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Simultaneous Quantitative Determination of Multiple Mycotoxins in Cereal and Feedstuff Samples by a Suspension Array Immunoassay

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Supporting Information

ABSTRACT: Mycotoxins produced by different species of fungi may coexist in single cereal and feedstuff samples, which could become highly toxic for humans and animals. In order to quantify four mycotoxins (zearalenone, fumonisin B1, deoxynivalenol, and aflatoxin B1) in cereal and feedstuff samples simultaneously, a new suspension array immunoassay was developed. Antimycotoxin monoclonal antibodies were conjugated to the surface of different encoding microspheres (19#, 37#, 39#, and 49#), and mycotoxin−protein conjugates were then coupled with biotin. Using streptavidin-phycoerythrin as a signal reporter protein, this direct competition multiple suspension array immunoassay was optimized. The results showed that the detection limits for zearalenone, fumonisin B1, deoxynivalenol, and aflatoxin B1 were 0.51, 6.0, 4.3, and 0.56 ng/mL, respectively, with detection ranges of 0.73−6.8, 11.6−110.3, 8.6−108.1, and 1.1−14.1 ng/mL, respectively. For the detection of the spiked samples, the recovery rates were between 92.3% and 115.5%. This method also shows a good correlation coefficient ($r = 0.99$, $P < 0.01$) with liquid chromatography−tandem mass spectrometry in the detection of toxins in commercial cereal and feedstuff samples. This suspension array immunoassay was high-throughput and accurate for the rapid quantitative detection of multiple mycotoxins in commercial cereal and feedstuff samples.

KEYWORDS: *zearalenone, fumonisin B1, deoxynivalenol, aflatoxin B1, suspension array*

INTRODUCTION

Mycotoxins are secondary metabolites of fungi. The most common and hazardous mycotoxins in feedstuffs, cereal grains, and foods are aflatoxins, zearalenone (ZEN), deoxynivalenol (DON), and fumonisins. These mycotoxins are produced by *Aspergillus* and *Fusarium* during the growth of cereal grains and other plants, and in the humid storage environment after harvest. The toxicities of these mycotoxins mainly include carcinogenicity (aflatoxin B1, AFB1),[1] potential carcinogenic and teratogenic agents, estrogenicity (zearalenone),[2] vomit inducing activity (deoxynivalenol),[3] equine leucoencephalomalacia,[4] and porcine pulmonary edema (fumonisin B1, FB1). On the basis of the previous studies, greater toxicities were observed when a multiplex of mycotoxins occurred in a single sample.[5,6] More importantly, these mycotoxins coexisted in individual samples from different countries.[7−10] Thus, a sensitive, rapid, and accurate test for multiplex mycotoxins is urgently needed to monitor cereal, feedstuff, and other samples.

To detect several mycotoxins simultaneously, researchers have developed many analytical methods, including liquid chromatography−mass spectrometry (LC-MS),[11] gas chromatography−mass spectrometry (GC-MS),[12] colloidal gold strips,[13] and solid-phase chips.[14,15] Chromatographic analyses (LC-MS and GC-MS) are accurate and reliable but costly and time-consuming. Since the development of arrays (chips), the technology has been widely applied in many areas, especially in the high-throughput detection fields, such as clinical medicine, molecular biology, cell biology, proteomics, and analytical chemistry. Solid-phase chips have been employed to evaluate or detect the binding of ligands and receptors. For the detection of mycotoxins, this solid-phase array immunoassay can detect four mycotoxins in one chip[14] but requires coating, blocking, and additional steps for each detection. In addition, it has low sensitivity and is time-consuming. Another solid-phase array based on the mycotoxin biosynthesis genes[15] can detect several mycotoxins simultaneously but also still requires coating, blocking, and 18 h to perform a complete test.

On the basis of the surface encoding of different fluorescent microspheres and the binding reaction of antigen/antibody, enzyme/substrate, ligand/receptor, and the nucleic acid hybridization reaction, suspension arrays have the advantages of sensitivity, rapidity, and accuracy. Signal responses are observed using red and green laser lights to achieve qualitative and quantitative detections. Using 100 different staining microspheres, a single sample may be tested for up to 100 different analytes. Moreover, the final signal response is calculated using a large number of microspheres for each analyte, which is more accurate than 3−5 repeats in a solid-phase array. The suspension array is more sensitive because a liquid system allows for greater potential interactions between immunoreagents compared to that in solid-phase immunoassays. This new generation of high-throughput detecting platform arrays including the suspension array have a great potential for applications in agriculture and food chemicals, such as pesticides,[16] antibiotics,[17] bacterial,[18] veterinary drugs,[19]

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and mycotoxins.[20] However, the suspension array technology based on a direct competition immunoassay and a biotin−streptavidin signal amplification system for mycotoxin detecting has not been reported. Compared to the indirect competition suspension array technology, this method is rapid and easier to perform.

In this study, specific monoclonal antibody coated microspheres, biotin-labeled mycotoxin conjugates, and streptavidin−phycoerythrin (signal reporter molecules) were used in suspension arrays to quantitatively detect four mycotoxins simultaneously. This method provides an excellent platform for the rapid detection of multiple low molecular weight analytes with high-throughput, accuracy, and reproducibility.

MATERIALS AND METHODS

Materials. Biotin, ZEN, and FB1 were purchased from Sigma Chemical (St. Louis, MO, USA). Ovalbumin (OVA) was obtained from Sangon Biotech (Shanghai, China). Tween-20 was obtained from Generay Biotech (Shanghai, China). Monoclonal antibodies against ZEN (2C9, mAb-ZEN) and FB1 (6H3, mAb-FB1) were generated in our laboratory.[21,22] DON, AFB1, DON-BSA conjugate, and AFB1-BSA conjugate, monoclonal antibodies against DON (mAb-DON) or AFB1 (mAb-AFB1) were obtained from Huaan megnech (Beijing, China). Sulfo-NHS-LC-biotin was purchased from Molecular Probes (Eugene, OR, USA). Streptavidin−phycoerythrin was obtained from Tellgen (Shanghai, China). The four mycotoxin (ZEN, FB1, DON, and AFB1)-free corn samples were provided by Shanghai Entry-Exit Inspection and Quarantine Bureau. The commercial samples were purchased from the nearby markets.

Equipment. Ninety-six-well microtiter plates were purchased from Greiner Bio (Würzburg, Germany). A Luminex 200 suspension array analyzer and control software and a computer (Luminex 100 IS) were provided by Luminex (Austin, TX, USA). The horizontal shaker (MX2) was from Finepcr (Seoul, Korea). The ultraperformance liquid chromatography (UPLC) system and UPLC BEH C18 column were supplied by Waters (Milford, MA, USA). The QTrap MS/MS system was obtained from Applied Biosystems (Foster City, CA, USA).

Methods. *Biotin-Labeled Fungal Toxin Conjugates and Antibody-Coated Microspheres.* The ZEN-OVA was conjugated with biotin as previously described[23] with slight modifications. ZEN-OVA was dissolved in reaction buffer (0.1 M Na_3PO_4 and 0.15 M NaCl, pH 7.2), and sulfo-NHS-LC-biotin was added to this solution slowly and mixed for 2 h. The biotinylated derivative (ZEN-OVA-biotin) was purified by gel filtration on a Sephadex G-25 column and detected by spectrophotometric measurement at 280 nm. Other biotin-labeled mycotoxins conjugates (ZEN-OVA, FB1-OVA, DON-BSA, and AFB1-BSA) were prepared as ZEN-OVA-biotin.

Antibody-coated microspheres were prepared as previously described.[24] The biotin-labeled mycotoxin conjugates and antibody-coated microspheres were measured by a direct competition suspension array method established in this study. The antibody−microspheres and biotin-labeled mycotoxin conjugates were considered sucsessfully synthesized when the signal response was of more than 200 mean fluorescence intensity (MFI).

Development and Optimization of Individual Mycotoxin Suspension Array Immunoassay. Suspension array immunoassay for ZEN: the mAb-ZEN-coated microspheres (49#) were diluted in 0.01 M phosphate buffered saline (PBS, pH7.4) to the concentration of 8×10^4 beads/mL. Twenty-five microliters of antibody−microsphere solution was added to a microplate well, then 10 μL of ZEN standard solution at varying concentrations (from 0 to 33.3 ng/mL) was added to the well. The solution was incubated at 37 °C for 30 min. Biotin-labeled ZEN-OVA conjugate was diluted to 0.25 μg/mL in PBS, and 25 μL was added to each well. The solution was incubated at 37 °C for 30 min. Streptavidin−phycoerythrin conjugate was diluted to 8 μg/mL in PBS, and 25 μL was added to each well. The solution was incubated at 37 °C for 30 min. One hundred microliters of stop solution was added to each well and incubated for 20 s. A suspension array analyzer (Luminex 200) and software program (Luminex 100 IS) were used to determine the signals of microsphere 49# and streptavidin−phycoerythrin.

In order to improve the sensitivity of detection, the concentrations of ZEN-OVA-biotin and streptavidin−phycoerythrin conjugate were optimized by the IC_{50} (50% inhibition) of each ZEN standard solution concentration (from 0 to 33.3 ng/mL) with the lower IC_{50} indicating higher sensitivity.

Suspension array immunoassay procedures for other individual mycotoxins (FB1, DON, and AFB1) were performed as described for ZEN. Microspheres 19#, 37#, and 39# were conjugated with mAb-AFB1, mAb-DON, and mAb-FB1, respectively. The concentrations of standard solution of mycotoxins were as follows: from 0 to 1000 ng/mL (FB1), from 0 to 500 ng/mL (DON), and from 0 to 50 ng/mL (AFB1). The concentrations of reagents (biotin−mycotoxin−OVA and streptavidin−phycoerythrin) in each detection were also optimized by IC_{50} as described ZEN above.

Development and Optimization of Multiple Mycotoxin Suspension Array Immunoassays. The mixtures in the multiple suspension array immunoassay were prepared as follows: Four monoclonal antibody-coated microspheres (19#, 37#, 39#, and 49#) were diluted to 3.2×10^5 beads/mL and mixed in a 1:1:1:1 (v/v/v/v) ratio. Different concentrations of four mycotoxins (ZEN, from 0 to 33.3 ng/mL; FB1, from 0 to 1000 ng/mL; DON, from 0 to 500 ng/mL; and AFB1, from 0 to 50 ng/mL) were prepared as antigen mixtures. The four biotin-labeled mycotoxin conjugates were mixed in the optimized concentrations in the above individual array.

In the multiple suspension array, 25 μL of antibody−microsphere mixture was added to the well of the microplate. Ten microliters of the four mycotoxin mixtures in different concentrations was added to the wells and incubated at 37 °C for 30 min. Twenty-five microliters of the mixture of four biotin-labeled mycotoxin conjugates was added to each well and incubated at 37 °C for 30 min. The streptavidin−phycoerythrin conjugate was diluted to 4 μg/mL in PBS, and 25 μL of solution was added to each well and incubated at 37 °C for 30 min. One hundred microliters of stop solution was added to each well and incubated for 20 s, before analyzing with the suspension array detector. The microsphere signals of 19#, 37#, 39#, and 49# were selected in the software, which corresponded to the four kinds of mycotoxins. The schematic diagram of the multiplex suspension array is shown in Figure 1. The standard curve of each mycotoxin was calculated by the

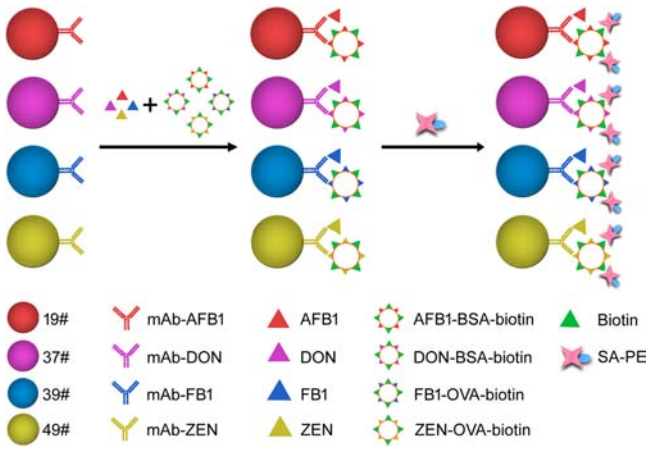

Figure 1. Schematic diagram of the multiplex suspension array. "SA-PE" is the abbreviated form of "streptavidin−phycoerythrin".

relative response (the percentage of response of different standard solutions divided by that of the response at 0 ng/mL, *Y*-axis) and log concentrations of different mycotoxins (*X*-axis) in triplicate.

Moreover, the maximum responses of MFI and the responses which added specific concentrations of mycotoxins (nearly 80% inhibition of each analysis, 10 ng/mL for ZEN, 100 ng/mL for FB1, 100 ng/mL for DON, and 15 ng/mL for AFB1) were determined by both individual

and multiplex suspension arrays to evaluate the differences between individual and multiplex arrays by Pearson correlation (SPSS, 11.5 version).

Specificity of the Suspension Array. To evaluate the cross-reactivity of the suspension array, different serial concentrations of ZEN (from 0 to 33.3 ng/mL), FB1 (from 0 to 1000 ng/mL), DON (from 0 to 500 ng/mL), or AFB1 (from 0 to 50 ng/mL) were used in an individual mycotoxin (ZEN, FB1, DON, and AFB1) suspension array. To evaluate the nonspecific binding of the mycotoxins, the mixture of four kinds of mycotoxins (1:1:1:1, v/v/v/v) were determined in each individual suspension array. Cross-reactivity of each antimycotoxin monoclonal antibody with other mycotoxin was evaluated according to the following formula (for example, the cross-reactivity of mAb-ZEN, the other mycotoxins including FB1, DON, or AFB1):

$$\text{cross-reactivity (\%)} = \frac{\text{IC}_{50}\text{ for ZEN in ZEN a suspension arrray}}{\text{IC}_{50}\text{ for other mycotoxin (FB1, or DON, or AFB1)}} \times 100\%$$

Recovery Studies. The mycotoxin (ZEN, FB1, DON, and AFB1)-free corn samples were ground and placed in 60 °C and incubated overnight. A 5 g sample was spiked with four mycotoxins at different concentrations of standard solution. The spiked samples were shaken for 10 min and incubated at room temperature overnight. Twenty milliliters of extraction solution (acetonitrile/water = 84:16, v/v) was added to each sample and shaken vigorously for 30 min at room temperature. The samples were centrifuged at 3,000*g* for 10 min, and supernatants were diluted four times in PBS. Ten microliters of sample extract was added to the microplate well and mixed with an antibody–microsphere mixture. The solution was incubated at 37 °C for 30 min. The remaining procedure was the same as that described above. Each sample was repeated in triplicate.

Detection of Commercial Samples by Multiple Suspension Array and LC-MS/MS. All samples (including corn, wheat, and feedstuff) were analyzed by a multiple suspension array and LC-MS/MS in parallel. Each sample was tested in triplicate to calculate standard deviation. For the detection by the suspension array, the commercial samples were extracted as the spiked samples. For LC-MS/MS, the extraction procedures were as follows: the samples were ground and dried overnight in a 60 °C incubator. The extraction solvent mixture (40 mL, acetonitrile/water/acetic acid, 79:20:1, v/v/v) was added to 10 g of samples and shaken vigorously on a horizontal shaker for 60 min at room temperature. The samples were allowed to stand for 10 min before centrifuging the samples at 2,500*g* for 10 min. The supernatants were then mixed with the same volume of mixture (acetonitrile/water/acetic acid, 20:79:1, v/v/v), and passed through a 0.22 μm filter before being injected into the LC-MS/MS instrument. Quantitative LC-MS/MS results were analyzed using Analyst software (AB SCIEX, Framingham, MA, USA). The correlation between the two methods was evaluated by Pearson correlation (SPSS, 11.5 version).

RESULTS AND DISCUSSION

Development and Optimization of the Individual Mycotoxin Suspension Array. In order to improve the sensitivity of the detection method, the concentration of biotin-labeled mycotoxin conjugates and streptavidin–phycoerythrin conjugate in each suspension array detection system was optimized. Lower IC_{50} indicates better sensitivity, and the lower concentration of the immunoreagent usually induces lower IC_{50}. However, in the suspension array, low concentrations of biotin-labeled mycotoxin conjugates and streptavidin–phycoerythrin resulted in no signal response. The optimization results of concentrations of the biotin-labeled mycotoxin conjugate and the streptavidin–phycoerythrin conjugate are shown in Supporting Information, Figure S1. The optimum concentrations of ZEN-OVA-Biotin, FB1-OVA-Biotin, DON-BSA-Biotin, and AFB1-BSA-Biotin were 0.25, 1, 0.5, and 0.25 μg/mL, respectively, and the concentrations of the streptavidin–phycoerythrin conjugate were 8, 4, 2, and 6 μg/mL, respectively.

Optimization of the Multiple Suspension Array. For the multiple suspension array, only one concentration of the

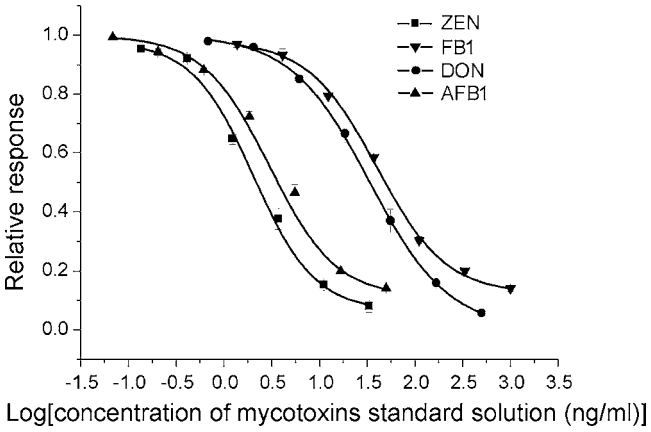

Figure 2. Standard curves of ZEN, FB1, DON, and AFB1 in suspension arrays. The *X*-axis is the log concentration of mycotoxins (ZEN, FB1, DON, and AFB1). The *Y*-axis is the ratio of the relative response, a ratio that represents the degree of competitive inhibition. The error bars indicate the standard deviation.

Table 1. Detection Characteristics of the New Multiplex Suspension Array

mycotoxins	detection limits (IC_{10}, ng/mL)	IC_{50} (ng/mL)	detection range (IC_{20}-IC_{80}, ng/mL)	regression equation
ZEN	0.51	2.1	0.73–6.8	$y = -0.5552x + 0.7037$ $R^2 = 0.9994$
FB1	6.0	41.5	11.6–110.3	$y = -0.4103x + 1.211$ $R^2 = 0.9841$
DON	4.3	32.0	8.6–108.1	$y = -0.497x + 1.2652$ $R^2 = 0.9895$
AFB1	0.56	4.6	1.1–14.1	$y = -0.5054x + 0.8335$ $R^2 = 0.9945$

Table 2. Recovery Rates of the Spiked Corn Samples

	concentrations (μg/kg)			
samples	spiked	detected	recovery rates (%)	RSD (%) ($n = 3$)
ZEN	20	19.3	96.5	6.4
	60	55.9	93.2	5.9
	100	106.5	106.5	7.3
FB1	200	221.4	110.7	8.1
	600	652.4	108.7	7.5
	1500	1732.9	115.5	8.6
DON	200	184.6	92.3	4.6
	1000	957.3	95.7	7.3
	1500	1683.2	112.2	7.9
AFB1	20	18.9	94.5	8.1
	100	104.3	104.3	6.3
	200	220.1	110.1	4.9

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Table 3. Mycotoxin Levels in the Commercial Samples Determined by the New Multiple Suspension Array and LC-MS/MS[a]

	suspension array (μg/kg, mean ± SD)				LC-MS/MS (μg/kg, mean ± SD)			
samples	ZEN	FB1	AFB1	DON	ZEN	FB1	AFB1	DON
Corn 1	42.2 ± 3.6	321.4 ± 25.4	-	-	45.3 ± 2.6	256.2 ± 15.3	-	39.1 ± 4.2
Corn 2	47.1 ± 4.1	326.5 ± 29.3	-	-	37.2 ± 21.5	350.7 ± 23.5	-	-
Corn 3	29.3 ± 1.7	364.8 ± 23.1	-	-	41.7 ± 5.2	325.9 ± 19.3	-	-
Corn 4	-	-	-	282.6 ± 31.5	-	-	-	257.9 ± 25.7
Wheat 1	48.3 ± 2.7	-	-	-	72.2 ± 5.2	-	-	-
Wheat 2	-	-	-	237.5 ± 19.8	-	-	-	211.4 ± 17.6
Feedstuff 1	294.6 ± 21.3	320.6 ± 26.4	-	-	326.1 ± 27.6	332.7 ± 24.3	-	-
Feedstuff 2	303.4 ± 26.7	331.3 ± 34.8	8.4 ± 0.5	-	232.4 ± 21.3	225.3 ± 17.7	10.5 ± 1.7	-
Feedstuff 3	593.5 ± 32.1	1657.9 ± 94.3	13.5 ± 0.6	-	572.5 ± 24.7	1573.2 ± 78.4	15.7 ± 1.5	-
Feedstuff 4	-	315.3 ± 23.1	-	-	-	424.7 ± 24.6	-	-
Feedstuff 5	87.9 ± 3.6	263.2 ± 13.5	-	-	72.5 ± 4.8	316.5 ± 19.4	-	-
Feedstuff 6	-	986.2 ± 69.5	12.6 ± 0.5	-	-	834.4 ± 45.6	14.4 ± 1.2	-
Feedstuff 7	-	341.4 ± 23.1	62.6 ± 2.7	-	-	185.3 ± 14.7	79.5 ± 5.1	-

[a]"-" means not detected.

streptavidin−phycoerythrin conjugate can be added as an individual reporter protein. However, the optimum concentrations of streptavidin−phycoerythrin for the individual suspension array were different among all four mycotoxins. Therefore, based on the optimum concentrations of streptavidin−phycoerythrin in the individual suspension array, different concentrations (2, 4, 6, and 8 μg/mL) of streptavidin−phycoerythrin were added in the multiple suspension arrays to assess the effect for the detection of the four mycotoxins. The results are shown in Table S1, Supporting Information. Four μg/mL streptavidin−phycoerythrin was selected as the lowest difference for IC_{50} when compared to those of the four individual suspension array immunoassays. Since the biotin-labeled mycotoxin conjugate can be adjusted to the optimum concentration before mixing, no optimization is needed.

Comparison of Individual and Multiple Mycotoxin Suspension Arrays. Because of the possibility of nonspecific binding between multiple analytes and immunoreagents, the signals and sensitivities of the multiple suspension array was compared with the individual suspension arrays. The maximum responses and the average responses of the specific concentrations of mycotoxins were determined by single and multiple suspension arrays. The results are shown in Supporting Information, Table S2 and S3. The maximum response and the average response had no difference when single suspension array or multiple suspension array was performed for each of the tested mycotoxins. These results indicate that the multiple suspension array has good correlations with the individual array (r = 0.99, P < 0.01, Pearson correlation, SPSS, 11.5 version) and that the nonspecific binding did not exist.

For the specificity of each antimycotoxin antibody, no cross-reactivity (<0.01%) was observed with other mycotoxins in the individual suspension array. For the mixture of four kinds of mycotoxins in the individual suspension array, there is no difference between the individual and mycotoxin mixtures for the IC_{50} of each mycotoxin, which indicates that there is no nonspecific binding among the mycotoxins. These results indicated that this multiple array immunoassay has good specificity for ZEN, FB1, DON, and AFB1.

Standard Curves of Four Mycotoxins in the Suspension Array. The standard curves were calculated according to the optimum conditions of the multiple suspension arrays. Four mycotoxin standard curves are shown in Figure 2. The detection limits, half inhibition values (IC_{50}), and detection linear ranges are shown in Table 1. This multiple suspension array was more sensitive for detecting ZEN and AFB1 with lower detection limits.

Recovery Studies. Myotoxin-free corn samples were spiked with ZEN, FB1, DON, and AFB1 with different concentrations to determine recovery rates. The results are shown in Table 2. The recovery rates of four mycotoxins were between 92.3% and 115.5%, and the relative standard deviations were 4.6−8.6%, which indicates that this multiple suspension array was accurate and has good reproducibility.

In addition, according to the comparison shown in Supporting Information, Table S4, this detection method can detect mycotoxins in certain samples below the standard limits of China or United States and most other countries.

Commercial Samples Analysis. The commercial samples were analyzed by the multiple suspension array and LC-MS/MS. The results are shown in Table 3 (only positive samples are listed). Thirteen samples including corn, wheat, and feedstuff contained mycotoxin when analyzed using the two methods. Furthermore, the concentrations of ZEN, FB1, and AFB1 in feedstuff samples were greater than that in the corn or wheat samples. These results showed that the two methods had a good correlation (r = 0.99, P < 0.01) evaluated by Pearson correlation (SPSS, version 11.5), indicating that the new multiple suspension array for ZEN, FB1, DON, and AFB1 was accurate and had good reproducibility. However, this suspension array immunoassay still needs improvement in detection sensitivity in further studies because the method had less sensitivity than LC-MS/MS (DON concentration of corn 1#, Table 3).

Several suspension arrays for the detection of mycotoxins have been reported using an indirect competition strategy.[20,25−27] The mycotoxin−protein conjugates and microsphere were synthesized, and the antibodies were biotinylated. The disadvantages of previous studies include complex procedures (8 or more steps), the necessity of carrying out reactions in Eppendorf tubes and transferring to microplates prior to determination, and time requirements (4 h). The direct competition procedure used in this research requires only 5 steps, and reactions are carried out in the microplate well and only require 2 h. Moreover, the direct competition strategy is

more suitable for commercial applications[28] since the antibodies have already been synthesized with microspheres.

In summary, the multiple direct competition suspension array can simultaneously determine four kinds of mycotoxins (ZEN, FB1, DON, and AFB1) in cereal and feedstuff. This method is rapid, accurate, sensitive, and meets the detection limits of most of countries. This study provides a good platform for the detection of mycotoxins and other low-weight analytes in samples of agriculture products to protect human and animal health.

■ ASSOCIATED CONTENT

Supporting Information

Optimization concentrations of biotin-labeled mycotoxin conjugates and streptavidin−phycoerythrin in an individual suspension array; optimization of streptavidin− phycoerythrin in a multiplex suspension array; maximum responses in individual and multiplex suspension arrays; average responses of the specific concentrations of mycotoxins in individual and multiplex suspension arrays; and comparison of the limit standard in China or US with this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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