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Rapid Simultaneous Quantification of Zearalenone and Fumonisin B1 in Corn and Wheat by Lateral Flow Dual Immunoassay

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Supporting Information

ABSTRACT: A lateral flow dual immunoassay (LFDIA) was developed for rapid quantitative detection of zearalenone (ZEN) and fumonisin B1 (FB1) in corn and wheat samples on a single test strip. Two test lines and the control line on the nitrocellulose membrane were coated with ZEN and FB1 conjugates and goat anti-mouse IgG, respectively. Colloidal gold nanoparticles were conjugated with monoclonal antibodies against ZEN or FB1. The intensity of the test lines was analyzed by a photometric strip reader to determine the concentrations of ZEN and FB1 based on the calibration curves of known concentrations versus intensity readings. Test parameters such as types of buffers, ratio of the two gold-labeled antibodies, and dilution of the sample extracts and the gold-labeled antibodies were optimized. The detection limit was 0.35 and 5.23 ng/mL for ZEN and FB1, respectively, and the corresponding detection ranges were 0.94−7.52 and 9.34−100.45 ng/mL, respectively. Spiked and natural samples were analyzed using both LFDIA and liquid chromatography−tandem mass spectrometry. The two methods had a good correlation (R^2 = 0.96). The dual quantitative LFDIA is sensitive, rapid, and easy-to-use for on-site testing of a large number of samples.

KEYWORDS: *Lateral flow dual immunoassay, zearalenone, fumonisin B1, quantification*

INTRODUCTION

Zearalenone (ZEN) and fumonisin B1 (FB1) are mycotoxins produced by *Fusarium* species that can be found in agricultural commodities, such as corn, wheat, and cereal products.[1,2] Both toxins have toxic effects on human and livestock.[3] ZEN is estrogenic and carcinogenic and also causes DNA damage and reproductive toxicity.[4] FB1 induces equine leukoencephalomalacia and porcine pulmonary edema and is a carcinogen in human and livestock.[5,6] Therefore, the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) has established the provisional maximum tolerable daily intake levels of 0.5 and 2 μg/kg of body weight per day for ZEN and fumonisins, respectively.[7,8]

A number of analytical methods have been developed for detection of individual and multiple mycotoxins in food samples. Chromatographic methods, including thin-layer chromatography,[9] liquid chromatography−tandem mass spectrometry (LC−MS/MS),[10] and high-performance liquid chromatography,[11] are sensitive and produce reliable results. However, the complex preparation steps, expensive equipment, and time-consuming procedures make such methods unsuitable for routine work in many laboratories and other locations, such as farms or factories.

ZEN, FB1, and other mycotoxins may coexist in single products.[12] To improve detection efficiency, some researchers have studied detection of multiple mycotoxins in a single assay, such as the microarray-based method.[13] The lateral flow immunoassay (LFIA) method based on indirect competition is widely used in detection of prohibited drugs, pesticides, viruses, and mycotoxins.[14−17] However, LFIA has been mostly used for qualitative detection of multiple mycotoxins in agricultural products in a single test.[16,18,19] With the increasing need for more sensitive detection of harmful substances in food, qualitative[16,18] and semi-quantitative[19] immunochromatographic assays are not sufficient in some situations. Therefore, quantitative immunochromatographic assays with improved sensitivity are of high demand and have been developed for single mycotoxins, such as deoxynivalenol,[20] fumonisins,[21] ochratoxin A,[22] and aflatoxin B1.[23] The detection limits of these tests are lower than those of qualitative methods.[14,18,24,25] Here, we present a novel format of LFIA [lateral flow dual immunoassay (LFDIA)] for simultaneous quantitative detection of ZEN and FB1 in foods.

MATERIALS AND METHODS

Materials. ZEN, FB1, and tetrachloroauric(III) acid were purchased from Sigma Chemical (St. Louis, MO). Bovine serum albumin (BSA) and ovalbumin (OVA) were obtained from Sangon Biotech (Shanghai, China). Tetronic 1307 was obtained from Pragmatic (Elkhart, IN). Monoclonal antibodies against ZEN (2C9, mAb-ZEN) and FB1 (6H3, mAb-FB1) were prepared in our laboratory.[26,27] The nitrocellulose (NC) membrane (Millipore 180) was obtained from Millipore (Bedford, MA). Glass fiber (SB08) was provided by Jiening Biotech (Shanghai, China). The horseradish peroxidase (HRP)-labeled goat anti-mouse antibody [used in enzyme-

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linked immunosorbent assay (ELISA)] and goat anti-mouse antibody (used in LFDIA) were obtained from ICL (Portland, OR). ZEN- and FB1-free corn and wheat samples were provided by Shanghai Entry−Exit Inspection and Quarantine Bureau.

Equipment. The following equipment were used: Bio-Tek microplate reader (Winooski, VT), JEM 2100 transmission electron microscope system (JEOL, Tokyo, Japan), Bio-Dot strip spray system (XYZ-3060, Irvine, CA), CHR-110R strip reader (Kaiwood, Taiwan, China), ultraperformance liquid chromatography (UPLC) system and UPLC BEH C18 column (Waters, Milford, MA), and QTrap MS/MS system (Applied Biosystems, Foster City, CA).

Preparation of ZEN and FB1 Conjugates. The ZEN conjugate (ZEN−BSA) was prepared using a previously reported method.[28,29] FB1 was conjugated to OVA (FB1−OVA) according to Christensen et al.[30] Conjugation of ZEN−BSA and FB1−OVA was evaluated by indirect ELISA, in which each of the conjugates was used as the coating antigen (with BSA or OVA coated as the negative control) and the monoclonal antibodies (2C9 or 6H3) specific against ZEN or FB1 were used as the probing antibodies, respectively.

Preparation of Colloidal Gold Particles. The colloidal gold particles were prepared according to a previously described method,[31] with minor modifications. Briefly, 100 mL of tetrachloroauric acid solution (0.01%, w/v) was stirred and heated until boiling. A volume of 0.7 mL of 1% sodium citrate (w/v) was then added to the solution. After gentle heating for 5 min, the mixture was cooled to room temperature and stored at 4 °C. The diameter of nanoparticles was determined using the transmission electron microscope.

Preparation of Colloidal Gold-Labeled Monoclonal Antibodies. The monoclonal antibodies mAb-ZEN and mAb-FB1 were evaluated by the qualitative test strip.[32] No cross-reactivity was observed with other mycotoxins, including FB1 (or ZEN), deoxynivalenol (DON), and aflatoxin B1 (AFB1) for mAb-ZEN or mAb-FB1 (see Figures S1 and S2 of the Supporting Information). The colloidal gold-labeled monoclonal antibodies were prepared according to a previously reported method.[33] To optimize the binding between colloidal gold particles and antibodies, colloidal gold solutions at different pH and antibody concentrations were evaluated. Initially, 1.0 mL of 2 mM borate buffer (BB, pH 7.4) containing mAb-ZEN (3.6 μg/mL) or mAb-FB1 (7.2 μg/mL) was added slowly with 1 min duration to 10 mL of colloidal gold solution (pH 6.5 for mAb-ZEN or pH 7.0 for mAb-FB1). After gentle stirring for 30 min, 1.0 mL of 10% BSA (w/v) was added to the mixture, which was again stirred for another 30 min. The mixture was then centrifuged at 1500*g* for 20 min. The supernatant sample was collected and centrifuged at 8000*g* for 30 min. Finally, the colloidal liquid was washed 3 times by adding 10 mL of 2 mM BB (pH 7.4) and centrifuging at 6000*g* for 30 min. The resulting colloidal gold-labeled mAb-ZEN (gold−mAb-ZEN) or mAb-Fb1 (gold−mAb-FB1) was then stored in 2 mM BB (pH 7.4) containing 6% trehalose (w/v), 4% sucrose (w/v), 1% BSA (w/v), and 0.05% sodium azide (w/v) at 4 °C.

Preparation of Lateral Flow Strips. ZEN−BSA (0.05 mg/mL), FB1−OVA (0.2 mg/mL), or goat anti-mouse antibody (0.07 mg/mL) was dissolved separately in 50 mM phosphate-buffered saline (PBS, pH 7.4) with 7% methanol (w/w). A volume of 1.0 μL/cm of each of the above solutions was sprayed onto the NC membrane to form two test lines (test 1 for ZEN and test 2 for FB1) and a control line, respectively. The distance between the two test lines and between test 1 and the control lines was 0.4 cm (Figure 1).

The sample pad made of glass fiber (SB08) was pretreated by 10 mM PBS (pH 7.4) containing 6% trehalose (w/v), 1% BSA (w/v), 0.5% Tetronic 1307 (w/v), and 0.05% sodium azide (w/v) and stored at 4 °C after freeze dehydration. To assemble the test strip, the antigen conjugates/secondary antibody-coated NC membrane was pasted onto the polyvinyl chloride (PVC) baseplate. Next, the sample pad in a 2 × 0.5 cm section and the absorption pad were pasted to the lower and upper portions of the baseplate, respectively, with 1−2 mm overlap to the NC membrane on both sides (Figure 1).

Optimization of LFDIA. To optimize LFDIA performance, PBS and BB with four different molar concentrations (2, 10, 20, and 50 mM) and two different pH values (7.4 and 8.2) were assessed (see

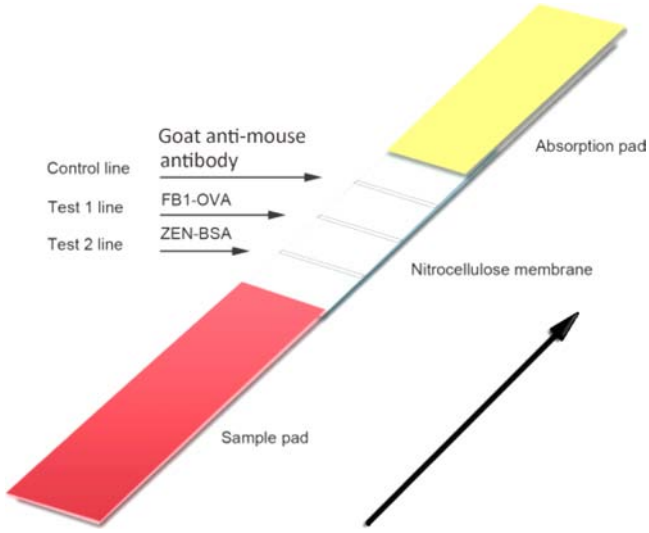

Figure 1. Schematic diagram of the quantitative LFDIA.

Table S1 of the Supporting Information). The buffers for antigen or antibody coating and sample pad pretreatment as well as for conjugation, storage, and dilution of gold-nanoparticle-labeled antibodies were investigated. Types and concentrations of components in the buffers, i.e., BSA (0, 0.5, and 1%, w/v), sucrose (0, 2, 4, 6, 8, and 10%, w/v), and trehalose (0, 2, 4, 6, 8, and 10%, w/v), were also investigated by assessing the detection limits of the test strip and the stability of gold-nanoparticle-labeled antibodies. The dilution ratios (1:2.5, 1:5, 1:10, 1:15, and 1:20, v/v) of the sample extracts were also optimized to decrease the methanol content in the extraction buffer and the influence of proteins or other components in the sample matrixes.

Mixtures of the two colloidal gold-labeled antibodies (gold−mAb-ZEN and gold−mAb-FB1) were prepared at different ratios (1:9, 1.5:8.5, 2:8, 2.5:8.5, and 3:7, v/v) to acquire similar product intensities in the test 1 and test 2 lines. Furthermore, different dilution ratios (1:1.5, 1:2, 1:2.5, 1:3, 1:3.5, and 1:4, v/v) of these mixtures were assessed using the 20 mM BB (pH 8.2) containing 6% trehalose (w/v), 1% BSA (w/v), and 0.05% sodium azide (w/v).

Sample Preparation. ZEN- and FB1-free corn and wheat samples were spiked by adding ZEN and FB1 standard solutions to the samples, which were then stored overnight at 4 °C. The concentrations used for spiked samples were 80, 150, and 300 μg/kg for ZEN and 600, 1000, and 2000 μg/kg for FB1.

For testing, 5 g of the spiked samples were extracted in a 20 mL methanol/water mixture (80:20, v/v) on a horizontal shaker for 15 min. The supernatant samples were then collected after centrifugation at 2000*g* for 10 min. The extracts were diluted 15-fold with 50 mM PBS (pH 7.4) containing 10% methanol (v/v) and 0.05% Tween 20 (v/v) (PBST). In addition, 40 natural corn and wheat samples were extracted as above for analysis.

Test Procedure. A 2-fold dilution series of ZEN (from 25 ng/mL) or FB1 (from 500 ng/mL) was prepared in 50 mM PBST. Aliquots (25 μL) of the gold−mAb-ZEN and gold−mAb-FB1 mixtures and 25 μL of the standard solutions of ZEN or FB1 were mixed and then added to the sample pad. During reaction in the LFDIA test strip, ZEN or FB1 in the mixture would compete for binding with the specific antibodies gold−mAb-ZEN or gold−mAb-FB1 against the coating antigen conjugates, thus altering the reaction intensity on the test lines (test 1 or test 2 lines, respectively). The representative results of the quantitative LFDIA were shown on Figure S3 of the Supporting Information.

The intensity of the test lines were captured with the strip reader after 30 min of reaction. Concentrations of ZEN or FB1 in the samples were quantified from the dose−response curves (band intensity versus concentrations of ZEN or FB1 in the standard solutions), which were run simultaneously in triplicate. The spiked and natural corn and

wheat samples were determined for ZEN or FB1 by quantitative LFDIA and LC–MS/MS. The recovery rates of the spiked samples were compared, and the correlation between LFDIA and LC–MS/MS results was investigated using linear regression (SPSS software, IBM, New York, NY).

LC–MS/MS Analysis. A previously described, the LC–MS/MS method was used,[34] with minor modifications. Briefly, samples (10 g each) were extracted with 40 mL of the extraction solvent (acetonitrile/water/acetic acid, 79:20:1, v/v/v) on a horizontal shaker for 60 min at room temperature and then centrifuged at 2000*g* for 10 min. The supernatant samples were mixed with the same volume of extraction solvent (acetonitrile/water/acetic acid, 20:79:1, v/v/v), and the mixtures were passed through a 0.22 μm filter before being injected into the LC–MS/MS instrument. Quantitative results were measured using the UPLC and QTrap MS/MS systems in Analyst software (AB SCIEX, Framingham, MA). The precursor ions of ZEN and FB1 were 319.0 and 722.05, and the product ions were 185 and 187 for ZEN and 334 and 352 for FB1, respectively.

RESULTS AND DISCUSSION

Optimization of the LFDIA. ZEN and FB1 conjugates were initially evaluated by indirect ELISA. The optical density

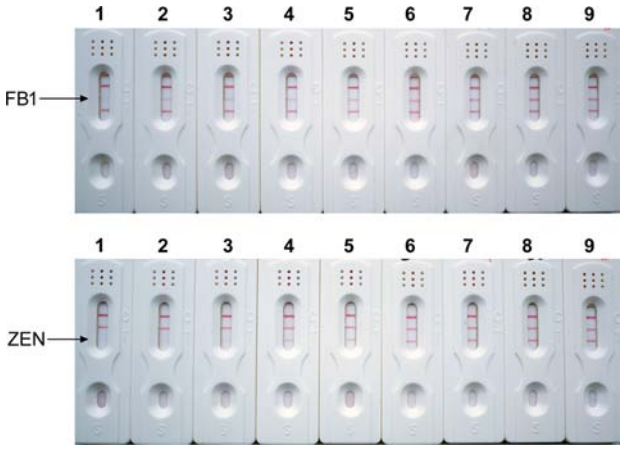

Figure 2. LFDIA strips for (top) FB1 and (bottom) ZEN. The concentrations of FB1 from 1 to 9 (top): 500, 250, 125, 62.5, 31.3, 15.6, 7.81, 3.91, and 0 ng/mL, and those of ZEN from 1 to 9 (bottom): 25, 12.5, 6.25, 3.13, 1.56, 0.781, 0.391, 0.195, and 0 ng/mL.

values (conjugates/negative control) were 1.12:0.04 for ZEN and 1.23:0.12 for FB1, indicating that the conjugation was successful.

The optimum ratio of the gold–mAb-ZEN and gold–mAb-FB1 was 1.5:8.5 (v/v) to generate a similar intensity of the two test lines. The colloidal gold nanoparticles were homogeneous, with about 25 nm in diameter. The optimum concentrations were determined for ZEN–BSA at 0.05 mg/mL, FB1–OVA at 0.2 mg/mL, and goat anti-mouse antibody at 0.07 mg/mL. A low concentration of colloidal gold-labeled antibodies results in higher sensitivity but narrows the detection range. Thus, the use of an optimum dilution ratio could balance the sensitivity and detection range in the LFDIA. The optimum dilution ratio of the colloidal gold-labeled antibodies mixture was 1:2.5 (v/v).

Methanol in the extraction buffer and the matrixes of sample extracts, including proteins and vitamins, are known to affect the stability of the colloidal gold-labeled antibodies.[14,17,19] Lower dilution ratios (1:2.5, 1:5, and 1:10, v/v) of sample extracts caused instability of the colloidal gold-labeled antibodies and flow disturbance in the LFDIA. Dilution at 1:15 was found optimal because the sensitivity was better than the 1:20 dilution.

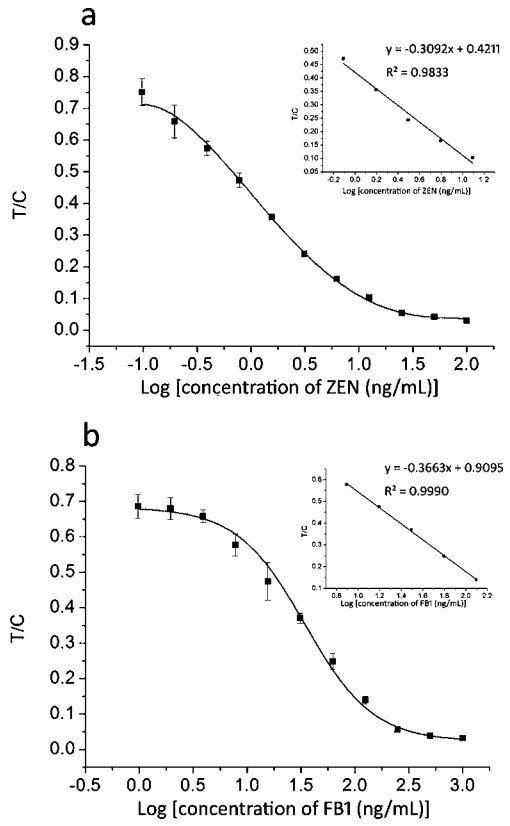

Figure 3. Calibration curves of (a) ZEN and (b) FB1 in the LFDIA. The *x* axes are the log concentrations of ZEN or FB1. The *y* axes are the ratio of the relative optical density of the test line to the control line, a ratio that represents the degree of competitive inhibition. The detection range was 0.94–7.52 ng/mL for ZEN and 9.34–100.45 ng/mL for FB1. The error bars indicate the standard deviation.

The use of inappropriate buffers for LFDIA can induce instability of the gold nanoparticle-labeled antibodies and affect sensitivity. The optimum buffers and optimum concentrations of buffer components were determined (see Table S1 of the Supporting Information) for lower detection limits, balance of intensities of the test and control lines, and stability of the mixtures (gold-nanoparticle-labeled antibodies and sample extraction).

Previously, different buffer types with different pH values were used for dilution of coating antigens or antibodies (e.g., 50 mM PBS,[11] 20 mM PBS,[16] and 10 mM PBS with pH 7.4[18]), for antibody–gold nanoparticle synthesis (e.g., 20 mM BB with pH 8.0,[16] 20 mM sodium borate with pH 9.0,[35] 10 mM PBS with pH 7.4[18] or pH 8.5,[36] and 50 mM potassium phosphate buffer with pH 7.4[37]), and for sample pad pretreatment (e.g., 20 mM BB with pH 8.0,[17] 50 mM BB with pH 7.4,[15] 20 mmol/L sodium borate with pH 8.0,[35] and 10 mM PBS with pH 7.4[36]). These buffers are mainly PBS- and BB-based, with pH values ranging from 7.4 to 9.0 and molar concentrations ranging from 10 to 50 mM. The components include sucrose and BSA. We found that intensities of the test lines, stability and release rate of the mixtures (gold-nanoparticle-labeled antibodies and sample extraction), and LFDIA sensitivity were markedly affected by the buffer types and concentrations of their components. For example, PBS could result in the appearance of deposits in gold nanoparticle and antibody conjugation. BB

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Table 1. Detection of ZEN-Spiked Samples by LFDIA and LC−MS/MS

		LFDIA			LC−MS/MS		
samples	spiked (μg/kg)	detected (μg/kg)	recovery rate (%)	RSD[a] (n = 3) (%)	detected (μg/kg)	recovery rate (%)	RSD[a] (n = 3) (%)
corn 1	80	75.3	94.1	9.2	76.5	95.6	6.7
corn 2	150	134.1	89.4	7.8	142.6	95.1	6.6
corn 3	300	283.9	94.6	5.6	283.9	94.6	5.7
wheat 1	80	72.5	90.6	8.3	86.3	107.9	9.2
wheat 2	150	162.6	108.4	6.8	153.2	102.1	3.8
wheat 3	300	285.2	95.1	7.1	332.5	110.8	7.8

[a]RSD indicates relative standard deviation.

Table 2. Detection of FB1-Spiked Samples by LFDIA and LC−MS/MS

		LFDIA			LC−MS/MS		
samples	spiked (μg/kg)	detected (μg/kg)	recovery rate (%)	RSD[a] (n = 3) (%)	detected (μg/kg)	recovery rate (%)	RSD[a] (n = 3) (%)
corn 1	600	560.3	93.3	7.3	585.2	97.5	4.9
corn 2	1000	898.5	89.9	6.8	934.2	93.4	5.1
corn 3	2000	1875.3	93.8	6.5	1904.3	95.2	4.2
wheat 1	600	573.3	95.6	6.9	642.5	107.1	9.6
wheat 2	1000	920.5	92.1	11.6	1104.1	110.4	10.6
wheat 3	2000	1943.8	97.2	7.4	1713.4	85.7	9.5

[a]RSD indicates relative standard deviation.

Table 3. Comparison of LFDIA and LC−MS/MS for Quantification of ZEN and FB1

	LFDIA (μg/kg)		LC−MS/MS (μg/kg)	
sample	ZEN	FB1	ZEN	FB1
corn	68.9	ND[a]	87.2	ND
corn	120.4	ND	80	ND
corn	296.7	ND	348.8	ND
corn	314.5	ND	257.6	ND
wheat	168.9	ND	214.4	ND
wheat	ND	753	ND	944
wheat	611.7	2313	573.6	1424

[a]ND means not detectable with the method used.

could reduce line intensities when used for coating of the antigens or antibody. Our results indicate that BB was more suitable than PBS for use in conjugation, dilution, and storage of gold-nanoparticle-labeled antibodies. Trehalose at 6% (w/v) was better than sucrose for stability of the gold-nanoparticle-labeled antibodies during storage.

Calibration Curves of LFDIA. The strip reader results from different concentrations of ZEN and FB1 (Figure 2) were used to determine the calibration curves of ZEN and FB1 (Figure 3). The regression equation for quantification of ZEN was $y = -0.309x + 0.421$ ($R^2 = 0.98$), and the detection range was 0.94−7.52 ng/mL, with the detection limit at 0.35 ng/mL. For FB1, the regression equation was $y = -0.366x + 0.909$ ($R^2 = 0.99$), and the detection range was 9.34−100.45 ng/mL, with the detection limit at 5.23 ng/mL. Relative standard deviations for these parameters were below 10% (ZEN; n = 3) and 12% (FB1; n = 3) (data not shown). At the dilution ratio of 1:60, the detection limits for ZEN and FB1 were 21 and 313.8 μg/kg in natural corn and wheat samples.

The detection limit for ZEN in this study (0.35 ng/mL) was lower than those reported in other LFIA studies, 2.5 ng/mL[15] or 5 ng/mL[18] in multiple qualitative LFIA studies and 1.0 ng/mL in an individual quantitative LFIA study.[38] The detection ranges of ZEN and FB1 in the present study were also wider than those of a semi-quantitative LFIA study, which used the cutoff values to classify the samples as "positive" or "negative".[19] For detection of mycotoxins produced by *Fusarium* species, including ZEN, FB1, FB2, FB3, and DON, which may coexist in food samples with enhanced toxicities,[12] multiple quantitative test methods, such as the dual test strip described here, can be more efficient.

Recovery Studies. Three different concentrations of ZEN- or FB1-spiked corn and wheat samples were analyzed using LFDIA and LC−MS/MS. Tables 1 and 2 show that the recovery rates by LFDIA were between 89.4 and 108.4%, while those by LC−MS/MS ranged from 85.7 to 110.8%.

Analysis of ZEN and FB1 in Natural Corn and Wheat Samples. A total of 40 samples were analyzed using both LFDIA and LC−MS/MS. The results from the 7 positive samples (4 for corn and 3 for wheat) are shown in Table 3. The relationship between LFDIA and LC−MS/MS results for ZEN and FB1 in natural samples was assessed by regression analysis, LFDIA = −43.64 + 1.13LC−MS/MS ($R^2 = 0.96$), indicating a good agreement between the two methods (see Figure S4 of the Supporting Information).

In summary, the LFDIA method described here is suitable for simultaneous quantification of ZEN and FB1 in corn and wheat samples with superior limits and ranges of detection over published immunochromatographic assay formats. In comparison to quantitative ELISA, it is rapid (30 min), easy to operate (a single step), and less expensive and may be used for monitoring ZEN and FB1 in the field, such as in food- or feed-processing factories or on farms.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Identification of control sample (PBS) and FB1, DON, and AFB1 standard solutions (100 ng/mL) for ZEN individual LFIA (Figure S1), identification of control sample (PBS) and ZEN, DON, and AFB1 standard solutions (100 ng/mL) for FB1 individual LFIA (Figure S2), representative results of the quantitative LFDIA (Figure S3), correlation of results obtained

by both LFDIA and LC−MS/MS for ZEN and FB1 detection on natural corn and wheat samples (Figure S4), and buffer types, optimum buffers, and optimum concentrations of additives in the quantitative LFDIA (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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