma cells were expanded and injected into BALB/c mice to produce mAbs. The ascites were harvested and purified by the caprylic acid-ammonium sulphate precipitation method (Chen et al., 2014), and the purified mAbs were stored at -20°C .

Development of the immunochromatographic strip

Preparation of colloidal gold nanoparticles

All the solvents were prepared with ultrapure water and passed through a 0.22 μm filter membrane. Chlorauric acid (0.1 g/L, 25 mL) was heated to boiling under constant stirring. Following the addition of 1.0 mL of 1% w/v sodium citrate tribasic dehydrate, the mixture was stirred for 30 min. When the colour of the solution turned wine-red, it was allowed to cool to room temperature, and stored at 4°C . Observations by transmission electron microscopy (TEM) revealed that the gold nanoparticles (GNPs) had a nearly uniform particle size of 15 nm.

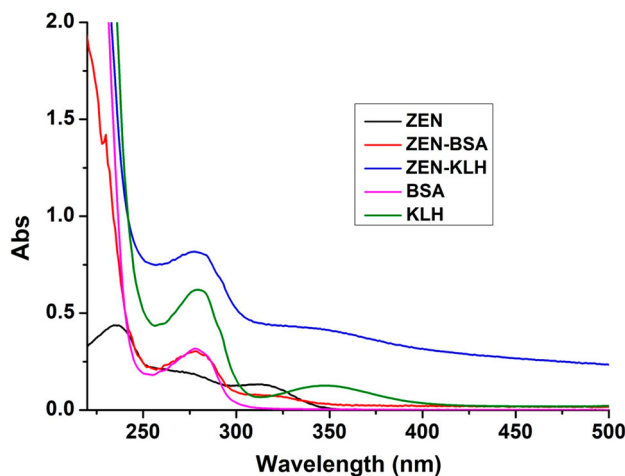


Figure 3. The UV spectra characterization for ZEN-KLH, ZEN-BSA, ZEN, KLH, and BSA.

Preparation of colloidal gold-labelled mAb

We used anti-ZEN mAb (no. #3) with a half-maximal inhibitory concentration (IC_{50}) of 0.1 ng/mL. Colloidal GNP solution (10 mL) was adjusted to pH 7.0 with 0.1 M K_2CO_3 (40 μ L). Briefly, mAbs (0.50 mL) were added to colloidal GNP solution dropwise. After 35 min, 1 mL of 10% (w/v) BSA was added, and the mixture was stirred for 2 h. The product was centrifuged at 8000 rpm for 45 min to remove any gold aggregates. The solution was separated into two layers; the lower layer (gold-labelled mAbs) was rinsed with 0.02 M phosphate buffer containing 5% sucrose, 1% BSA, and 0.5% polyethylene glycol (PEG) 6000 (pH 7.4). The conjugation products were reconstituted to 1 mL using gold-labeling resuspension buffer (0.02 M PBS, 5% sucrose, 2% sorbitol, 1% mannitol, 0.1% PEG, 0.1% tween, and 0.04% NaN_3) and stored at 4 °C.

Test procedure and principle

Prior to the test, 50 μ L of gold-labelled mAbs is mixed with 150 μ L of the sample solution, allowed to react for about 5 min, and added onto the sample pad. The solution migrates to the absorbent pad, and the test results are visualized within 5 min. In ZEN-positive samples, ZEN competes with ZEN-BSA conjugates embedded in the test (T) line for the finite amount of anti-ZEN mAb. When a sufficient amount of ZEN is present, free ZEN binds to gold-labelled mAb, preventing mAb from binding to ZEN-BSA in the T line. Therefore, the higher the ZEN contents in the sample, the weaker the colour of the T line. In ZEN-negative samples, the limited amount of colloidal gold-labelled mAb is trapped by the immobilized ZEN-BSA conjugate, and a clearly visible red T line appears.

The sample must reach the control (C) line, which contains goat anti-mouse IgG antibody. Therefore, the C line must always appear in a successful test, whereas the T line will only appear when the sample is negative (Figure 4(A)). The appearance of the C line alone is indicative that ZEN is present (Figure 4(B)), whereas if neither the C line nor the T line appears (Figure 4(C)), the test procedure is incorrect or the strip is invalid, indicating that the test should be repeated with a new strip.

Determination of performance

Sensitivity of the test strip

The sensitivity of the test strip was determined by testing ZEN, α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZEA-containing samples. ZEN, α -ZAL, and β -ZAL standard was diluted to 0, 0.01, 0.025, 0.05, 0.1, 0.25, and 0.5 ng/mL, meanwhile α -ZOL, β -ZOL, and ZEA standard was diluted to 0, 0.05, 0.1, 0.25, 0.5, and 1 ng/mL in 0.01 M PBS (pH 7.4) and the detection limit was determined. The sample solution (150 μ L) was mixed with 50 μ L gold-labelled mAb, allowed to react for 5 min, and added onto the sample pad. After 5 min, the test strip reader was used to measure the colour intensities. The lowest detection limit (LDL) that could be detected by the naked eye was defined as the amount of ZEN, α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZEA that produced a colour reaction on the strip that was visibly different in intensity from that obtained from negative (0 ng/mL). Six replicates for each concentration were analysed on the same day.

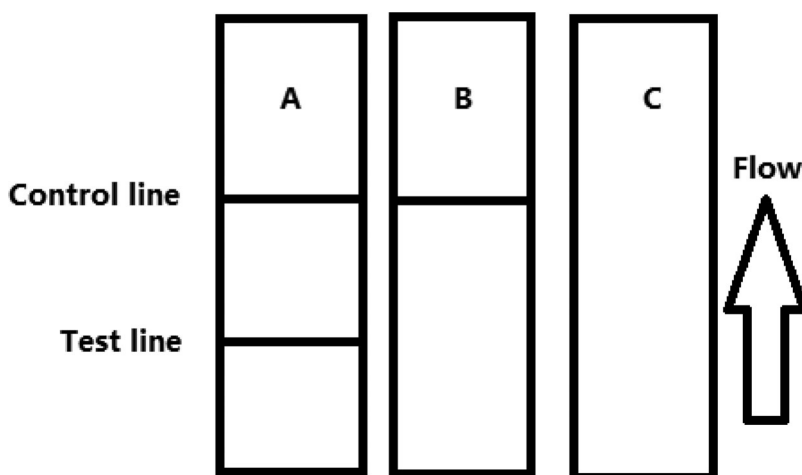


Figure 4. Illustration of typical strip test results. If the sample is negative (A), a positive result could be indicated only if the control line appears (B), and if the control and test line does not appear (invalid) (C).

Detection of ZEN, α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZEA in corn flour

We obtained corn flour from a local market at Wuxi, China. Corn flour (2 g) was mixed with 5 mL of 60% methanol/water (60:40, v/v). The solution was centrifuged at 4000 G for 5 min. After centrifugation, 1 mL of the supernatant was mixed with 4 mL 0.01 M PBS containing 0.1% tween (PBST). one hundred and fifty microlitres of the sample solution was obtained as the matrix for detection. The ZEN, α -ZAL, and β -ZAL concentrations in corn flour were 0 (control), 2.5, 5, 10, 25, and 50 ng/mL, meanwhile α -ZOL, β -ZOL, and ZEA concentrations were 2.5, 5, 10, 25, 50, and 75 ng/mL. Six replicates for each concentration were analysed using the test strips.

Results and discussion

Preparation of antibody-AuNPs (mAb-GNPs) conjugate

In the production of AuNPs, the particle size is inversely proportional to the sodium citrate volume. In this study, we choose 1 mL of 10% sodium citrate. The colour of HAuCl_4 changed from yellow to wine-red. Colloidal GNPs with a diameter 15 nm were selected because of their stability, simplicity, and bright colour. The GNPs with size 5 nm do not have the bright red colour, hence they give only a very weak to no signal at the detection line. Amount of 0.1 M KC_2CO_3 was added into the AuNPs solution to ensure the AuNPs solutions are stable.

The concentration of mAbs in GNPs of the coating antigen affects the sensitivity of the assay. In this study, two different concentrations of mAbs (8 and 10 $\mu\text{g/mL}$) were allowed to react with ZEN-negative (0 ng/mL) and ZEN-positive spiked in 0.01 M PBS (1 ng/mL). Based on Figure 5, there were significant differences in colour intensity between 8 and 10 $\mu\text{g/mL}$. We observed that the optimum concentration of mAb in GNP was 10 $\mu\text{g/mL}$. Additionally, there was a significant difference in the colour intensity at 0.05 and 0.1 mg/mL. It was found that the coating antigen with a concentration of 0.05 mg/mL

had a weaker colour intensity than at 0.1 mg/mL, suggesting that more sensitive detection could be achieved at 0.1 mg/mL. Therefore, the optimum conditions for our developed assay consisted of 0.1 mg/mL coating antigen and 8 μ g/mL.

Optimization of the strip test

The coating antigen and surfactants were optimized. The strip materials must be hydrophilic and have consistent flow characteristics in the immunochromatographic assay. NC must be hydrophobic. ICA with different coating concentrations (0.05 and 0.1 mg/mL) on the T line were evaluated with 0.01 M PBS (pH 7.4) containing negative (0 ng/mL) or positive (0.5 ng/mL). Figure 6 shows that T red lines appeared with different colour intensities at 0.05 and 0.1 mg/mL. At 0.1 mg/mL the T red lines had a deeper colour intensity than 0.05 mg/mL, suggesting that a more sensitive detection could be achieved at 0.1 mg/mL coating antigen. It is important to choose the appropriate surfactant when developing these assays because not every protein is compatible with every surfactant. In this study, suspension buffer (0.02 M PBS, 5% sucrose, 2% sorbitol, 1% mannitol, 0.1% PEG, 0.1% tween, and 0.04% NaN_3) was mixed with two reagents including polyvinylpyrrolidone (PVP) and PEG. Based on Figure 5, the suspension buffer mix with 5% PEG gave best signal colour on the T line. Therefore, the optimum conditions of our developed assay consisted of 0.1 mg/mL coating antigen and suspension buffer mixed with 5% PEG was carried out in the ICA assay.

We investigated the sensitivity of the assay using a series of ZEN, α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZEA standards diluted in 0.01 M PBS (pH 7.4). The LDL was defined as the amount of ZEN, α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZEA that produced

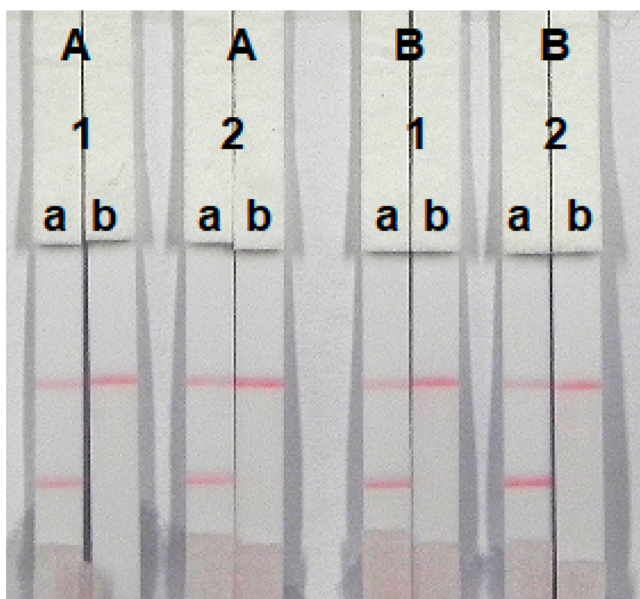


Figure 5. Optimization of the immunochromatographic strip. Concentration of coating antigen (A) 0.05 mg/mL; (B) 0.1 mg/mL. The dosage of the mAb that add in GNP: (1) 8 μ g/L; (2) 10 μ g/L. The standard concentration: (a) 0 ng/mL and (b) 1 ng/mL.

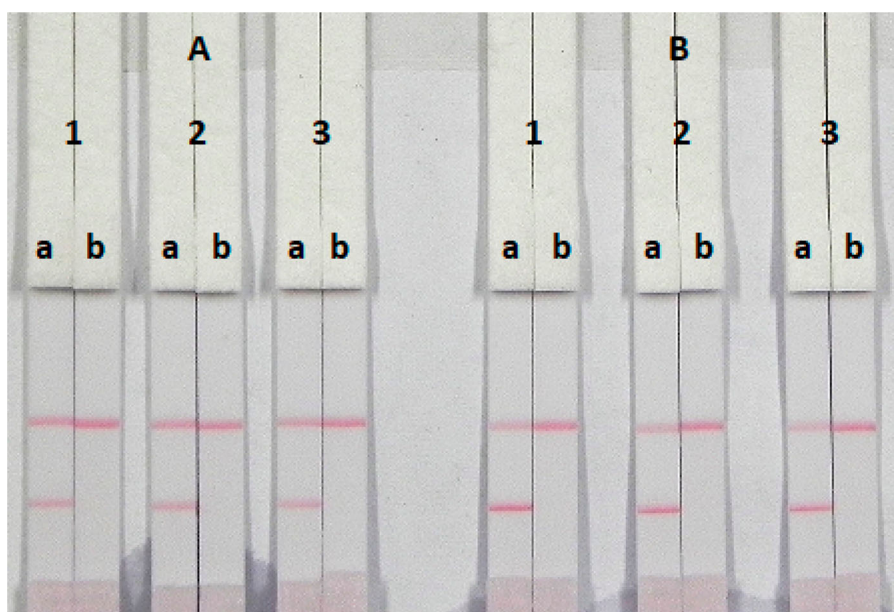


Figure 6. Optimization of the ICA test. (A) Coating antigen concentration (0.05 mg/mL) and (B) coating antigen concentration (0.1 mg/mL). (1) suspension buffer; (2) PVP and (3) PEG. (a) Negative (0 ng/mL) and (b) positive (0.5 ng/mL).

a colour reaction on the strip that was visibly different in intensity from that obtained from ZEN, α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZEA-negative samples. Figure 7 shows that the signal colour on the T lines changed from strong (0 ng/mL) to weak and completely disappeared at 0.5 ng/mL ZEN, α -ZAL, and β -ZAL, meanwhile 1 ng/mL α -ZOL, β -ZOL, and ZEA.

Detection of ZEN, α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZEA in corn flour

One of the main effects to be considered in developing these assays is the choice of surfactant reagents because the correct reagent is required for the success of the test. In this study, suspension buffer (0.02 M PBS, 5% sucrose, 2% sorbitol, 1% mannitol, 0.1% PEG, 0.1% tween, and 0.04% NaN_3) was added to four kinds of reagents, tween-20, brij-35, triton X-100, and Rhodasurf® On-870 (an ethoxylated oleyl alcohol). As shown in Figure 8, triton X-100 which produced good and stable colour reactions was used for subsequent experiments.

In this study, we determined the matrix effects using corn flour. One of the major advantages of the immunochromatographic strip assay is its rapidity and ease of use. The samples were spiked with ZEN, α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZEA standard solution (10 $\mu\text{g/mL}$, prepared with 0.01 M acetonitrile pH 7.4) at final ZEN, α -ZAL, and β -ZAL concentrations of 2.5, 5, 10, 25, and 50 ng/mL, meanwhile, ZEA concentrations of 2.5, 5, 10, 25, 50, and 75 ng/mL, and α -ZOL and β -ZOL concentrations of 5, 10, 25, 50, and 75 ng/mL. Six replicates were determined at each concentration. Figure 9 shows that the colour intensity decreased with an increase in the standard concentrations.

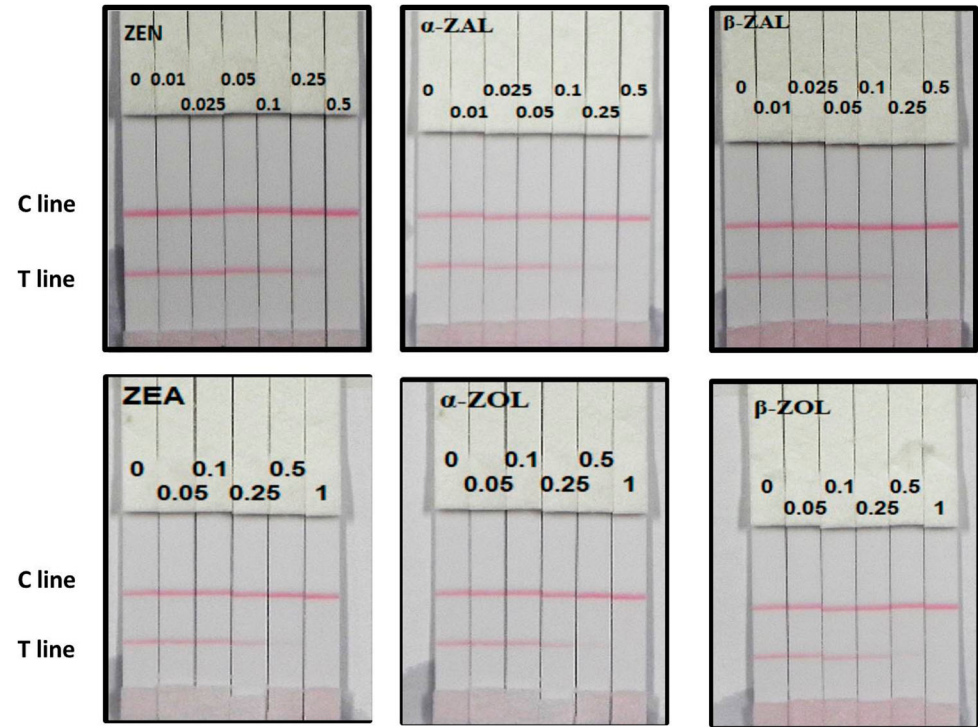


Figure 7. Colloidal gold immunochromatography assay for ZEN, α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZEA in 0.01 M PBS (pH 7.4). C line: control line; T line: test line.

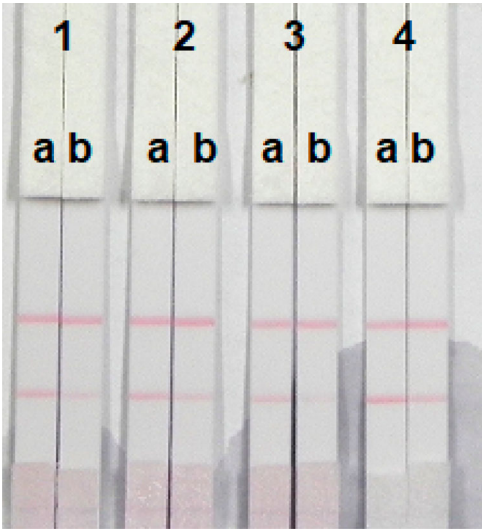


Figure 8. Result of using four kinds of reagent. (1) tween-20; (2) Brij-35; (3) Triton X-100; and (4) On-870. (a) negative (0 ng/mL); (b) positive (20 ng/mL).

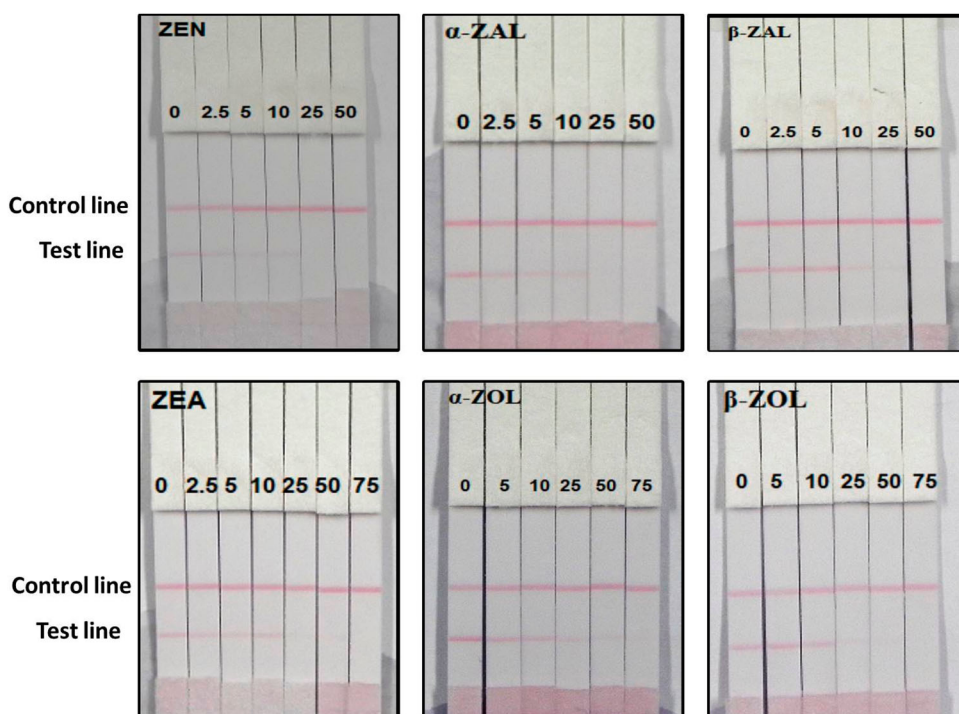


Figure 9. Result of ZEN, α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZEA in corn flour sample.

Conclusions

A simple and rapid analytical method for simultaneous determination of ZEN, α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZEA was developed by the lateral-flow immunochromatography assay. Our results demonstrate that quantitative and semi-quantitative utility of the immunochromatography strip assay, with cut-off limits for the semi-quantitative test strip as low as 0.5 ng/mL in 0.01 M PBS (pH 7.4) and 50 ng/mL in corn flour samples for ZEN, α -ZAL, β -ZAL, meanwhile 1 ng/mL in 0.01 M PBS (pH 7.4) and 75 ng/mL in corn flour samples for α -ZOL, β -ZOL, and ZEA and 50 ng/mL. The results were obtained in 10 min. The lateral-flow immunochromatographic assay represents a sensitive and rapid detection method of ZEN, α -ZAL, β -ZAL, α -ZOL, β -ZOL, and ZEA in corn flour.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work is financially supported National Key R & D Program (2016YFD0401101) and grants from Natural Science Foundation of Jiangsu Province and MOF (BE2016307, BK20150145, BX20151038, BK20140003, BE2014672, CMB21S1614, 201513006), and Taishan Industry Leading Talent Special Funds.

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