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A quadruple-label time-resolved fluorescence immunochromatographic assay for simultaneous quantitative determination of three mycotoxins in grains†

Xiaoxi Chang,‡[a] Yaqing Zhang,‡[a] Hebing Liu[b] and Xiaoqi Tao *[ac]

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A multiplex quadruple-label time-resolved fluorescence immunochromatographic assay (QL-TRFICA) for simultaneous quantitative detection of aflatoxin B_1 (AFB_1), zearalenone (ZEN) and ochratoxin A (OTA) in grains was established in this paper. This assay was developed based on quadruple-label immunoprobes, one of which was a chicken IgY-specific antibody coupled with time-resolved fluorescent nanobeads (TRFN) for the test C line signal while the other three were specific to AFB_1, ZEN and OTA monoclonal antibodies coupled with TRFN for the test T line signal. Quantitative relationships between the concentrations of the three mycotoxins and T/C ratios were established to determine the analyte concentration through a portable multi-channel fluorescence reader. The limits of detection (LODs) for AFB_1, ZEN and OTA were 0.04 μg kg^{-1}, 0.20 μg kg^{-1} and 0.10 μg kg^{-1} in 6 grains (corn, soybean, sorghum, wheat, rice and oats), respectively. Recoveries ranged from 71.60% to 119.98% at fortified concentrations of LOD, 2LOD and 4LOD, with a coefficient of variation <15%. Analysis of field grain samples by QL-TRFICA was in accordance with that of LC-MS/MS. Therefore, this proposed multiplex QL-TRFICA was a promising method for on-site screening of multiple mycotoxins in grains.

Introduction

Mycotoxins are secondary fungi metabolites that are produced by a variety of molds during their growth.[1] Among these mycotoxins, aflatoxin B_1 (AFB_1), zearalenone (ZEN) and ochratoxin A (OTA) are found predominantly in plant-based products (corn, soybean, sorghum, wheat, rice, oat and their products) and also as residues and metabolites in foods of animal origin.[2–7] AFB_1, OTA and ZEN were listed as group 1, 3 and 3 carcinogens by the International Agency for Research on Cancer (IARC), respectively.[8] Maximum residue limits for AFB_1, OTA and ZEN are regulated in many countries[9] including China, the USA and the European Union (EU) (Table S1†).[10–12] It has been reported that grain is often contaminated with a mixture of various mycotoxins.[13] Moreover, mixed pollution has significant additive effects and synergistic effects by toxicological tests, which can lead to increased toxicity.[14] Therefore, it is essential to establish a sensitive, convenient and simple method to simultaneously detect coexisting mycotoxins in grains.

Several instrumental methods[15–17] for simultaneously detecting coexisting mycotoxins have been developed. Although the results are accurate and reliable, instrumental methods require expensive equipment and skilled personnel. Biosensor methods based on antigen–antibody and antigen–aptamer[18–22] reactions have become a research hotspot for detecting toxins, among which immunoassays are widely used in on-site testing due to their high sensitivity, simplicity and cost effectiveness. Enzyme-linked immunosorbent assay (ELISA)[23] and fluorescence-linked immunosorbent assay (FLISA)[24] enable detection of multiple-mycotoxin residues, but all are heterogeneous immunoassays that require washing steps to isolate antibodies and antigens in reactions. Recently, immunochromatographic assays such as colloidal gold immunochromatography,[25] quantum dot fluorescence immunochromatography[26,27] and time-resolved fluorescence immunochromatography (TRFICA)[28] have been developed to detect multiple mycotoxins in grains or in food samples. However, colloidal gold immunochromatography has low sensitivity and quantum dot immunochromatography is affected by matrix interference. Lanthanides, such as Eu(III), Tb(III), Sm(III), and Dy(III) chelate labels, were usually used in TRFICA to provide an ultrasensitive result for detection. As a lanthanide, it is easy to use europium particles to perform, rapid, sensitive, and quantitative detection. They can eliminate

[a]College of Food Science, Southwest University, No. 2 Tiansheng Road, Beibei District, Chongqing, 400715, China. E-mail: 77179000@qq.com; Tel: +86-18306008102
[b]Beijing WDWK Biotech Co., Ltd, Beijing, 100095, China
[c]Key Laboratory of Luminescence and Real-Time Analytical Chemistry, Ministry of Education, Southwest University, Chongqing, 400715, China

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‡ These authors contributed equally to this work and should be considered co-first authors.

the interference of fluorescence background and the ability to resist matrix interference is greatly enhanced.[29] In recent years, several TRFICA methods[28,30,31] with high sensitivity and anti-interference ability for simultaneous detection of AFB_1 and ZEN mycotoxins have been reported. However, the reported methods can only detect two types of mycotoxins. Therefore, to find new methods for the multiplex detection of three or more types of mycotoxins is the future development direction for the detection of coexisting mycotoxins in grains.

In this study, we used three mycotoxins (AFB_1, ZEN and OTA) as target analytes to develop a rapid and quantitative quadruple-label time-resolved fluorescence immunochromatographic assay (QL-TRFICA) for detecting multi-class mycotoxins in grains, which may provide a new immune research approach for other hazardous chemical substances (Fig. 1). To the best of our knowledge, this is the first report of a time-resolved fluorescence immunochromatographic assay which could achieve simultaneous quantitative detection of three mycotoxins in grains.

Materials and methods

Chemicals and equipment

AFB_1, ZEN, OTA and other structurally related mycotoxin standard solutions were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemical substances were purchased from Beijing Chemical Reagent Company (Beijing, China). All solvents and other chemicals were of analytical reagent grade. Cocktail working standards of AFB_1, ZEN and OTA were prepared from the 2 mg mL^{-1} stock solution by serial dilution in sample buffer solution (0.3 M Tris–HCl containing 0.5% polyvinyl pyrrolidone and 0.4% tetronic 1307, pH 8.0). The corresponding concentrations were 0, 0.001, 0.005, 0.05, 0.1, 0.5 and 1.0 μg L^{-1} for AFB_1, ZEN and OTA, respectively.

Europium nanobeads (EuNPs) were purchased from Bangs Laboratories, Inc. (Indiana, USA). A monoclonal antibody

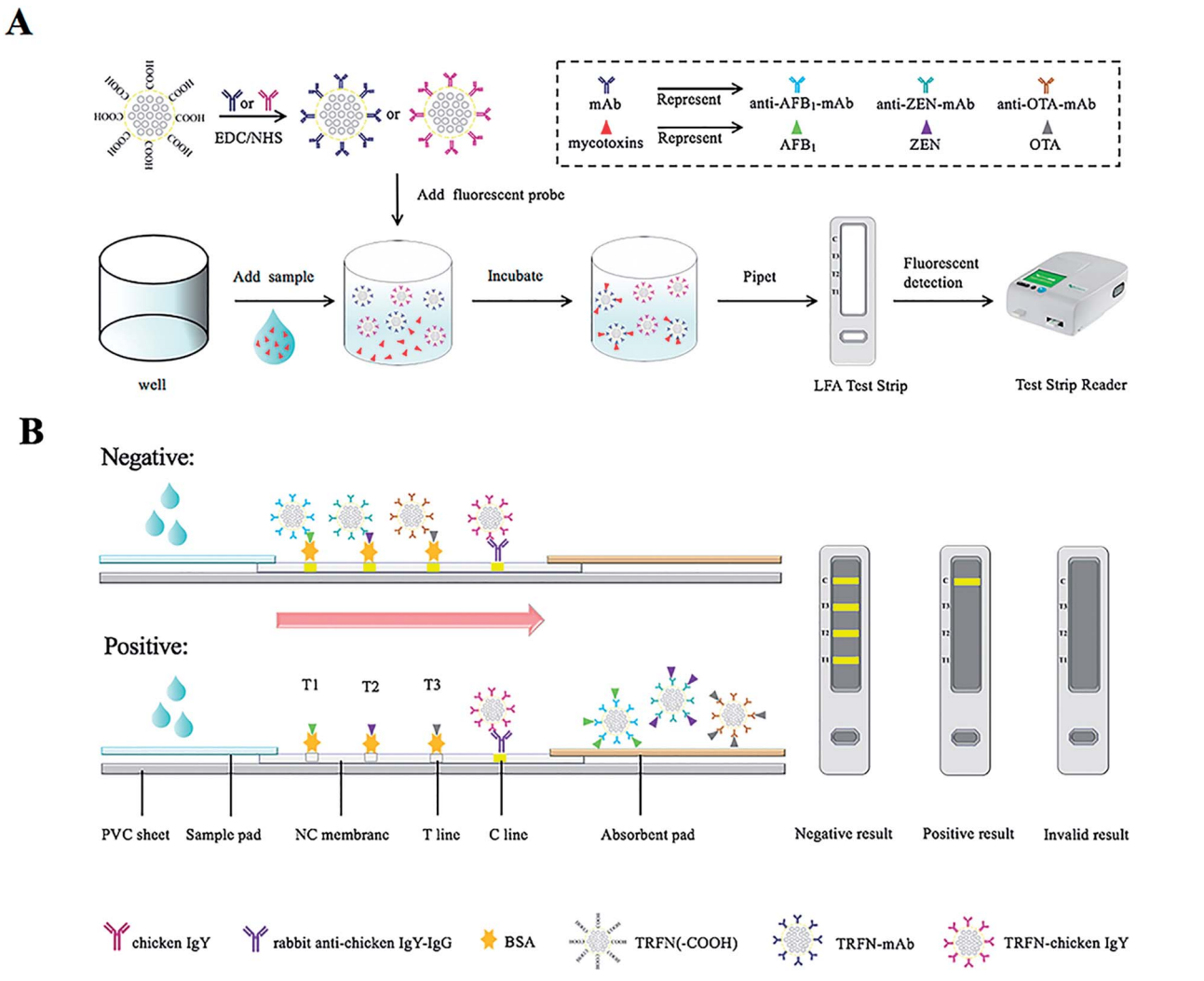

Fig. 1 Schematic demonstration of (A) detection procedure of QL-TRFICA (B) assembly of immunochromatographic strips for AFB_1, OTA and ZEN detection using multiplex QL-TRFICA.

against AFB_1 (anti-AFB_1 mAb), monoclonal antibody against ZEN (anti-ZEN mAb), and monoclonal antibody against OTA (anti-OTA mAb), and antigens of AFB_1 (AFB_1-CMO-BSA), ZEN (ZEN-CMO-BSA) and OTA (OTA-CMO-BSA) were all from WDWK Bio Co., Ltd. (Beijing, China). Rabbit anti-chicken IgG and chicken IgY were obtained from Beijing Biodragon Immunotechnologies Co., Ltd. (Beijing, China). The NC membrane (Unistart CN95) was acquired from Sartorius Stedim Biotech GmbH (Gottingen, Germany). The sample pad (glass fiber) and the absorbent pad were supplied by Shanghai Liangxin Co., Ltd. (Shanghai, China). The microtiter plates were supplied by Guangzhou JET BIOFIL Co., Ltd. (Guangzhou, China).

An XYZ3060 dispensing platform was purchased from Bio Dot Inc. (Irvine, CA, USA). The CM4000 guillotine-cutting module was purchased from Kinbio Tech Co., Ltd. (Shanghai, China). The multiplex fluorescence immunochromatographic strip reader (FIC-S2011-C36) was purchased from Shanghai Femdetection Bio-Tech Co., Ltd (Shanghai, China). Ultrapure water was purified with a Milli-Q system from Millipore Corp. (Bedford, MA, USA).

Preparation of time-resolved fluorescent probes

Quadruple immunoprobes were prepared using the above four antibodies (anti-AFB_1 mAb, anti-ZEN mAb, anti-OTA mAb and chicken IgY) conjugated to EuNPs, respectively (Fig. 1A). The EuNPs with an average size of 100 nm have a carboxyl acid group which can bind to a protein through a dehydration reaction. For conjugation, the EuNPs (5 μL, 1.0%, w/v) were mixed with 45 μL of 0.05 M 4-morpholine ethane sulfonic acid (MES) buffer solution (pH 6.0), and centrifuged at 20 000*g* for 15 min, and then the supernatant was discarded. The rinsed EuNPs were resuspended with 40 μL of 0.05 M MES buffer solution and sonicated for 1 min at room temperature. Then 1 mM NHS solution (5 μL) and 1 mM EDC solution (5 μL) were subsequently added, mixed fully, and then shaken for 15 min at room temperature. The activated EuNPs were then centrifuged at 20 000*g* for 15 min at 10 °C. After the supernatant was removed, 50 μL borate buffer (0.04 M, pH 8.0) was added and the precipitate was resuspended. Then, 20 μL of anti-AFB_1 mAb, anti-ZEN mAb, anti-OTA mAb or anti-chicken IgG (2.0, 2.1, 2.5 or 3.3 μg mL^{-1} in borate buffer at pH 8.0) was added. The mixture was shaken at 200 rpm at room temperature for 2 h, and then centrifuged at 20 000*g* for 15 min at room temperature. After the supernatant was removed, 100 μL 0.02 M PBS with 1.0% BSA was added to resuspend the precipitate. The resuspended nanobeads were shaken at 200 rpm at room temperature for 2 h, and then centrifuged at 20 000*g* for 15 min at room temperature. After the supernatant was removed, 50 μL Tris–HCl solution (0.01 M, pH 8.5) with 1.0% BSA, 2.0% sucrose and 2.0% trehalose was added to resuspend the precipitate, and the final solution was stored at 4 °C for use.

Preparation of immunochromatographic strips

The immunochromatographic strips were assembled as illustrated in Fig. 1B, consisting of the sample pad, NC membrane, absorbent pad and PVC sheet. AFB_1-CMO-BSA, ZEN-CMO-BSA and OTA-CMO-BSA were separately sprayed onto the NC membrane as capture reagents to form three test lines (T1 line for AFB_1, T2 line for ZEN and T3 line for OTA), and rabbit anti-chicken IgY IgG was sprayed onto it to form the control line (C line). The dispensing rate was 0.7 μL cm^{-1} and the interval between two lines was 4 mm. The prepared NC membrane was then dried at 45 °C for 2 h. Finally, the NC membrane, absorbent pad and sample pad were assembled with a 2 mm overlap on the PVC sheet and cut into 4.72 mm wide test strips and installed in a plastic card. Then the prepared strips were stored under dry conditions at 4 °C and kept sealed until use.

The procedure of QL-TRFICA for the three mycotoxins

Sample preparation. The samples (corn, soybean, sorghum, wheat, rice and oat) were ground into powder using a pulveriser. Each ground sample (1 g) was added into a 10 mL centrifuge tube and extracted with 4 mL of methanol/water solution (70/30, v/v). The mixture was vortexed for 5 min and centrifuged at 4000 rpm for 10 min at room temperature. The supernatant (1 mL) was diluted with 9 mL of sample buffer solution (0.3 M Tris–HCl containing 0.5% polyvinyl pyrrolidone and 0.4% tetronic 1307, pH 8.0) before measurement using QL-TRFICA.

QL-TRFICA detection. Initially, a volume of 200 μL standard or sample extraction solution was incubated with 1 μL of mixed time-resolved fluorescent probes for 5 min at room temperature (25 °C) in microwells (Fig. 1A). Then, 120 μL of the mixture was added into the sample pad and allowed to migrate. Finally, the strip was inserted into a multiplex fluorescence immunochromatographic strip reader after incubation for 9 min, and the signal of T/C was collected for quantification.

Establishment of standard curves. For the quantitative assay, the T line to C line fluorescence intensity ratio was defined as F_T/F_C. B_0 represents F_T/F_C value when there is 0 μg L^{-1} analyte, and B means F_T/F_C value when the concentration of the analyte is at other concentration. Calibration curves were obtained by plotting B/B_0 against the logarithm of analyte concentration and fitted to a four-parameter logistic equation using Origin (version 2017, OriginLab, Northampton, MA, USA) software packages.

$$y = (A - D)/[1 + (x/C)^B] + D$$

where A is the response value at high asymptote, B is the slope at the inflection point, C is the x value at the inflection point (corresponding to the concentration resulting in 50% inhibition), and D is the response value at low asymptote.

Validation of QL-TRFICA. For validation of QL-TRFICA, 6 grains (corn, soybean, sorghum, wheat, rice and oat) were confirmed to be free of AFB_1, ZEN and OTA by LC-MS/MS.[32–34] The limit of detection (LOD) was calculated as the mean value of 20 blank samples plus three times standard deviation (mean + 3SD). The accuracy of the method was investigated by spiking blank samples with single or multiple analytes at three certain concentrations (LOD, 2LOD, and 4LOD). The recovery was calculated by the following equations: recovery (%) = (measured concentration/fortified concentration) × 100%. The intra-assay and inter-assay precisions of this method were represented by

the coefficient of variation (CV), and each sample was tested 10 times in duplicate and on three consecutive days. Furthermore, 60 field grain samples (10 samples for each of corn, soybean, sorghum, wheat, rice and oat) were analyzed by the QL-TRFICA and LC-MS/MS method.[32–34]

Results and discussion

Principle of the QL-TRFICA strip for simultaneous quantification of the three mycotoxins

Fig. 1 shows the strategy of QL-TRFICA. EuNP-labeled chicken IgY was specifically captured by rabbit anti-chicken IgG on the C line as a reference signal. Immunoprobe 1 (corresponding to the T1 line) is an AFB_1-specific mAb conjugated to EuNPs, immunoprobe 2 (corresponding to the T2 line) is a ZEN-specific mAb conjugated to EuNPs, and immunoprobe 3 (corresponding to the T3 line) is an OTA-specific mAb conjugated to EuNPs, while immunoprobe 4 is a nonspecific chicken IgY conjugated to EuNPs (corresponding to the C line). In particular, different from the previously reported immunochromatographic assay which has only one labeled antibody (immunoprobes), which is a single-label assay, immunoprobes usually need to be slightly excessive to create a clearly visible or fluorescence control line for qualitative analysis.[35] For example, AFB_1-CMO-BSA (coating antigen) is immobilized on the T1 line and the rabbit anti-chicken IgG is immobilized on the C line. Immunoprobe 4 will be consistently captured by the C line while immunoprobe 1 that is not bound to free AFB_1 will be captured by the T1 line. Immunoprobe 1 bound to free AFB_1 will flow past both T (T1, T2 and T3) and C lines. The QL-TRFICA exhibited a constant C line fluorescence signal since the independent immunoprobe 4 (EuNP-chicken IgY) was used for the C line. Similarly, the fluorescence intensity of T2 and T3 lines decreased with increasing amounts of the analytes ZEN and OTA, but the C line fluorescence intensity showed no significant alteration. Therefore, quantitative relationships (inversely proportional) can be established between the concentrations of the three mycotoxins and T/C ratios and put into a portable reader for quantitative calculations.

Characterization of the three fluorescent probes

In this study, we have developed a new quadruple-label time-resolved fluorescence immunochromatographic assay, in which EuNP-anti-AFB_1 mAb, EuNP-anti-ZEN mAb, and EuNP-anti-OTA mAb were used as detection probes for simultaneous detection of AFB_1, ZEN and OTA. First, the three fluorescent probes, EuNP-anti-AFB_1 mAb, EuNP-anti-ZEN mAb and EuNP-anti-OTA mAb, were synthesized. The changes in particle size and UV absorption spectra of the three probes were explored. Fig. S1A† shows that the size of the mAbs was 25 nm (anti-AFB_1 mAb), 37 nm (anti-ZEN mAb) and 37 nm (anti-OTA mAb) respectively. After labeling the antibodies, the particle size of EuNPs was significantly increased from 91 nm to 115 nm (EuNP-anti-AFB_1 mAb), 122 nm (EuNP-anti-ZEN mAb) and 122 nm (EuNP-anti-OTA mAb). This proved the successful synthesis of the three fluorescent probes. Fig. S1B† shows that there was no significant red-shift or blue-shift of the UV absorption peak after labeling the antibodies, probably because of the large particle size of EuNPs. And the shape of the absorption peak was not significantly changed which means that the synthesized fluorescent probes were stable and uniformly dispersed in the solution.

Optimization and establishment of QL-TRFICA

To achieve the best analytical performance of QL-TRFICA, several factors were investigated, including most appropriate working concentrations of coating antigens (AFB_1-CMO-BSA, ZEN-CMO-BSA and OTA-CMO-BSA), working concentrations of immunoreagents of the immunoprobes (EuNP-anti-AFB_1 mAb, EuNP-anti-ZEN mAb and EuNP-anti-OTA mAb) and lateral flow reaction time (3–10 min at a time interval of 1 min). The optimal inhibition rate was obtained by investigating appropriate fluorescence intensity of T lines and C line. In this study, the fluorescence intensity of the C line (F_C) was almost constant under the same reaction conditions (1.6 μg mL^{-1} of rabbit anti-chicken IgG as the coating antigen and 3.4 μg mL^{-1} of EuNP-chicken IgY as the immunoprobe). Hence, the fluorescence intensity of the T lines (F_T) and the inhibition rate were chosen to estimate the effect of a certain factor on the QL-TRFICA

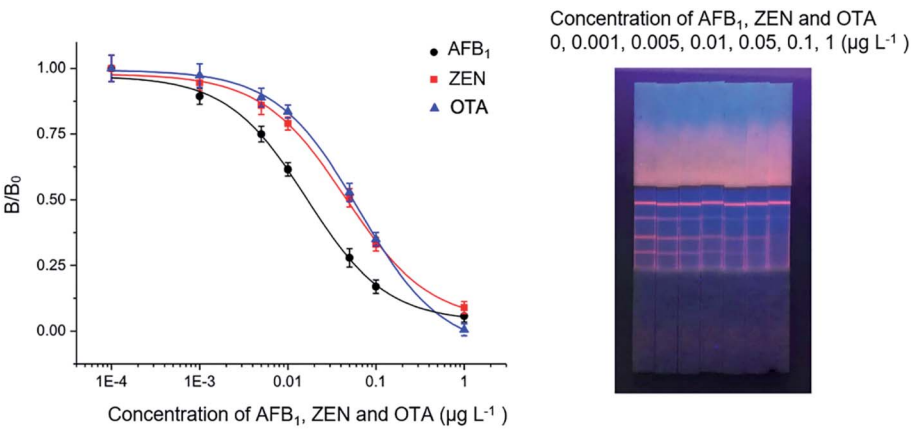

Fig. 2 Establishment of the AFB_1, ZEN and OTA standard curves in buffer.

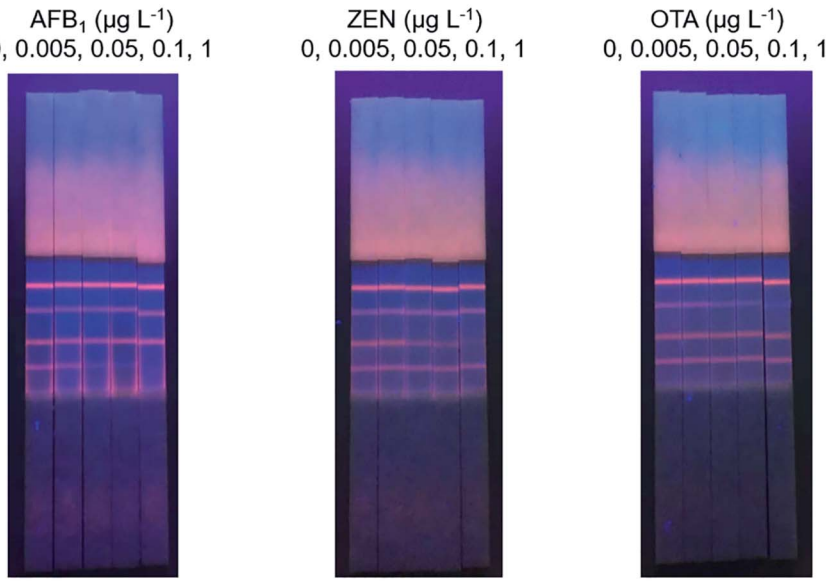

Fig. 3 Specificity of QL-TRFICA.

performance. The higher F_T value and inhibition rate indicated better sensitivity. In this study, optimum parameters of the established QL-TRFICA were 0.3, 1.25, and 0.65 μg mL^{-1} of coating antigen (AFB_1-CMO-BSA, ZEN-CMO-BSA and OTA-CMO-BSA) (Fig. S2A†), 3.0, 4.5 and 3.0 μg mL^{-1} of immunoprobes (EuNP-anti-AFB_1 mAb, EuNP-anti-ZEN mAb and EuNP-anti-OTA mAb) (Fig. S2B†) and 9 min for the lateral flow reaction time (Fig. S2C†). Under these optimized conditions, the QL-TRFICA achieved required fluorescence and optimal sensitivity.

Sensitivity

With increasing concentration of the three mycotoxins, the fluorescence intensity of each corresponding test line gradually decreased. The calibration curves were respectively established using the three mycotoxins (AFB_1, ZEN and OTA) diluted in sample buffer solution, rather than a matrix matched calibration standard. The sensitivity of QL-TRFICA was evaluated using the values of IC_{50} obtained from the calibration curves (Fig. 2), which were 0.01638, 0.06427 and 0.04496 μg L^{-1} for AFB_1, ZEN and OTA respectively. The dynamic linear range determined as the concentrations causing 20–80% inhibition of B/B_0 was 0.00387–0.06924, 0.01435–0.28789 and 0.0099–0.20423 μg L^{-1}, respectively.

Specificity

There was no mutual interference in the simultaneous detection of the three mycotoxins at serial concentrations (0.0005, 0.05, 0.1

Table 1 Comparison with immunoassays for the determination of AFB_1, ZEN and OTA

Detection method	Target substance	Sample	Time[a] (min)	LOD
TRFICA[28]	AFB_1 and ZEN	Chinese herbal medicines Semen Coicis, Rhizoma Dioscoreae, and *Platycodon grandiflorus*	15 + 15	0.60 and 0.40 g kg^{-1}
TRFIA[30]	AFB_1 and OTA	Buffer solution	5 + 95	0.02 and 0.05 μg L^{-1}
AIdnb-TRFICA[31]	AFB_1 and ZEN	Buffer solution	16 + 8	0.05 and 0.07 μg L^{-1}
GICA[36]	OTA and ZEN	Cereal	5 + 10	0.7697 and 1.2000 μg kg^{-1}
MNP-UCNPs-FICA[37]	AFB_1 and OTA	Maize	5 + 63	0.01 and 0.01 μg L^{-1}
GICA[38]	AFB_1, OTA and ZEN	Buffer solution	5 + 20	0.25, 0.50, and 1.0 μg L^{-1}
Dual-wavelength fluorescence polarization immunoassay[39]	Total amount of AFs, and the total amount of six ZEN analogues	Maize flour	10 + 20	4.98 and 11.03 μg kg^{-1}
QD-LFIA[40]	DON, ZEN and T_2/HT_2	Barley	30 + 15	1000, 80, and 80 μg kg^{-1} (cut off value)
QL-TRFICA (this study)	AFB_1, ZEN, and OTA	6 grains (corn, soybean, sorghum, wheat, rice and oats)	15 + 9	0.04, 0.20, and 0.10 μg kg^{-1}

[a] The time including pretreatment and immunoassay was appropriately estimated from the procedure in the report.

and 1.0 μg L^{-1}) around their respective cut-off values (Fig. 3). This indicated the high specificity of the developed QL-TRFICA. Furthermore, other structurally related mycotoxins, such as AFB_2, AFM_1, AFG_1 OTB, ZAN, α-ZOL and β-ZAL, and non-structurally related mycotoxins including DON and T-2 were tested individually by the QL-TRFICA to evaluate the specificity (Table S2†). The

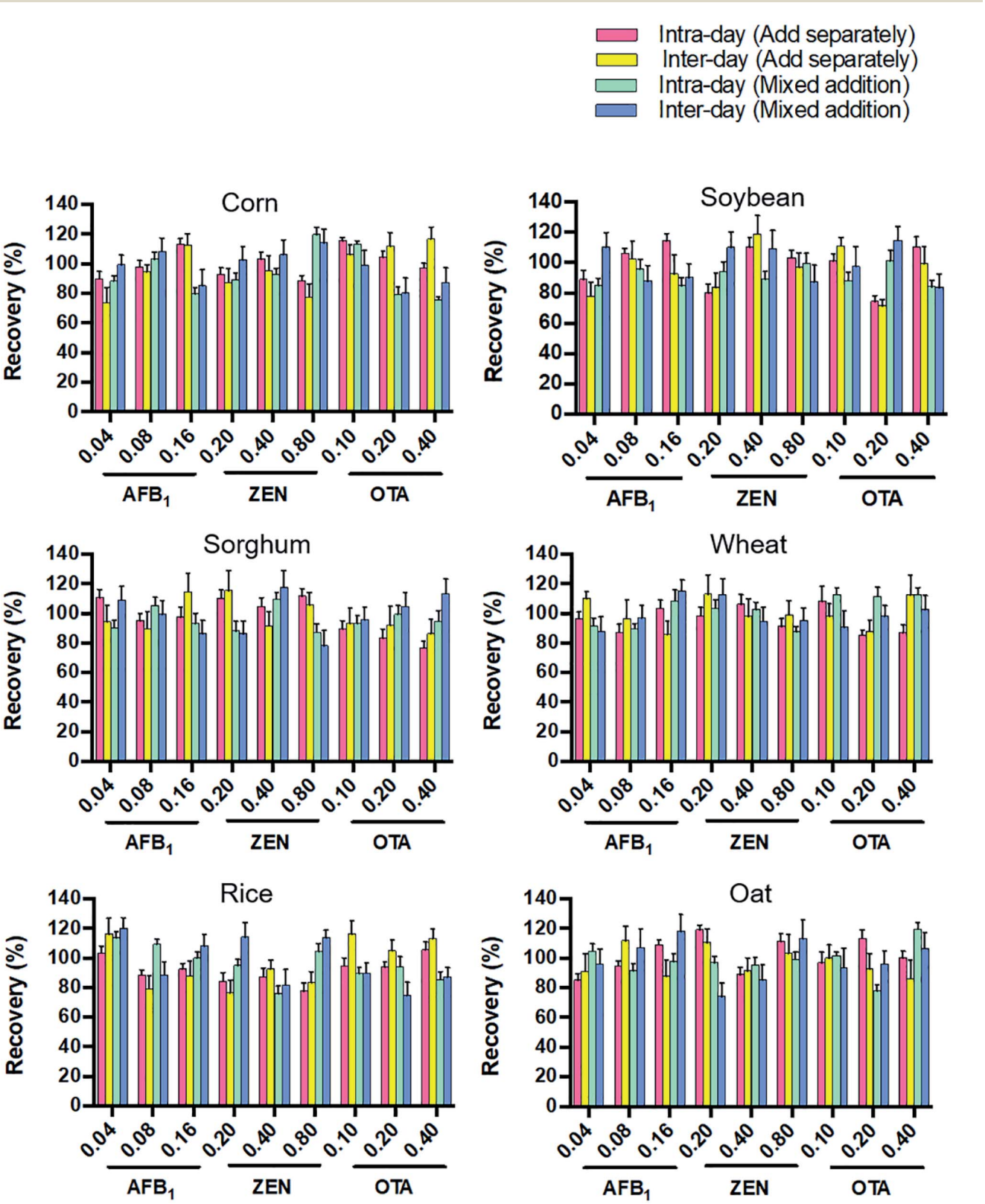

Fig. 4 The accuracy and precision of the QL-TRFICA in spiked samples.

results suggested that the other mycotoxins have negligible cross-reactivity (CR < 20%) with AFB_1, ZEN and OTA. All the results indicated that the high specificity of the LB-ICA for the three mycotoxins was acceptable.

Validation of QL-TRFICA

Limit of detection. The LOD was calculated as the mean value of 20 blank grain samples plus three times standard deviation (mean + 3SD). Each of the 20 blank grain samples (corn, soybean, sorghum, wheat, rice and oat) was extracted and analyzed according to the QL-TRFICA procedure. The LODs for AFB_1, ZEN and OTA were 0.04, 0.20 and 0.10 μg kg^{-1} in 6 grains, respectively. The Chinese standard (Table S1†) stipulates that the LOD of AFB_1 is 5 μg kg^{-1} in wheat and 20 μg kg^{-1} in corn, 60 μg kg^{-1} for ZEN in wheat and corn, and 5 μg kg^{-1} for OTA in cereals. The LODs of QL-TRFICA were far lower than the Chinese standard for the three mycotoxins (AFB_1, ZEN and OTA). The results exhibited comparable or higher sensitivity than conventional immunoassay methods (Table 1). Specifically, the established QL-TRFICA could detect three types of mycotoxins in 6 grain samples, while the TRFICA,[28] TRFIA,[30] AIdnb-TRFICA,[31] GICA[36] and MNP-UCNPs-FICA[37] could only detect two toxins. And the LOD of the developed QL-TRFICA was one order of magnitude lower than that of TRFICA,[28] GICA[36] and GICA,[38] and two orders of magnitude lower than that of the dual-wavelength fluorescence polarization immunoassay.[39] Although the reaction times (including pretreatment and immunoassay times) of TRFICA,[28] GICA[38] and dual-wavelength fluorescence polarization immunoassay[39] were similar, the immunoassay time of QL-TRFICA was reduced by almost half. Moreover, the QL-TRFICA reaction time was reduced 1 time compared with QD-LFIA,[40] 2 times compared with MNP-UCNPs-FICA[37] and 3 times compared with TRFIA.[30] In summary, the QL-TRFICA method we developed was superior to previously reported methods.

Accuracy and precision. To evaluate the accuracy of the QL-TRFICA, 6 blank grain samples (corn, soybean, sorghum, wheat, rice and oat) confirmed by LC-MS/MS were fortified with AFB_1 and/or ZEN and/or OTA standards (added separately and mixed addition of the three mycotoxins) at different known concentrations (LOD, 2LOD, 4LOD), respectively. Each sample was tested 6 times in duplicate and on three consecutive days to verify the repeatability. The average intra-assay and inter-assay recoveries of the three mycotoxins ranged from 71.60% to 119.98% with a CV value less than 15% (Fig. 4). This confirms that the QL-TRFICA is an accurate, sensitive and effective detection method and suitable for multiplex detection of mycotoxins in grains.

Application to field grain samples. For validation of the developed QL-TRFICA, 60 field samples (corn, soybean, sorghum, wheat, rice and oat) were analyzed by the QL-TRFICA and LC-MS/MS methods with a LOD of 0.03 μg kg^{-1}, 5.0 μg kg^{-1} and 1.0 μg kg^{-1} for AFB_1, ZEN and OTA.[32–34] No false-negative or false-positive results were given by the QL-TRFICA. All the results from these two methods were coincident (Table 2), proving that the QL-TRFICA method is a reliable and efficient method for the simultaneous screening of trace AFB_1, ZEN and OTA residues in grains.

Table 2 Simultaneous determination of commercially available grain samples by QL-TRFICA and LC-MS/MS methods ($n = 3$)

		QL-TRFICA (μg kg^{-1})			LC-MS/MS (μg kg^{-1})		
Sample	Number	AFB_1	ZEN	OTA	AFB_1	ZEN	OTA
Corn	6	3.11	ND	1.70	3.02	ND	1.83
	9	ND	ND	3.07	ND	ND	3.21
	1–5, 7, 8, 10	ND	ND	ND	ND	ND	ND
Soybean	5	4.66	ND	2.07	4.70	ND	1.74
	6	ND	7.24	ND	ND	7.51	ND
	1–4, 7–10	ND	ND	ND	ND	ND	ND
Sorghum	4	1.16	ND	ND	0.95	ND	ND
	1–3, 5–10	ND	ND	ND	ND	ND	ND
Wheat	2	ND	ND	6.07	ND	ND	5.93
	9	1.07	5.81	3.94	0.99	5.74	3.55
	1, 3, 4–8, 10	ND	ND	ND	ND	ND	ND
Rice	8	ND	6.39	ND	ND	6.03	ND
	1–7, 9–10	ND	ND	ND	ND	ND	ND
Oat	5	1.04	ND	5.99	1.36	ND	6.14
	1–4, 6–10	ND	ND	ND	ND	ND	ND

Conclusion

In this study, a multiplex QL-TRFICA for simultaneous quantitative determination of three mycotoxins in 6 types of grain (corn, soybean, sorghum, wheat, rice and oat) was successfully developed. Quadruple-label time-resolved fluorescent nanobeads (europium nanobeads) were employed as immunoprobes in the multiplex QL-TRFICA, achieving spatial resolution. This strategy has advantages of sensitivity, specificity, accuracy, rapidity, and cost-effectiveness. This newly developed QL-TRFICA strategy is a promising approach for high-throughput and on-site screening of multiple mycotoxins in grains. It can be applied on a large scale and used in quality inspection departments such as food inspection institutes to provide a practical reference for detecting more mycotoxins.

Conflicts of interest

Xiaoxi Chang declares that she has no conflict of interest. Yaqing Zhang declares that she has no conflict of interest. Hebing Liu declares that he has no conflict of interest. Xiaoqi Tao declares that he has no conflict of interest.

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