Original Paper

Development of Enzyme-Linked Immunosorbent Assay for Analysis of Total Aflatoxins Based on Monoclonal Antibody Reactive with Aflatoxins B₁, B₂, G₁ and G₂

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A direct competitive enzyme-linked immunosorbent assay (dc-ELISA) was developed for the determination of total amount of aflatoxin B₁, B₂, G₁ and G₂ (AFB₁, AFB₂, AFG₁ and AFG₂), using a mouse monoclonal antibody that shows similar reactivity to each of these AFs. The working range of the developed dc-ELISA was 50–230 pg/mL for AFB₁, 50–270 pg/mL for AFB₂, 60–390 pg/mL for AFG₁ and 65–700 pg/mL for AFG₂. The recovery of AFs from spiked roasted peanuts was 98%. Further, when 4 samples actually contaminated with AFB₁, AFB₂, AFG₁ and AFG₂ were examined, the results of dc-ELISA were highly correlated with the values assigned by the Food Analysis Performance Assessment Scheme. The developed dc-ELISA appears to be suitable for the determination of total AFs at concentrations around the maximum permitted level (10 µg/kg for all foods) in Japan.

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Introduction

Aflatoxins (AFs) are toxic secondary metabolites produced by fungi of the genus *Aspergillus*, mainly *Aspergillus flavus* and *Aspergillus parasiticus*, which are found in agricultural crops such as peanuts, maize, tree nuts, and cottonseed in tropical and subtropical regions.^{1), 2)} AFs have high acute toxicity: the 50% lethal dose is 0.3 mg/kg body weight in rabbits and 18 mg/kg body weight in rats.³⁾ Intake of foods contaminated with a high concentration of AFs can cause severe acute hepatitis.⁴⁾ Two acute human aflatoxicosis outbreaks have been reported, one resulting in 106 deaths out of 397 patients in India in 1974, and the other resulting in 125 deaths out of 317 patients in Kenya in 2004.^{5), 6)} Chronic intake of low concentrations of

AFs is also associated with an increased risk of hepatic carcinoma. The International Agency for Research on Cancer (IARC) classifies AFs as a human carcinogen (Group 1).^{7), 8)} It is therefore extremely important to prevent the intake of AFs through contaminated foods.

The main naturally occurring AFs are AFB₁, AFB₂, AFG₁ and AFG₂. AFB₁ is not only the most toxic and carcinogenic, but it also occurs most frequently in agricultural products, although AFB₂, AFG₁ and AFG₂ are also found in various agricultural products.^{9)–12)} AFG₁ contamination, at higher concentrations than that of AFB₁, was reported in maize and ground nuts in 2010 and 2011 in Malawi.¹³⁾ The total amount of AFB₁, AFB₂, AFG₁ and AFG₂ (total AFs) in foods is therefore regulated in many countries. The Ministry of Health, Labour and Welfare in Japan set the maximum level of total AFs to 10 µg/kg for all foods^{*1}. The Co-

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dex Alimentarius Commission (Codex) set the maximum levels of total AFs to 10 or 15 µg/kg for some nuts^{*2}. The European Union (EU) set the maximum levels of total AFs to 4–15 µg/kg for nuts, dried fruits, cereals, and spices^{*3}. The USA set the maximum level of total AFs to 20 µg/kg for all foods^{*4}. Thus, total AFs must be monitored in these foods, and AF-contaminated products must be removed from the market to avoid potential risks to consumers' health.

AFs are generally detected by instrumental analysis, such as high-performance liquid chromatography (HPLC) with fluorescence detection, liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS).^{14)–17)} The Codex recommends HPLC with post column derivatization and immunoaffinity column clean-up for analysis of AF contamination in cereals, shell-fruits and their products^{*5}. Although such instrumental analyses are sensitive and accurate, they require expensive equipment, a lot of time to prepare samples for measurement, and sophisticated techniques, and are not suitable for on-site analysis in food production and distribution facilities.

As an alternative, enzyme-linked immunosorbent assay (ELISA) is a simple, rapid, and cost-effective method based on antigen-antibody interaction, and is suitable for the analysis of a large number of samples in a short time. Many kinds of ELISA had been developed for determination of AFB₁ since Chu *et al.* reported the preparation of anti-AFB₁ antibody in 1977.^{18)–27)} ELISA methods for total AFs have also been developed since regulation of total AFs was introduced in many countries.^{22)–25), 27)} However, their performance is not as good as that of other total AFs determination methods because of low reactivity to AFG₂ compared to the other AFs. Thus, an antibody with high sensitivity and specific reactivity against the common structure of AFB₁, AFB₂, AFG₁ and AFG₂ is required.

A mouse monoclonal antibody that reacts almost equally to AFB₁, AFB₂, AFG₁ and AFG₂ was previously prepared for the development of an immunoaffinity column for AF analysis.²⁸⁾ In this present study, we used this monoclonal antibody to develop a direct competitive ELISA (dc-ELISA) for the determination of total AFs. We confirmed that the developed dc-ELISA has sufficient reactivity to AFG₂ as well as AFB₁, AFB₂ and AFG₁, and also demonstrated its applicability to determination of total AFs in several foods.

Materials and Methods

1. Materials

AFB₁, AFB₂, AFG₁ and AFG₂, which were of analytical grade for mycotoxin analysis, and *N*-hydroxysuccinimide (NHS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 1-Ethyl-3-(3-dimethyl-

aminopropyl)carbodiimide hydrochloride (EDC) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Bovine serum albumin (BSA; product no. A7888) was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Ninety-six-well microtiter plates (Nunc MaxiSorp) and goat anti-mouse IgG (H+L) antibody (Pierce) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Horseradish peroxidase (HRP) was purchased from Toyobo Co., Ltd. (Osaka, Japan).

Roasted peanuts produced in Kanagawa prefecture, Japan, were obtained from the market, and it was confirmed by LC-MS/MS that they were not contaminated with AFs. Ground food samples contaminated with AFB₁, AFB₂, AFG₁ and AFG₂ were provided by the Food Analysis Performance Assessment Scheme (FAPAS): peanuts (T04118), almond (T04114), pistachio (T04283) and chili powder (T04286). Commercial peanut butter from the USA (17 samples), India (2 samples), or Japan (4 samples) was purchased from markets in the Kansai area of Japan.

All other chemicals and reagents, which were of analytical grade, were purchased from Wako Pure Chemical Industries, Ltd. or Nacalai Tesque, Inc. (Kyoto, Japan).

2. Preparation of anti-AF monoclonal antibody

The monoclonal antibody MoAb2-3, showing specific reactivity to AFB₁, AFB₂, AFG₁ and AFG₂, was provided by Horiba Ltd. (Kyoto, Japan) as mouse ascites fluid.²⁸⁾ MoAb2-3 was purified from the fluid by precipitation with saturated ammonium sulfate solution (33% concentration). The resulting precipitate was dissolved in phosphate-buffered saline (PBS: 10 mmol/L phosphate, 150 mmol/L sodium chloride; pH 7.0) and the solution was dialyzed against PBS.

3. Preparation of AF-HRP conjugate

AFB₁ was derivatized to AFB₁-1-(*o*-carboxymethyl)oxime (AFB₁-oxime) according to the method of Chu *et al.*^{18), 19)} The carboxy residue of AFB₁-oxime was then covalently conjugated with the amino residue of HRP by the activated ester method, as described previously.^{29), 30)} In brief, 100 µL of AFB₁-oxime (5 µmol) was mixed with 5 µL of NHS (6 µmol) and 10 µL of EDC (6 µmol), in anhydrous dimethyl sulfoxide. The mixture was left to stand at room temperature for 1.5 hr. Then, 1 mL of HRP (10 mg/mL) dissolved in PBS was added, and the mixture was stirred for 1.5 hr, then dialyzed against PBS, and subjected to gel filtration chromatography to obtain the pure AFB₁-HRP conjugate. The prepared AFB₁-HRP conjugate solution was diluted as required in PBS containing 0.2% BSA immediately before use. The AFB₂-HRP conjugate was prepared in the same way.

4. dc-ELISA

The dc-ELISA was conducted as described previously.^{29), 30)} In brief, each well of a 96-well microtiter plate was coated with 100 µL of anti-mouse IgG goat antibody (5 µg/mL) in PBS at 4°C overnight, and then blocked

*² CODEX STAN 193–1995

*³ Commission Regulation EC No. 1881/2006

*⁴ The U.S. FDA, Compliance Policy Guideline 555.400

*⁵ CODEX STAN 234–1999

with 300 μ L of PBS containing 0.4% BSA at 25°C for 1 hr. One hundred microliters of MoAb2-3 (40–150 ng/mL) in PBS containing 0.2% BSA was added to each well, and the plate was incubated at 25°C for 1 hr. The wells were washed once with 350 μ L of PBS containing 0.02% Tween 20. Standard solutions of AFB₁, AFB₂, AFG₁, AFG₂ in 10% (v/v) methanol were prepared at various concentrations (12.5 pg/mL to 10 ng/mL). A mixture of AFs (AFB₁:AFB₂:AFG₁:AFG₂=8:1:6:1) was also prepared for measurement of the standard curve of the dc-ELISA. This ratio is the same as that used in the recovery test when the immune-affinity column based on MoAb2-3 was developed.²⁸⁾ The standard solutions or measurement samples were mixed with AFB₁-HRP (2.5–5.0 ng/mL) or AFB₂-HRP (30–100 ng/mL) at 1:1 ratio, and 100 μ L of the mixture was added to the wells coated with MoAb2-3. The plates were incubated to allow competitive reaction at 25°C for 1 hr, then washed three times in the same manner as described above, and 100 μ L/well of the HRP substrate solution consisting of 3,3',5,5'-tetramethylbenzidine (100 μ g/mL) and H₂O₂ (0.006%) dissolved in acetate buffer (100 mmol/L; pH 5.5) was added. Incubation was continued at 25°C for 10 min. The color development reaction was stopped by addition of 100 μ L of sulfuric acid (0.5 mol/L) to the wells. The absorbance at 450 nm was measured using a microplate reader (xMark) from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

5. Preparation of measurement samples

Roasted peanut samples were used for the recovery test. The samples were sufficiently ground in a food processor (MILLSER IFM-700G; Iwatani, Tokyo, Japan). Each of the samples (2.5 g) was transferred to a plastic centrifuge tube (15 mL). AF mixture (AFB₁:AFB₂:AFG₁:AFG₂=8:1:6:1) for spiking the samples was prepared at the same ratio as in the previous report.²⁸⁾ One hundred μ L of the mixture was added to the samples to give a final concentration of 2.0 or 10 ng/g. After vigorous shaking, the samples were left to stand at room temperature for 30 min.

The roasted peanut samples for the recovery test, 4 kinds of AF-contaminated samples, and 23 kinds of peanut butter samples were extracted according to the Japanese notification method^{*6} with partial modification, as described below.

For the dc-ELISA, each of the samples (2.5 g) was mixed with sodium chloride (0.25 g) and 10 mL of methanol–water (4:1, v/v) solution, and the mixture was vigorously shaken (Shaker SA320; Yamato Scientific Co., Ltd., Tokyo, Japan) for 30 min to extract the AFs. The extract was centrifuged at 3,000 rpm for 10 min, and the supernatant was diluted 8-fold with distilled water to prepare the measurement sample containing 10% (v/v) methanol equivalent. The prepared sample was subject-

ed to dc-ELISA. If necessary, further dilution of the measurement sample was carried out to adjust the concentrations of the AFs to the measurement range, using a methanol–water (1:9, v/v) solution.

For LC-MS/MS, each of the samples (25 g) was mixed with sodium chloride (2.5 g) and 100 mL of methanol–water (4:1, v/v) solution, and the mixture was homogenized for 5 min to extract the AFs. The homogenate was centrifuged at 3,000 rpm for 15 min, and 25 mL of the supernatant was diluted to 50 mL with distilled water. Then, 10 mL of this solution was applied to an immunoaffinity column (AFLAKING; Horiba Ltd.) to clean up the AFs. The column was washed 3 times with PBS and 2 times with deionized water. The water was blown out with air, and the AFs were eluted from the column with 3 mL of acetonitrile. The solution was evaporated under N₂ and the residue was dissolved in 2 mL of 10 mmol/L ammonium acetate solution–methanol (6:4, v/v) mixture. Five microliters of the solution was injected into the LC-MS/MS system.

6. LC-MS/MS analysis

The LC system consisted of an AQUITY UPLC (Waters Corporation, Milford, MA, USA), with an AQUITY UPLC BEH C18 column (1.7 μ m, 2.1 i.d. $\times$ 50 mm; Waters Corporation). Mobile phase A was ammonium acetate solution (10 mmol/L; pH 5.0) and mobile phase B was methanol. Elution was carried out as follows (A:B): 0–5 min (95:5), 5–5.1 min (20:80) and after 5.1 min (95:5). The column oven temperature was set at 40°C, the flow rate was 0.5 mL/min, and the injection volume of the sample was 5 μ L. The LC-MS/MS system consisted of UPLC and Quattro Premier XE (Waters Corporation). The electrospray-ionization source was used in the positive mode. The measurement mode was multiple reaction monitoring. The precursor ion (m/z), product ion (m/z), cone voltage (V), and collision energy (eV) were as follows: 313/241/40/35 for AFB₁, 315/259/40/35 for AFB₂, 329/243/40/30 for AFG₁, and 331/245/40/35 for AFG₂, respectively. The source temperature was set at 120°C, and the desolvation temperature was set at 400°C. The flow rate of the desolvation gas, the cone gas, and the collision gas were set at 800, 50 L/hr and 0.20 mL/min, respectively.

7. Safety

All items that had come into contact with AFs were detoxified by using a commercial chlorine-based bleach before being discarded or reused.

Results and Discussion

1. dc-ELISA constitution

Among the various types of immunoassays, dc-ELISA is a comparatively sensitive and precise method and has been widely used for mycotoxin analysis. Therefore, we chose dc-ELISA to determine total AFs in this study, using a combination of MoAb2-3 with AFB₁-HRP or AFB₂-HRP. Four kinds of candidate conditions were evaluated by checkerboard titration, based on an absorbance value

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Fig. 1. Reactivity of AFB₁ with MoAb2-3 in dc-ELISA under various conditions

(●) shows inhibition curve under conditions of unsaturated MoAb2-3 (40 ng/mL) and AFB₁-HRP (5.0 ng/mL), (▲) shows MoAb2-3 (40 ng/mL) and AFB₂-HRP (100 ng/mL), (○) shows saturated MoAb2-3 (150 ng/mL) and AFB₁-HRP (2.5 ng/mL), and (△) shows MoAb2-3 (150 ng/mL) and AFB₂-HRP (30 ng/mL).

Each data point is the mean of duplicates in independent experiments.

Fig. 2. Inhibition curve of the AFs in dc-ELISA

(●) shows inhibition curve of AFB₁, (○) AFB₂, (▲) AFG₁, (△) AFG₂, and (◇) AF mixture.

Each data point is the mean of four replicates in independent experiments.

Error bars indicate $\pm$ S.D.

of 1–1.5 at 450 nm, which was about half of the maximum absorbance. The obtained inhibition curves of AFB₁ are presented in Fig. 1. Because saturation with the antibody can determine the antigens with less variation, MoAb2-3 (150 ng/mL) was initially examined. The dc-ELISA with AFB₂-HRP was 4-fold more sensitive than that with AFB₁-HRP, but unfortunately both con-

Table 1. IC values in dc-ELISA

AFs	IC values (pg/mL)		
	IC ₂₀	IC ₅₀	IC ₈₀
B ₁	50	90	230
B ₂	50	100	270
G ₁	60	130	390
G ₂	65	190	700

ditions were insufficiently sensitive to determine total AFs at the maximum permitted levels in foods. In order to increase the sensitivity, we examined an unsaturated condition (MoAb2-3 40 ng/mL) with the HRP-conjugated AFs. The IC₅₀ value with AFB₁-HRP was 90 pg/mL, while that with AFB₂-HRP was 85 pg/mL. These sensitivities are high enough to measure total AFs at around the maximum permitted levels in foods. When the AFB₁-HRP- and AFB₂-HRP-based dc-ELISAs were compared, 20-fold more AFB₂-HRP (100 ng/mL) was necessary to obtain the same absorbance as AFB₁-HRP (5.0 ng/mL). Therefore, the combination of 40 ng/mL MoAb2-3 and 5.0 ng/mL AFB₁-HRP was finally chosen for the dc-ELISA.

The inhibition curves of AFB₁, AFB₂, AFG₁ and AFG₂ obtained by the developed dc-ELISA are shown in Fig. 2. The 20%-inhibitory concentration (IC₂₀) values, the 50%-inhibitory concentration (IC₅₀) values, and the 80%-inhibitory concentration (IC₈₀) values calculated from the inhibition curves are summarized in Table 1. The IC₅₀ values, which are generally used for comparison of the sensitivity of dc-ELISAs, were 90, 100, 130 and 190 pg/mL for AFB₁, AFB₂, AFG₁ and AFG₂, respectively. The working range, which is defined as the concentration range between the IC₂₀ and IC₈₀ values, was 50–230 pg/mL for AFB₁, 50–270 pg/mL for AFB₂, 60–390 pg/mL for AFG₁ and 65–700 pg/mL for AFG₂. The lower limit of the working range of AFG₂ (65 pg/mL), which had the lowest sensitivity among the AFs in this assay, is 153-fold lower than the maximum level permitted in Japan (10 µg/kg), assuming the AFs in foods are extracted into methanol at 100% yield. These results indicate that the dc-ELISA has sufficiently high sensitivity to measure total AFs after 32-fold dilution during sample preparation.

Contaminated food samples often contain not only AFB₁, but also AFB₂, AFG₁ and/or AFG₂. Therefore, AF mixture (AFB₁ : AFB₂ : AFG₁ : AFG₂ = 8 : 1 : 6 : 1) was used to prepare the standard curve of this dc-ELISA, as shown in Fig. 2.

2. Recovery of AFs spiked into roast peanut samples

The applicability of the dc-ELISA for determination of total AFs was examined in a recovery test using roasted peanut samples that were not contaminated with AFs. The AF mixture was added to the samples at the same ratio as the standard (AFB₁ : AFB₂ : AFG₁ : AFG₂ = 8 : 1 : 6 : 1), and the total AFs concentration was determined by the dc-ELISA. As shown in Table 2, the recovery was 98% for total AFs spiked at 2 and 10 ng/g. These results indicate that the dc-ELISA would be applicable

Table 2. Recovery of total AFs spiked into roasted peanuts

Spiked conc. (ng/g)	Recovery (%)
2.0	98±7.2*
10.0	98±3.5

* Values are the mean of five replicates±S.D.

Table 3. Determination results of total AFs in contaminated food samples

Samples	dc-ELISA	Assigned values*				
	Total	AFB ₁	AFB ₂	AFG ₁	AFG ₂	Total
Peanuts	10.9	6.52	1.81	2.66	1.84	12.82
Pistachio	10.9	7.98	1.72	1.66	0.776	11.9
Almond	10.3	2.85	1.22	1.70	0.92	6.82
Chili powder	12.2	4.70	2.41	2.31	0.843	10.3

* The values were assigned by FAPAS.

The values are the mean of two replicates.

to determine total AFs in roast peanuts.

3. Determination of total AFs in other food samples

Various food samples may be contaminated with AFB₁, AFB₂, AFG₁ and AFG₂. Therefore, it is important that real samples contaminated with all of them are examined to confirm the applicability of the dc-ELISA with the AF mixture standard. Four different contaminated food samples (peanuts, almond, pistachio and chili powder) containing assigned concentrations of AFs were available from FAPAS. As shown in Table 3, the results of dc-ELISA showed a good correlation with the corresponding assigned values, except for almond. Nevertheless, because the result for almond was false positive but not false negative, we consider that the dc-ELISA can be used to test food samples, although care is needed in the case of almonds, for which the assay should be limited to screening tests.

In addition, 23 commercial products of peanut butter were examined, and the results were compared to those obtained by LC-MS/MS. None of these samples contained AFG₁ or AFG₂. The results were well correlated with the results obtained by LC-MS/MS (Table S1).

4. Comparison of the developed dc-ELISA with reported ELISAs

Five kinds of ELISAs have been reported for determination of total AFs^{22)–25), 27)}, although they cannot adequately determine total AFs (Table 4), because the same reactivity for AFB₁, AFB₂, AFG₁ and AFG₂ would be necessary for this purpose. The ratio of the IC₅₀ values of AFB₁ and AFG₂ among them ranged from 5.2- to 26-fold, except for the ic-ELISA reported by Li *et al.*, which gave a value of 1.5-fold.²²⁾ However, the sensitivity of this assay is too low for application to the determination of total AFs.

The developed dc-ELISA in this study showed sufficient sensitivity and the ratio between the highest and the lowest IC₅₀ values was only 2.1-fold: 90 pg/mL for

Table 4. Comparison of specificity for aflatoxins among reported ELISAs

IC ₅₀ value (pg/mL)				ELISA type	Reference
AFB ₁	AFB ₂	AFG ₁	AFG ₂		
2090	2230	2190	3210	ic-ELISA*, MoAb	22
1.2	1.3	2.2	18	ic-ELISA, MoAb	23
150	550	320	3830	dc-ELISA, PoAb	24
130	220	81	420	ic-ELISA, MoAb	25
31	67	48	280	dc-ELISA, MoAb	27
90	100	130	190	dc-ELISA, MoAb	Newly developed

* ic-ELISA: indirect competitive ELISA.

AFB₁ and 190 pg/mL for AFG₂. These results suggest that the developed assay is suitable for the determination of total AFs in foods at concentrations around the maximum permitted levels in Japan.

Conclusion

A dc-ELISA employing MoAb2-3 and AFB₁-HRP conjugate was developed and shown to be suitable for determination of total AFs in several foods at around the maximum permitted concentration. AFB₁ and AFG₂, however, showed an approximately 2-fold difference in reactivity. Thus, AFG₂ concentration is determined as half the value of AFB₁. We propose that the developed dc-ELISA would be applicable for determination of total AFs in foods, e.g., for on-site screening, if the cut off value is set at 5 µg/kg, which is a half of the maximum permitted level in foods.

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References

- 1) Woloshuk, C. P., Shim, W. B. Aflatoxins, fumonisins, and trichothecenes: A convergence of knowledge. *FEMS Microbiol. Rev.*, **37**, 94–109 (2013).
- 2) Perrone, G., Gallo, A. *Aspergillus* species and their associated mycotoxins. *Methods Mol. Biol.*, **1542**, 33–49 (2017).
- 3) Trucksess, M. W. "Aflatoxins". *Bad Bug Book, Food-borne Pathogenic Microorganisms and Natural Toxins*, 2nd ed. Lampel, K. A., Al-Khalidi, S. Cahill, S. M., eds. Maryland, USA, Food and Drug Administration, 2012, p. 230–235. (ISBN 1-4952-0361-1)
- 4) Marin, S., Ramos, A. J., Cano-Sancho, G., Sanchis, V. Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food Chem. Toxicol.*, **60**, 218–237 (2013).
- 5) Krishnamachari, K. A., Bhat, R. V., Nagarajan, V., Tilak, T. B. Hepatitis due to aflatoxicosis. An outbreak in western India. *Lancet*, **1**, 1061–1063 (1975).
- 6) Probst, C., Njapau, H., & Cotty, P. J. Outbreak of an acute aflatoxicosis in Kenya in 2004: Identification of the causal agent. *Appl. Environ. Microbiol.*, **73**, 2762–2764 (2007).

- 7) IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC Monogr. Eval. Carcinog. Risks Hum., **82**, 171–300 (2002).
- 8) IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Chemical agents and related occupations. A review of human carcinogens. IARC Monogr. Eval. Carcinog. Risks Hum., **100**, 225–244 (2012).
- 9) Kensler, T. W., Roebuck, B. D., Wogan, G. N., Groopman, J. D. Aflatoxin: A 50-year odyssey of mechanistic and translational toxicology. Toxicol. Sci., **120**, S28–S48 (2011).
- 10) Rubert, J., Sebastia, N., Soriano, J. M., Soler, C., Manes, J. One-year monitoring of aflatoxins and ochratoxin A in tiger-nuts and their beverages. Food Chem., **127**, 822–826 (2011).
- 11) Habibipour, R., Tamandegani, P. R., Farmany, A. Monitoring of aflatoxin G₁, B₁, G₂, and B₂ occurrence in some samples of walnut. Environ. Monit. Assess., **188**, 669 (2016).
- 12) Kabak, B. Aflatoxins in hazelnuts and dried figs: Occurrence and exposure assessment. Food Chem., **211**, 8–16 (2016).
- 13) Matumba, L., Sulyok, M., Njoroge, S. M., Njumbe Ediage, E., Van Poucke, C., De Saeger, S., Krska, R. Uncommon occurrence ratios of aflatoxin B₁, B₂, G₁, and G₂ in maize and groundnuts from Malawi. Mycotoxin Res., **31**, 57–62 (2015).
- 14) Nonaka, Y., Saito, K., Hanioka, N., Narimatsu, S., Kataoka, H. Determination of aflatoxins in food samples by automated on-line in-tube solid-phase microextraction coupled with liquid chromatography-mass spectrometry. J. Chromatogr. A, **1216**, 4416–4422 (2009).
- 15) Quinto, M., Spadaccino, G., Palermo, C., Centonze, D. Determination of aflatoxins in cereal flours by solid-phase microextraction coupled with liquid chromatography and post-column photochemical derivatization-fluorescence detection. J. Chromatogr. A, **1216**, 8636–8641 (2009).
- 16) Shejoooni-Fumani, N., Hassan, J., Yousefi, S. R. Determination of aflatoxin B₁ in cereals by homogeneous liquid-liquid extraction coupled to high performance liquid chromatography-fluorescence detection. J. Sep. Sci., **34**, 1333–1337 (2011).
- 17) Tamura, M., Uyama, A., Mochizuki, N. Development of a multi-mycotoxin analysis in beer-based drinks by a modified QuEChERS method and ultra-high-performance liquid chromatography coupled with tandem mass spectrometry. Anal. Sci., **27**, 629–635 (2011).
- 18) Chu, F. S., Hsia, M. T., Sun, P. S. Preparation and characterization of aflatoxin B₁-1-(O-carboxymethyl) oxime. J. Assoc. Off. Anal. Chem., **60**, 791–794 (1977).
- 19) Chu, F. S., Ueno, I. Production of antibody against aflatoxin B₁. Appl. Environ. Microbiol., **33**, 1125–1128 (1977).
- 20) Lee, N. A., Wang, S., Allan, R. D., Kennedy, I. R. A rapid aflatoxin B₁ ELISA: Development and validation with reduced matrix effects for peanuts, corn, pistachio, and soybeans. J. Agric. Food Chem., **52**, 2746–2755 (2004).
- 21) Kolosova, A. Y., Shim, W. B., Yang, Z. Y., Eremin, S. A., Chung, D. H. Direct competitive ELISA based on a monoclonal antibody for detection of aflatoxin B₁. Stabilization of ELISA kit components and application to grain samples. Anal. Bioanal. Chem., **384**, 286–294 (2006).
- 22) Li, P., Zhang, Q., Zhang, W., Zhang, J., Chen, X., Jiang, J., Xie, L., Zhang, D. Development of a class-specific monoclonal antibody-based ELISA for aflatoxins in peanut. Food Chem., **115**, 313–317 (2009).
- 23) Zhang, D., Li, P., Zhang, Q., Zhang, W., Huang, Y., Ding, X., Jiang, J. Production of ultrasensitive generic monoclonal antibodies against major aflatoxins using a modified two-step screening procedure. Anal. Chim. Acta, **636**, 63–69 (2009).
- 24) Liu, B., Hsu, Y., Lu, C., Yu, F. Detecting aflatoxin B₁ in foods and feeds by using sensitive rapid enzyme-linked immunosorbent assay and gold nanoparticle immunochromatographic strip. Food Control, **30**, 184–189 (2013).
- 25) Jiang, W., Wang, Z., Nölke, G., Zhang, J., Niu, L., Shen, J. Simultaneous determination of aflatoxin B₁ and aflatoxin M₁ in food matrices by enzyme-linked immunosorbent assay. Food Anal. Methods, **6**, 767–774 (2013).
- 26) Yu, F. Y., Gribas, A. V., Vdovenko, M. M., Sakharov, I. Y. Development of ultrasensitive direct chemiluminescent enzyme immunoassay for determination of aflatoxin B₁ in food products. Talanta, **107**, 25–29 (2013).
- 27) Oplatowska-Stachowiak, M., Sajic, N., Xu, Y., Haughey, S. A., Mooney, M. H., Gong, Y. Y., Verheijen, R., Elliott, C. T. Fast and sensitive aflatoxin B₁ and total aflatoxins ELISAs for analysis of peanuts, maize and feed ingredients. Food Control, **63**, 239–245 (2016).
- 28) Uchigashima, M., Saigusa, M., Yamashita, H., Miyake, S., Fujita, K., Nakajima, M., Nishijima, M. Development of a novel immunoaffinity column for aflatoxin analysis using an organic solvent-tolerant monoclonal antibody. J. Agric. Food Chem., **57**, 8728–8734 (2009).
- 29) Hirakawa, Y., Yamasaki, T., Watanabe, E., Okazaki, F., Murakami-Yamaguchi, Y., Oda, M., Iwasa, S., Narita, H., Miyake, S. Development of an immunosensor for determination of the fungicide chlorothalonil in vegetables, using surface plasmon resonance. J. Agric. Food Chem., **63**, 6325–6330 (2015).
- 30) Hirakawa, Y., Yamasaki, T., Harada, A., Ohtake, T., Adachi, K., Iwasa, S., Narita, H., Miyake, S. Analysis of the fungicide boscalid in horticultural crops using an enzyme-linked immunosorbent assay and an immunosensor based on surface plasmon resonance. J. Agric. Food Chem., **63**, 8075–8082 (2015).