Analytical Methods

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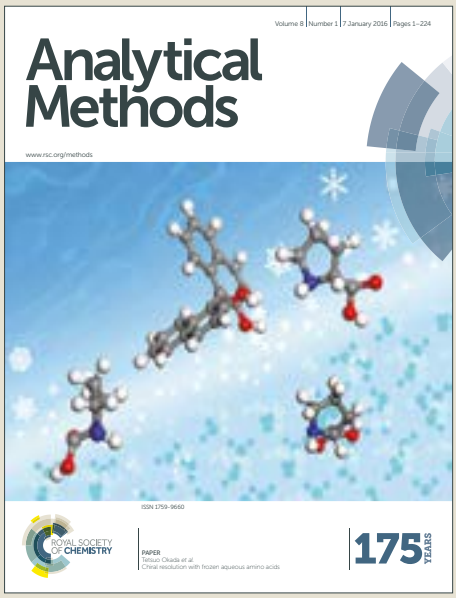

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Analytical Methods

ARTICLE

A competitive immunoassay for zearalenone with integrated poly (dimethysiloxane) based microarray assay

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A monoclonal antibody (mAb) (2B10) against zearalenone (ZEN) was first prepared in this study, which was class specific and showed no cross reactivity to other groups of mycotoxins. Then a competitive microarray assay based on a novel solid supporting material, integrated poly (dimethylsiloxane) (iPDMS), is proposed for qualitative and/or semiquantitative determination of ZEN in cereal samples. The limit of detection (LOD) and limit of quantification (LOQ) of the iPDMS based method were 0.53 μg kg^{-1} and 1.02 μg kg^{-1} for ZEN, respectively, which was lower than the EU maximum limit (ML). Meanwhile recoveries ranged from 93% to 114 % and the coefficients of variation were below 15%. The developed method was successfully applied to analyse 29 cereal samples and the results showed that 3 corn flour samples were ZEN positive, while 2 corn flour samples were determined as suspicious, which was in a good agreement with LC-MS/MS. It is worth noting that, compared with ELISA, the developed microarray assay is rapid, simple and free of blocking step. In addition, due to its advantage of zero background, appropriately increasing the concentration of coating antigen and horseradish peroxidase (HRP)-labeled secondary antibody can enhance the sensitivity of the membrane and reduce the matrix effect of the sample.

Introduction

Mycotoxins belong to a diverse group of toxic secondary metabolites produced by fungi, which can broadly contaminate the plant and threaten the safety of food or feed, thus affecting the health of human as well as animals.[1] Among the mycotoxins that have been widely documented, zearalenone (ZEN) is a frequently occurring compound that is prone to contaminate kinds of cereals crops, such as corn, barley, wheat, oats and cereal products.[2] Due to its similarity to oestrogens, ZEN can cause central precocious puberty and other reproduction problems such as infertility, abortion and other breeding problems.[3] Besides, ZEN has other toxic effects on the organism, such as teratogenesis, neurotoxicity and carcinogenicity.[4, 5] Therefore ZEN has been listed as a group III carcinogen by the International Agency for Research on Cancer.[6] Monitoring the presence of ZEN in food matrices is of great importance for securing the health of human and livestock. So far, MLs for ZEN in different matrices have been set up for its regulation.[7, 8] For instance, in Europe, the ML for ZEN is 200 μg kg^{-1} in unprocessed maize, maize flour, meal, grits, and refined maize oil, and 20 μg kg^{-1} in processed cereal-based or maize-based foods for infants and young children. The Ministry of Agriculture of China has set the ML at 60 μg kg^{-1} in cereal and cereal-based products.

To minimize the health risk caused by ZEN, many analytical methods have been developed for ZEN determination in food samples. Among these methods, chromatographic-based methods, such as gas chromatography-mass spectrometry (GC-MS),[9] and high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) are recommended as confirmatory method and commonly used for ZEN confirmation.[10, 11] However chromatographic-based methods are hampered by their unavoidable limitations. First, these instruments are unaffordable for most of laboratories. Second, the analyses are time consuming and complicated, so skilled technicians are needed. Last but not least, these analyses are unsuitable for on-site application.[12] The immunoassays, such as enzyme-linked immunosorbent assay (ELISA),[13-15] fluorescence polarization immunoassay (FPIA),[16, 17] fluorescent-labelled immunosorbent assay (FLISA) [18] and immunochromatographic test strip (ICTS),[19, 20] have emerged as a valuable alternative for mycotoxin detection with the availability of monoclonal or polyclonal antibodies. ELISA allows quantitatively detecting with high sensitivity, however, it is time consuming and not suitable for multi-analyte detection. Although ICTS has been widely used for simple sample analysis, most of the assays are qualitative showing low sensitivity and throughput.[21]

The development of a microarray with high-throughput by coupling molecular recognition element, such as antibody, on some

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substrate has acquired great attention in the field of mycotoxins detection.[22, 23] Immunoassays of small molecules are mainly based on the competitive mode,[24] in which the target molecule competes with the antigen attached on the substrate for the labelled antibody. Therefore, the detected signal is inversely proportional to the amount of target. Compared to the sandwich mode, the competitive mode of microarray is intrinsically of low sensitivity.[25, 26] Therefore, to achieve high sensitivity and throughput of low molecular weight analytes, the microarray should have high probe loading capacity, low signal variation and low non-specific protein absorption. In addition, antibodies with high affinity and specificity are very important.

Recently a novel solid supporting material, polymer-coated initiator integrated poly (dimethylsiloxane) (iPDMS), has been used for microarray assay, which was prepared by a facile surface modification of poly (dimethylsiloxane) (PDMS).[21] With property of intrinsic non-fouling and high immobilization capacity, the iPDMS could reduce background noises, achieve high sensitivity and improve the throughput in immunoassays. Next to that, the iPDMS based immunoassay requires no blocking step and the washing frequency can be reduced from 3 times to 1 time, in addition, the capture antibodies consumed in the assay can be saved up to 8-folds.[27] Up to now some immunoassays have been developed with iPDMS, such as for tumour markers analysis and the serodiagnosis of infectious diseases.[27, 28] However, as we know, no iPDMS based immunoassays have been developed for low molecular weight compounds, such as mycotoxins, antibiotics or pesticides.

In the present study, with the availability of a novel anti-ZEN mAb with high sensitivity and specificity, a sensitive microarray assay for ZEN determination based on iPDMS was developed and validated. Due to the excellent properties of iPDMS, the microarray assay provides a rapid, sensitive and inexpensive screening for ZEN with a lower limit of quantification (LOQ) of 1.02 μg kg^{-1}. More importantly, it is promising for development of multiplex screening of mycotoxins and other compounds based on iPDMS.

Experimental

Reagents and materials

ZEN, zearalanone (ZAN), α-zearalenol (α-ZEL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL) and β-zearalenol (β-ZEL) (HPLC grade, purity≥ 98%), T-2 toxin, deoxynivalenol (DON), aflatoxin B1 (AFB1), fumonisin B1 (FB1) and ochratoxin A (OTA) were obtained from Fermentek (Jerusalem, Israel). Mice (BALB/c) were purchased from Vital River Laboratory Animal (Beijing, China). All the animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Agricultural University, and the National Institutes of Health guidelines for the performance of animal experiments were followed. Carboxymethoxylamine (CMO), N-hydroxysucciniimide (NHS), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), dimethylformamide (DMF), bovine serum albumin (BSA) and tween-20 were provided by Solarbio (Beijing, China). Complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), ovalbumin (OVA), horseradish peroxidase labelled goat anti-mouse IgG (IgG-HRP), HAT media supplement (50×), HT media supplement (50×) and polyethylene glycol 1450 (PEG 1450, 50%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chemiluminescence substrate was purchased from Thermo Fisher Scientific (Rockford, USA). Fetal bovine serum was from Gibco (USA). Polymer-coated initiator integrated poly (dimethylsiloxane) (iPDMS) was provided by Suzhou SJ Biomaterials (Suzhou, China). Unless otherwise stated, all other inorganic chemicals and organic solvents were of analytical reagent grade or better. SP2/0 myeloma cells were obtained from the cell bank of the Chinese Academy of Sciences (Wuhan, China).

Instruments

The absorbance of conjugates was analyzed by a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). Chemiluminescence was recorded by Amersham Imager 600RGB (GE, USA). The results of the analysis were processed using GenePix Pro 6.0 software. Microplate reader was purchased from Biotek (ELX800, USA). LC–MS/MS analysis was performed on Agilent LC-MS-6410 Tandem Quadrupole LC-MS/MS equipped with an Agilent LC-1200 Series Liquid Chromatography. The analytical column was a C_{18} column (Agilent Poroshell 120, 150 mm × 2.1 mm, 3.5 μm). Water used in all experiments was purified with a MilliQ purification system (Millipore, Bedford, USA). LC-20AT (Shimadzu, Japan) was used for HPLC-UV analysis at the wavelength of 236 nm. The mobile phase was methanol-water (70: 30, v/v) and the flow rate of the mobile phase was 0.5 mL min^{-1}. The column (Diamonsil, 5 μm, C_{18} 150×4.6 mm) was kept at 30 °C.

Synthesis of hapten and hapten-protein conjugates

The ZEN-oxime (ZENO) was synthesized by reaction of ZEN with CMO.[29] Briefly ZEN (1 mg) was dissolved into 1.2 mL pyridine, then 2 mg CMO was added. The mixture was stirred for 16 h at room temperature, then dried with N_2 at 45 °C. The resultant was redissolved in distilled water (1 mL), and alkalized to pH 8.0 with 1 M NaOH. After ultrasonication for 2 min, the solution was extracted with benzene (3×1 mL). The aqueous phase was adjusted to pH 3.0 with 1 M HCl and extracted with ethyl acetate (4×2.5 mL). Ultimately the extract was processed with anhydrous Na_2SO_4 and dried with N_2 at 45 °C. Identity of ZENO was confirmed by HPLC-UV using methanol-water (70: 30, v/v) as mobile phase, and MS, with electrospray ionization (ESI^-) and full scan mode. The scan ranges of MS were from 100 amu to 700 amu.

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ZEN-BSA (immunogen) and ZEN-OVA (coating antigen) were synthesized using carbodiimide condensation with activated N-hydroxysuccinimide ethers.[30] For ZEN-BSA, ZENO (0.8 mg), NHS (0.6 mg), and EDC (0.5 mg) were first dissolved in 1 mL DMF and incubated for 1 h at room temperature. Then the reaction mixture was added drop by drop in 0.05 M carbonate buffer (4.15 mL, pH 9.5) containing 11 mg BSA. The mixture was stirred at room temperature for 16 h, followed by dialysis against 0.01 M phosphate buffer (pH 7.4) for 3 days in 4 °C. The conjugates were analyzed by a NanoDrop 2000 Spectrophotometer at a wavelength of 240-440 nm. After confirmation with SDS-PAGE,[31] ZEN-conjugates were stored at −20 °C before use. ZEN-OVA was synthesized similarly.

Immunization, cell fusion and antibody screening

Four six-week-old female BALB/c mice were immunized with 100 μg ZEN-BSA emulsified in an equal volume of CFA. At 14-day intervals mice received booster injections with the same amount of immunogen emulsified in IFA. To check the immune effect of the immunogen, antiserums were analysed by indirect ELISA (iELISA) and indirect competitive ELISA (icELISA) after the third booster injections. The mice with the highest titer and sensitivity with ZEN were sacrificed. Three days before cell fusion, mice were intraperitoneally injected with ZEN-BSA conjugates dissolved in PBS without adjuvant.

The fusion protocol was based on a standard method[32] with some modifications. Briefly, the spleen cells (1×10^8) and SP2/0 myeloma cells (5×10^7) in exponential phase were fused in the presence of 1 mL of 50% PEG1450. After two weeks of HAT selection, the supernatants were assessed by iELISA and the positive well was screened by icELISA using ZEN as a competitor. The positive hybridomas were subcloned by limiting dilution followed by cultivation for 1 week in HT medium. The stable high sensitivity clones after four times subcloning were expended and frozen in liquid nitrogen. For large-scale production of mAbs, the cell-line was inoculated into paraffin treated BALB/c mice. After 7-10 days, ascites fluid was collected and centrifuged at 5 000 g for 15 min. The supernatant solution was saved in 100 μL aliquots at -80 °C after purification.

Indirect competitive ELISA

The icELISA was performed as follows. High-binding polystyrene 96-well microplates were coated with 100 μL ZEN-OVA diluted in pH 9.6 carbonate buffer. After incubation at 4 °C overnight, the plates were blocked with 200 μL 5% skim milk in PBS for 2 h. Then a mixture of ZEN standard solutions and anti-ZEN mAb (diluted in 5% skim milk-PBS) (1:1, v/v) was added (100 μL/well). After incubation for 1 h, 100 μL of IgG-HRP (1: 5 000) was added and incubated for another 1 h. Finally, 100 μL of TMB substrate solution was added. The reaction was stopped after 10 min by 50 μL of 2 M H_2SO_4, and the absorbance was measured by a Biotek microplate reader (ELX800, USA) at 450 nm.

Characterization of antibodies

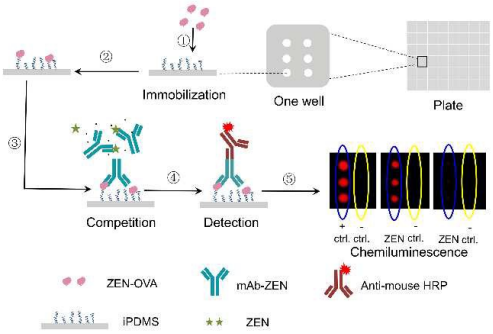

Fig. 1 Schematic illustration of microarray assay with chemiluminescence detection.

The sensitivities of the antibodies to ZEA were determined by icELISA. The isotype identification of mAb was performed using a commercial ELISA kit (Biodragon, China). Specificity was defined as the ability of the antibody to distinguish different mycotoxins from each other.[33] The specificity of the anti-ZEN mAbs was evaluated by the cross-reactivity (CR) with ZEN and its analogue, ZAN, α-ZEL, α-ZAL, β-ZAL, β-ZEL, also with AFB1, DON, FB1, OTA and T-2 due to their common presence in cereals. CR was calculated as: CR (%) = [(IC_{50} of ZEN/IC_{50} of analyte)] × 100%

Development of iPDMS based immunoassay

A 7.7 × 6.6 cm^2 sheet of iPDMS was divided into 48 wells, and 6-24 microarray spots were arranged in each well. Firstly, ZEN-OVA and spotting buffer (pH7.4, 0.01 M PBS, 0.3% mannitol, 0.005% Tween-20, and 0.5% glycerin) were respectively jet printed in 1×3 arrays (3 replicates). Then ZEN standard solution or sample extract (50 μL/well) was added together with diluted ZEN mAbs (50 μL/well), and incubated at 37 °C for 30 min. The plates were washed three times with PBST. IgG-HRP (diluted at 1: 20 000, 100 μL/well) was then added, and the plates were incubated for another 30 min at 37 °C. Next to it, 15 μL of chemiluminescence substrate was added, and images were taken at a wavelength of 645 nm using GE Amersham Imager 600 RGB. Microarray image was processed using GenePix Pro 6.0 software. The competitive inhibition rates (CIR) were obtained by the formula: CIR= (1-B/B_0) × 100%, where B_0 and B represented chemiluminescence intensity of the negative sample and ZEN solution. The schematic diagram of the developed microarray assay is shown in Fig. 1. Different levels of ZEN (0, 0.14, 0.41, 1.23, 3.70, 11.11, 33.33 and 100 μg kg^{-1}) standards were detected with the microarray assay for standard competitive curves preparation. The statistical analysis was conducted by Origin 8.0.

Sample preparation

Totally 15 corn flour and 14 wheat flour samples were collected from different local supermarkets in China. The samples were processed as follows. An amount of 1 g of ground cereals powder was extracted with 5 mL of methanol-water (8:2; v/v) for 15 min on a horizontal shaker at room temperature. After centrifuging at 3500 g for 15 min, the supernatant was filtered through a 0.45 μm membrane. Then 1 mL of the solution was dried with N_2 and dissolved into 200 μL of phosphate buffer (0.01 M) for microarray assay with 3 replicates.

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Method validation

In order to validate the iPDMS based immunoassay, the ZEN free corn and wheat samples, confirmed by LC-MS/MS, were spiked at different concentration (10, 20 and 40 μg kg^{-1}) and analysed. The detection results were compared with the results obtained with LC–MS/MS.[34]

Results and discussion

Characterization of hapten and antigen

Due to its small molecular mass (MW=318), ZEN cannot be used as an immunogen directly. Thus, the conjugates of ZEN with a carrier protein are necessary for the antibody production. ZEN contains an active keto and hydroxyl group, which can be modified to conjugate with a protein. In this study, ZEN was first derived to ZENO by introducing a carboxyl group into its 6' ketone. ZENO was confirmed by HPLC-UV and the result indicated that the purified hapten appeared at 6.3 min, while the peak of ZEN appeared around 19.3 min (Fig. 2A, B). Furthermore, the UV absorbance spectrum of ZEN (λ max, 232.5, 275, 315 nm) was almost similar with ZENO (λ max, 232.5, 275, 315 nm) (the insert of Fig. 2A and Fig. 2B), which meant that introducing the oxime group did not affect absorbance of ZEN.

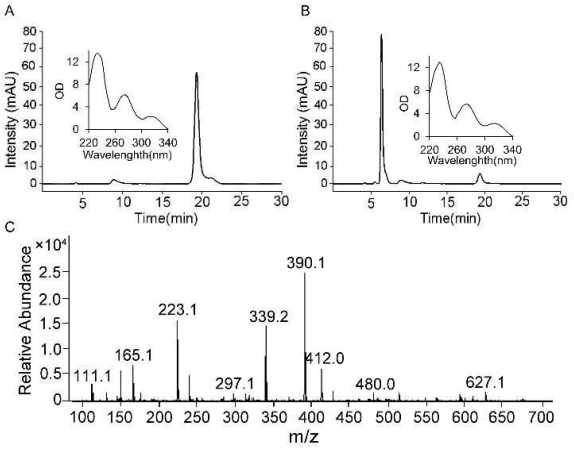

Fig. 2 Identification of ZENO hapten. (A) HPLC-UV chromatogram and UV spectrum of ZEN, (B) HPLC-UV chromatogram and UV spectrum of ZENO, (C) mass spectrum of ZENO.

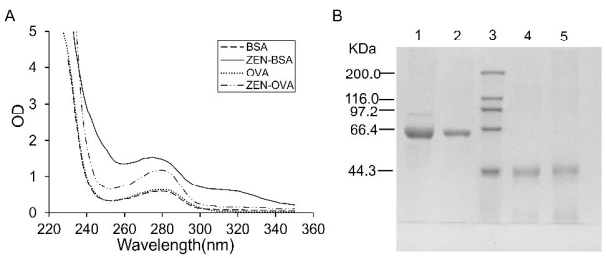

Fig. 3 Identification of hapten-protein conjugates. (A) The UV spectrum of protein and hapten-protein conjugates, (B) Analysis of protein and hapten-protein conjugates by SDS-PAGE (1: ZEN-BSA; 2: BSA; 3: Protein Marker; 4: OVA; 5: ZEN-OVA).

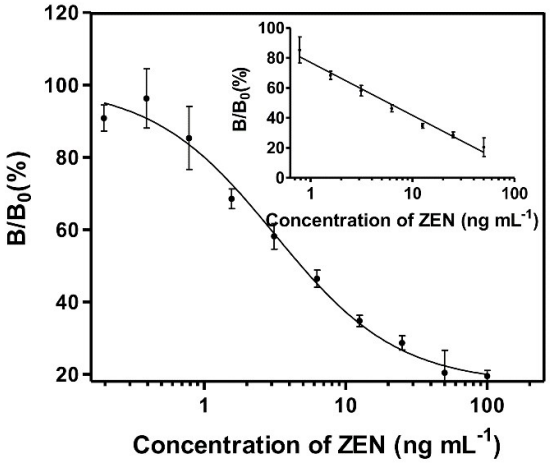

Fig. 4 Standard calibration curve of 2B10 for ZEN characterized by icELISA, the regression equation: y = -35.3×lgx + 77.0 (R^2 = 0.98). "B" and "B_0" means the absorbance at 450 nm in presence or absence of ZEN, respectively. Each point represents the mean of three wells.

Additionally, MS further identified the resultant synthetic product had the typical spectrum of ZENO ($[M-1]^-$), with m/z of 390.1 (Fig. 2C).

The ZEN-BSA and ZEN-OVA conjugates were further confirmed using UV spectrum scanning. As shown in Fig. 3A, the absorption changed after proteins conjugation and the value increased dramatically (obvious at 280 nm), which proved that the conjugation was successful. In addition, the conjugates were analyzed by SDS-PAGE, which indicated that the synthesized antigens had a little shift in molecular mass after conjugation (Fig. 3B).

Hybridoma cell selection and characterization of antibodies

In the study, after the fourth immunization, the sera titers of mice recorded a positive value >1/10 000. The icELISA further demonstrated that sera from four mice could be inhibited by free ZEN. Then the mouse showing the highest sensitivity was selected

Table 1 Parameters of the three monoclonal antibodies.

Clone	Titers of mAb	ZEN(ng mL^{-1})		
		IC_{20} [a]	IC_{50}	IC_{80} [b]
1C5	5.12×10^4	3.82	17.2	77.8
2B10	1.02×10^5	0.82	5.81	41.1
4F3	2.56×10^4	6.21	26.9	116

a Concentration at which the binding of the antibody to the coating antigen is inhibited by 20%.
b Concentration at which the binding of the antibody to the coating antigen is inhibited by 80%.

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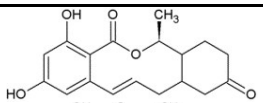
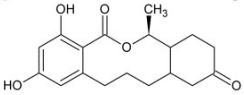
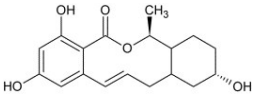
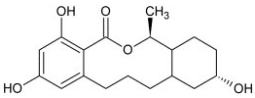
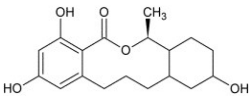
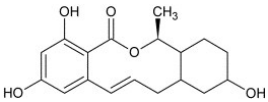
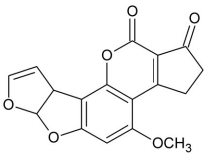
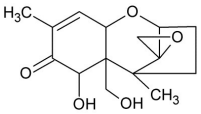
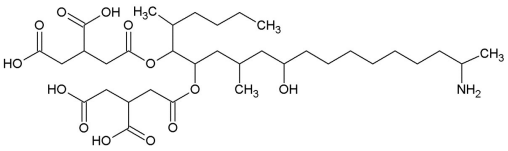
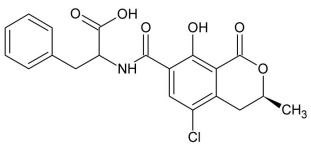
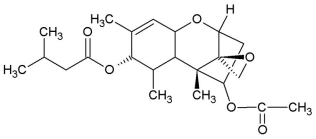
Table 2 Cross-reactivity and IC_{50} values for 2B10-mAb against ZEN, its metabolites and others mycotoxins.

Compounds	IC_{50} (ng mL^{-1})	Cross-reactivity (%)	Chemical structure
ZEN	5.81	100	
ZAN	25.26	27	
α-ZEL	8.18	71	
α-ZAL	12.10	48	
β-ZAL	38.73	15	
β-ZEL	41.50	14	
AFB1	>1000	<0.58	
DON	>1000	<0.58	
FB1	>1000	<0.58	
OTA	>1000	<0.58	
T-2	>1000	<0.58	

ZEN: zearalenone, ZAN: zearalanone, α-ZEL: α-zearalenol, α-ZAL: α-zearalanol, β-ZAL: β-zearalanol, β-ZEL: β-zearalenol, AFB1: aflatoxin B1, DON: deoxynivalenol, FB1: fumonisin B1, OTA: ochratoxin A.

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as the spleen donor for the fusion. Normally the positive cell after fusion could be selected by indirect ELISA (iELISA) or direct ELISA (dELISA). However, it has been reported that iELISA could lead to a considerable number of false positives (about 50%) and dELISA could classify the cells with high-affinity mAbs into false negative.[32] Hence, we used a two-step screening, including iELISA and icELISA, to examine positive strains in this study. Finally, three stable hybridoma clones (1C5, 2B10, 4F3) were selected to produce mAbs, which showed the highest sensitivity to ZEN. Among these mAbs, mAb produced by 2B10 (named 2B10 thereafter) had the highest sensitivity for ZEN with the lowest IC_{50} value of 5.81 ng mL^{-1} and the titer of 1.02 $\times 10^5$ (Fig. 4, Table 1), therefore it was selected for further application. The CRs of 2B10 toward other mycotoxins was also evaluated. The result indicated that this antibody showed high cross reactivity with ZEN (100%) and α-ZEL (71%), while the values for other ZEN analogues, including α-ZAL, ZAN, β-ZEL and β-ZAL, were 48%, 27%, 14% and 15%, respectively. More importantly, 2B10 exhibited no cross reactivity towards other mycotoxins commonly found in cereals (Table 2). In addition, mAb 2B10 belongs to IgG1 family and its light chain isotype was kappa.

Optimization of iPDMS based microarray assay

To achieve the best sensitivity and high chemiluminescence signals on microarray, a similar checkerboard titration was performed with a series of mAbs concentrations and ZEN-OVA concentrations. The result showed that the dilutions of mAbs at 1:3000 and concentration of ZEN-OVA at 100 μg mL^{-1} were the optimal combination for the microarray assay, with the highest CIR of 83.6% (Table 3). The calibration curve for ZEN is presented in Fig. 5. As showed in Fig. 5A, the optimal experimental conditions, with increasing concentration of the target ZEN in corn, the test spots would diminish, even disappear eventually. In detail, when the ZEN concentrations were below 1.2 μg kg^{-1}, chemiluminiscence intensity of test spots was almost invariable compared with the control. Nevertheless, chemiluminiscence intensity reduced sharply when ZEN was more than 1.2 μg kg^{-1}, and disappeared in the end. A

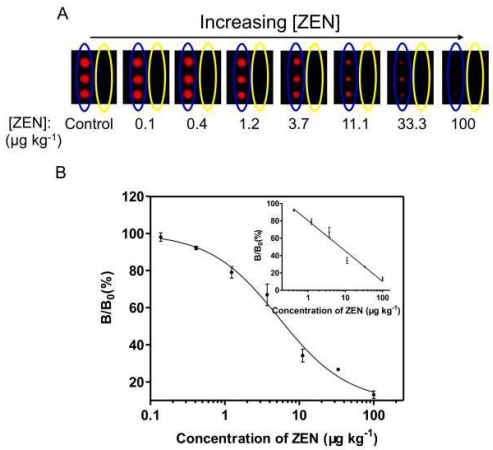

Fig. 5 Images of the microarray assay in sequential experiments with increasing ZEN concentration. In blue circle，the spots were coated with ZEN-OVA, and in yellow circle, the spots were coated with spotting buffer (A). Standard curve for quantitative detection of ZEN by iPDMS based method, the regression equation: y = -35×lg(x) +80.3 (R^2 = 0.97). "B" and "B_0"means chemiluminescence intensity in presence or absence of ZEN. Each point represents the mean of three replicates (B).

standard curve was also constructed by plotting the ratio of B/B_0 (%) against the logarithm of ZEN concentrations (Fig. 5B). By calculation, a dynamic linear range from 1.02 μg kg^{-1} to 52.6 μg kg^{-1} was obtained, with an IC_{50} of 7.32 μg kg^{-1} (n=3). Furthermore, LOQ was 1.02 μg kg^{-1} and the limit of detection (LOD) was 0.53 μg kg^{-1}, which were far below the ML of ZEN in samples set by the European Union. Therefore, the method developed in the study is promising for quantification of ZEN in real samples. Compared with ELISA, the iPDMS based microarray assay is rapid and simple, it can reduce incubation time of primary antibody and secondary antibody from 1 h to 0.5 h and shorten the washing time from half an hour to 5 min.

Table 3 Optimization of the concentrations of ZEN-OVA and anti-ZEN mAbs using a checkerboard titration.

Number	The dilution of anti-ZEN mAbs	The concentrations of ZEN-OVA (μg mL^{-1})	B_0[a]	B[ab]	CIR (1-B/B_0 (%))
1	1:1500	100	57509±19	10929±5	80.99±0.00
2	1:1500	33.3	13716 ±9	2590±7	81.12±0.04
3	1:1500	11.3	3525±34	1281±11	63.66±0.67
4	1:1500	3.7	2139±5	1138±24	46.81±1.00
5	1:3000	100	27053±16	4441±13	83.58±0.04c
6	1:3000	33.3	5825±1	1716±10	70.55±0.18
7	1:3000	11.3	2066±10	1117±5	45.91±0.50
8	1:3000	3.7	1439±9	1123±18	21.97±0.73
9	1:6000	100	9727±24	1710±3	82.42±0.02
10	1:6000	33.3	3039±16	1122±1	63.09±0.24
11	1:6000	11.3	2337±11	1027±20	56.07±0.68
12	1:6000	3.7	1260±4	1119±4	11.19±0.04

a The means of the B_0 and B values are based on three duplicate measurements.
b The B values are obtained from the 65 ng mL^{-1} ZEN standard solutions.
c The optimal parameters of microarray assay.

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Table 4 Precision and accuracy calculated by analyzing replicate samples spiked in corn and wheat samples at the concentrations of 10, 20 and 40 μg kg^{-1} of ZEN (n=3).

Samples	Theoretical level (μg kg^{-1})	Microarray assay			LC-MS/MS		
		Measured	Recovery (%)	CV (%)	Measured	Recovery (%)	CV (%)
corn	10	9.27±1.33	93	13	11.2±1.07	112	11
	20	19.0±1.22	95	6	19.8±1.31	99	7
	40	39.5±0.96	99	2	41±1.66	102	4
wheat	10	11.4±1.01	114	10	9.23±1.26	92	13
	20	21±1.6	105	8	19.3±1.15	97	6
	40	38.9±1.86	97	5	40.3±2.76	101	7

Besides, it does not require any blocking step. It is worth noting that, due to the zero background advantage of the membrane, appropriately increasing the concentration of coating antigen and HRP-labelled secondary antibody can enhance the sensitivity of assay and reduce the matrix effect of the sample.[27]

Determination of ZEN in spiked samples

To evaluate the recoveries of the method, spiked blank corn and wheat were investigated. The parameters were acquired by spiking the samples at three concentrations (10, 20 and 40 μg kg^{-1}). The samples were also analysed in parallel by LC-MS/MS. The results demonstrated good agreement between the measured values and the fortified concentration in both iPDMS based microarray assay and LC-MS/MS, with recoveries ranging from 93% to 114% for microarray analysis and from 92% to 112% for LC-MS/MS. Besides, acceptable CVs below 13% were also obtained for these analyses (Table 4).

Application of the proposed method

Once the method was validated, it was used to investigate the occurrence of ZEN in real cereal samples. As shown in Table 5, among the 15 corn flour samples, 3 samples were ZEN positive, with the concentration of 13.02, 7.02 and 18.7 μg kg^{-1}, respectively. ZEN was also found in other 2 samples, but cannot be quantified. In the 14 wheat flour samples, no ZEN were determined. Furthermore, the results of the iPDMS based microarray assay were in good agreement with the LC–MS/MS method. Therefore, this newly developed method could be used for ZEN analysis in various cereal sample matrices.

Conclusions

In this study, a ZEN specific mAb was produced and characterized, which displayed an IC_{50} value of 5.81 ng mL^{-1} for ZEN. Based on the mAb, an iPDMS based microarray assay was developed and validated. The application of the method in spiked samples and actual samples showed that the developed approach was reliable and could be used for ZEN detection in cereals. The novel platform offers multiple advantages for its simplicity, rapidity, sensitivity, high throughput and time-efficiency. It should be noted that, based on the method, there is a large prospect of developing immunoassay formats for multiplex screening of analytes. Besides, it also feasible to replace the chemiluminescence substrate by TMB chromogenic reagent substrate, by which, visualized and semi-quantitative detection can be realized.

Conflicts of interest

All authors declare that they have no conflict of interest.

Table 5 Analysis of ZEN in natural cereal samples by microarray assay and LC-MS/MS.

Matrices	Sample	Microarray assay (μg kg^{-1})	LC-MS/MS(μg kg^{-1})
corn flour	1	n/d[a]	n/d
	2	n/d	n/d
	3	n/d	n/d
	4	13.02	12.43
	5	n/d	n/d
	6	7.02	7.58
	7	n/d	n/d
	8	n/d	n/d
	9	n/d	n/d
	10	18.7	21.30
	11	n/d	n/d
	12	n/d	n/d
	13	<LOD	n/d
	14	n/d	n/d
	15	<LOD	n/d
wheat flour	1	n/d	n/d
	2	n/d	n/d
	3	n/d	n/d
	4	n/d	n/d
	5	n/d	n/d
	6	n/d	n/d
	7	n/d	n/d
	8	n/d	n/d
	9	n/d	n/d
	10	n/d	n/d
	11	n/d	n/d
	12	n/d	n/d
	13	n/d	n/d
	14	n/d	n/d

[a] n/d: Not detected.

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Development and application of an integrated poly (dimethylsiloxane) (iPDMS) based microarray assay for zearalenone