WFL Publisher
Science and Technology
Meri-Rastilantie 3 B, FI-00980
Helsinki, Finland
e-mail: info@world-food.net

Journal of Food, Agriculture & Environment Vol.11 (1): 190-194. 2013 *www.world-food.net*

Preparation of aflatoxin B_1 polyclonal antibody and development of an indirect competitive enzyme-linked immunosorbent assay

Hao Zhang [1, 2], Hai-zhen Mo [2]* and Hua Li [3]*

*[1] College of Food Science and Engineering, Northwest A&F University, Yangling 712100, China. [2] School of Food Science, Henan Institute of Science and Technology, Xinxiang 453003, China. [3] College of Enology, Northwest A&F University, Yangling 712100, China. *e-mail: mohaizhen008@126.com, lihuawine@nwsuaf.edu.cn*

Received 29 September 2012, accepted 20 January 2013.

Abstract

This article aimed to prepare polyclonal antibodies (pAbs) against aflatoxin B_1 (AFB_1) and develop an immunoassay for detecting aflatoxin residues in tea matrix. After derivation, AFB_1 hapten was conjugated to carrier proteins to synthesize the artificial antigen, and rabbits were employed to produce pAbs. Based on the bidimensional checkerboard titration results, two indirect competitive ELISA (icELISA) standard curves have been established. For the traditional two-step assay, the linear range was from 0.12 to 103 ng/ml, with the half maximal inhibitory concentration (IC_{50}) and limit of detection (LOD) values of 2.97 and 0.05 ng/ml, respectively; while the rapid one-step icELISA had a working range from 0.14 to 126 ng/ml, with IC_{50} and LOD values of 3.85 and 0.06 ng/ml. Of all the competitive analogues, this one-step assay exhibited a high cross-reactivity to AFB_2 (26.2%), AFG_1 (61.3%), AFG_2 (25.5%), AFM_1 (18.6%) and AFM_2 (3.4%). It also indicated that a 10-fold dilution in tea extracts gave an inhibition curve almost the same as that in PBS buffer. These results suggest that the immunoassay provided was a promising alternative for screening aflatoxin residues in tea sample, even in other agro-products such as peanuts, feedstuffs and vegetable oils.

***Key words*:** Aflatoxin B_1, artificial antigen, polyclonal antibody, indirect competitive ELISA, tea sample.

Introduction

Agricultural products are often contaminated with fungi that can produce toxic metabolites referred to as mycotoxins. Aflatoxins are a group of secondary metabolites mainly produced by *Aspergillus flavus* and *A. parasiticus* and have assumed economic importance because of their influence on the health of human beings and livestock, and on the marketability of agricultural products [1]. Aflatoxins are hepatotoxic and carcinogenic, and numerous epidemiological studies have linked consumption of aflatoxin-contaminated food with liver cancer, hepatic failure resulting in death and impaired child growth [2, 3]. Among the aflatoxins identified, aflatoxin B_1 (AFB_1) is the predominant form, which presents the highest toxic potential, and is classified as carcinogenic to humans by the International Agency for Research on Cancer [4]. AFB_1 mainly decreases lymphoid cell populations especially circulating activated lymphocytes, suppresses lymphoblastogenesis and impairs both cutaneous delayed-type hypersensitivity (DTH) and graft versus host reaction. AFB_1 also decreases natural killer cytolysis and several macrophage functions, such as phagocytic activity, intra-cellular killing or production of oxidative radicals [5].

Therefore, legal limits varing from country to country have been established. European Union legislation has established maximum permissible levels of 2-8 µg/kg for AFB_1 and 4-15 µg/kg for total aflatoxins, depending on the different foods used for direct human consumption or as ingredients in other food products [6]. Some developing countries like China and Mexico have set up regulations compatible with those in the United States for human consumption. For example, the current maximum allowed level (MAL) set by United states and China is 20 ng/ml (20 µg/kg) in foodstuffs [7].

This has resulted in the development of a broad range of methods to detect AFB_1 and its metabolites in biological matrices. Conventional analytical methods include thin-layer chromatography (TLC) [8], high-performance liquid chromatography (HPLC) [9], liquid chromatography-tandem mass spectrometry (LC-MS) [10] and so on. These methods, applied to food or feed samples, yield results within hours or days. Competition within the food and feed industry is forcing manufacturers to reduce costs, so that rapid, less expensive, and easier-to-use analysis have become increasingly important. Therefore, techniques such as radioimmunoassay, enzyme immunoassay, fluroimmunoassays, and test strip immunoassay have been reported for the measurement of AFB_1 and its metabolites in various biological and non-biological samples [1, 7, 11-13].

However, commercial kits using immunological methods are expensive and can be problematic to import in many developing countries. Morever, most of the reports that have appeared so far for aflatoxins have involved in grain, dry fruits, fermentation products, and other foodstuffs. Therefore, the aim of this study was to generate sensitive polyclonal antibodies for the precise analysis of AFB_1 residues in tea samples. In this paper, we also investigated the matrix effects and established different standard curves.

Materials and Methods

Materials and reagents: Aflatoxin B_1 (AFB_1) was obtained from Sigma (St. Louis, MO, USA), while other structurally related aflatoxins (AFB_2, AFG_1, AFG_2, AFM_1 and AFM_2) were purchased from Fluka (Switzerland). Bovine serum albumin (BSA), ovoalbumin (OVA) and GaRIgG-HRP were purchased from Sino-American Biotechnology Company (Shanghai, China). 1-Ethyl-3-(3-dimethylaminopropy) carbodiimide (EDC), Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) were obtained from Pierce (USA). N-hydroxysuccinimide (NHS) was obtained from Sigma-Aldrich (USA). O-(carboxymethyl) hydroxylamine hemihydrochloride and succinic anhydride were supplied by Sigma while dialysis bag was from Solarbio Company. 3,3,5,5-tetramethylbenzidine (TMB), carboxymethyl hydroxylamine hemihydrochloride (CMO), phenacetin and urea peroxide were obtained from Sigma Company. All other solvents and reagents were of analytical or higher grade, unless otherwise stated.

Phosphate buffered saline (PBS) consisted of NaCl (137 mM), $Na_2HPO_4.12H_2O$ (10 mM), KCl (2.68 mM) and KH_2PO_4 (1.47 mM), pH 7.4; carbonate buffer saline (CBS) contained Na_2CO_3 (15 mM), and $NaHCO_3$ (35 mM), pH 9.6. Washing buffer consisted of PBS containing 0.05% Tween-20 (PBST); blocking buffer contained BSA (1%, w/v) in PBS; assay buffer consisted of adding part A (500 ml) and part B (500 ml) solutions together. Part A contained (per litre of water) 3.15 g of citric acid, 6.97 g of anhydrous sodium acetate, 0.08 g phenacetin and 0.05 g of urea peroxide adjusted to pH 5.0 with HCl. Part B contained 1.27 g of TMB dissolved in 500 ml of methanol and 500 ml of glycerol. The stopping solution was 2 M H_2SO_4.

Synthesis of AFB_1 artificial antigen: AFB_1 was conjugated to BSA to prepare the immunizing conjugate and to OVA for preparation of the coating antigen via the linker CMO. To a solution of 2 mg AFB_1 in 3 ml solution (0.5 ml anhydrous pyridine + 2 ml methanol + 0.5 ml distilled water), 4 mg CMO was added, and then the mixture was stirred in the absence of light, kept at 86°C for 5 h. After pyridine was removed with nitrogen evaporator, the remainders were dissolved with 10 ml of ethyl acetate and washed with water three times. After the organic solution was desiccated by anhydrous sodium sulfate, ethyl acetate was removed by a rotary evaporator under reduced pressure and the synthesized yellow creamy product was AFB_1-CMO oxime hapten (AFB_1O).

Optimized EDC method was employed to synthesize the artificial antigen of AFB_1-BSA, and the procedure is presented in Fig. 1. Briefly, 1 mg AFB_1-CMO oxime hapten was suspended in 2 ml solution of DMF and water (v/v, 6:9), and then 0.598 mg NHS and 1.073 mg EDC were added. During the following 24 h incubation in dark, the mixture was stirred with a HY-4 Reciprocal Shaker at 37°C. After centrifuged (4000 r/min, 5 min), the supernatant was added dropwise to 8.6 mg of BSA, which was dissolved in 5 ml of 0.13 M $NaHCO_3$ (pH 7.6). The resulting mixture was stirred by rotor in a dark champer for 1 h and incubated with a reciprocal shaker for 3 h at 37°C. After centrifuged, the obtained supernatant was dialyzed against distilled water and followed by PBS for 4 days. When the micromolecule absorption peak disappeared, stored the AFB_1-BSA immunogen in ampoule, kept at 20°C. AFB_1-OVA coating antigen was prepared in a similar way.

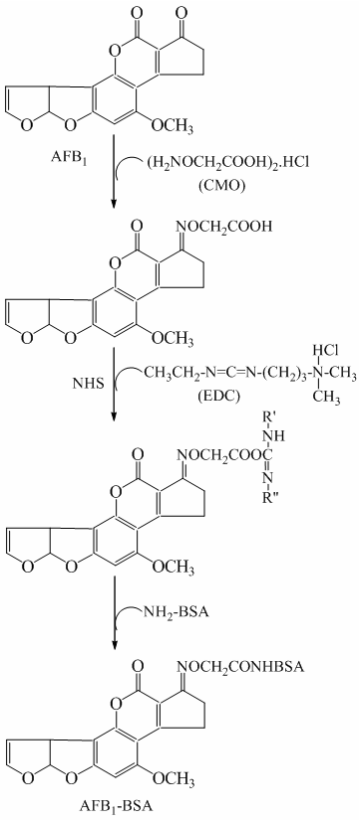

Figure 1. Synthesis procedure for AFB_1 artificial antigen through modified EDC method.

Production of AFB_1 pAb: New Zealand white rabbits were subcutaneously immunized with AFB_1-BSA conjugate to produce the pAb. FCA was employed in the first immunization and FIA was used in the subsequent boost injections. Rabbits were immunized every 30 days with 500 µg of immunogen, and blood samples from the marginal vein of the ear were taken for ELISA identification. Ten days after the final boost, all rabbits were exsanguinated by heart puncture and the serum was separated from blood cells by storing at 4°C overnight. Purification of pAb was performed according to the modified caprylic acid ammonium sulphate precipition method.

Indirect ELISA protocol: The protocol used was similar to that described in previous studies [14]. Briefly, coating antigen of AFB_1-OVA was appropriately diluted in CBS and 100 µl was added to each well of the flat-bottom polystyrene ELISA plates. After 2 h incubation at 37°C, the plates were washed with PBST three times and unbound active sites were blocked with 250 µl/well of blocking buffer, followed by overnight incubation at 4°C. After the blocking solution was removed, appropriate dilution of the antiserum (50 µl/well) was added across the plate, which was incubated for 15 min at 37°C and followed by washing. Then GaRIgG-HRP (1:1000, 50 µl/well) was added, followed by incubation for 25 min at 37°C. After three washings, 60 µl/well of TMB substrate solution was added, followed by 15 min incubation at room temperature. The enzymatic reaction was inhibited with sulfuric acid (2 M, 100 µl/well) and absorbance was spectrophotometrically read in a single wavelength mode at 450 nm. Pre-immune withdrawal serum (the serum before immunisation) and PBST were used as a negative

control and blank control, respectively. The antibody titer was defined as the reciprocal of the dilution that resulted in an absorbance value that was twice of the blank value.

Indirect competitive ELISA (icELISA): The bidimensional checkerboard titration was used to obtain the optimized concentrations of coating antigen and antibody, and the protocol used for icELISA was also carried out according to the general assay conditions [15]. After being coated and blocked, 50 µl of pAb and 50 µl of varying concentrations of standard analyte or target samples were added to each well. During the traditional two-step icELISA procedure, GaRIgG-HRP (1:1000, 50 µl/well) was added 15 min later, and the following steps were similar to that of the indirect ELISA. In this study, we also tried the one-step icELISA procedure and analyzed the competitive inhibition curves against AFB_1. In this way, 50 µl antibody, 50 µl target analyte, and 50µl GaRIgG-HRP were added simultaneously. The plates were then incubated, washed, and measured with a microplate reader at 450 nm as described above.

The calibration curves were fitted based on the average of 3 separate assays in triplicate and sensitivity was evaluated according to the IC_{50} values, which represented the concentration of AFB_1 that produced 50% inhibition of antibody binding to the hapten conjugate. The detection of limit (LOD) was defined as the lowest concentration of AFB_1 that exhibits a signal of 15% inhibition [16]. The working range for icELISA was calculated as the concentration of the analyte providing a 20-80% inhibition rate (IC_{20}-IC_{80} values) of the maximum signal. Specificity was defined as the capability of structurally related chemicals to bind to the antibody and cross-reactivity (CR) was calculated as: (IC_{50} of AFB_1)/(IC_{50} of competitors) ×100.

The pretreatment of tea samples: Freshly collected XinYangMaoJian tea samples (Henan, China) with known backgrounds and certified as free of AFB_1 were used as blank sample and fortified with AFB_1 to give the final concentrations needed. Five gram of homogeneous and nearly ground material was weighed into a centrifugal screw cap vial, and 5 ml of acetonitrile: water (v/v, 80:20) was added. The homogenate was mixed on a vortex mixer for 30 s, vigorously shaken for 15 min, and centrifuged for 30 min (3500 r/min at 25°C). The upper aqueous layer was removed completely and filtered through a folded paper filter. The clear filtrate was collected and compensated to original 5 ml by distilled water. Then, the obtained 5 ml aqueous solution was transferred into a calibrated flask, and diluted in assay buffer (total 2-, 4-, 6-, 10- and 20-fold dilution) before applied to the microtiter plate. Typical experimental response curves were plotted by the absorbance values, against the different concentrations of AFB_1. B and B_0 values from each diluted curve were compared with that generated from the PBS buffer to determine the appropriate dilution factor.

Results and Discussion

Preparation of artificial antigen: Generally, for the production of high-quality antibodies, a good option is to place a linking group opposite from the characteristic group and the idealed space arm of a hapten should be 3-6 chain length. If space arm is too short, the hapten will be submerged into the "sea" of the carrier protein molecule. Conversely, if the space arm is too long, it will be folded. Following this approach, AFB_1 derivative was synthesized by introducing a three-atom spacer length at the pyridinyl ring of the parent compound (Fig. 1). This hapten preserves the specific moieties of AFB_1, and thus achieves the highest antigenicity, which was also the common method for preparation of AFB_1 derivative in other publications [11, 13]. Moreover, the proteins were treated to convert carboxylic acid groups on the carrier proteins to primary amine groups. The use of cationized carriers has the advantage that more amino groups on the carrier become available for coupling and therefore generate an increased immune response compared with their native forms.

Two-step and one-step indirect competitive ELISA standard curves: It is well known that working concentrations of antibody and coating antigen are crucial factors for the sensitivity of ELISA methods. For this reason, checkerboard titrations were performed, taking into account the optimal dilutions. The optimum reagent concentrations were determined when the A_{max} was between 1.5-2.0 and response curve of inhibition ratio versus the AFB_1 concentration pursued the lowest IC_{50} values. From the checkerboard assays, primary antigen concentrations were 1.0 and 1.2 µg/ml of the two-step and one-step assays, respectively. The secondary reaction with the polyclonal antibody was allowed at 0.6 µg /ml (1/10,000 dilution) for the two-step assay and 0.8 µg /ml (1/8000 dilution) for the rapid one. Other assay conditions were as described under Materials and Methods. The organic solvents added to the solution to dissolve AFB_1 were also evaluated. Increasing the concentration of pyridine usually decreased and then increased the IC_{50} value, while increasing the methanol concentration caused a continuous decrease in the A_{max} value but increase in the time for color development. Therefore, the combination of pyridine/methanol/PBS (v/v/v, 10:20:70) was selected, where A_{max}/IC_{50} was the highest.

Under these conditions, two representative standard curves (two-step and one-step) are shown in Fig. 2. The competitive curve obtained with the two-step ELISA allowed the detection of AFB_1 from 0.12 to 103 ng/ml, with an IC_{50} value of 2.97 ng/ml. The limit of detection (LOD) of the assay was determined to be 0.05 ng/ml. Using the one-step ELISA format, the analyte could be determined in the range of 0.14-126 ng/ml, with IC_{50} and LOD values of 3.85 and 0.06 ng/ml, respectively, indicating high sensitivity and stability of our developed ELISAs. Though IC_{50} and LOD values of two assay formats were comparable, the one-step ELISA method was more rapid. The developed one-step assay

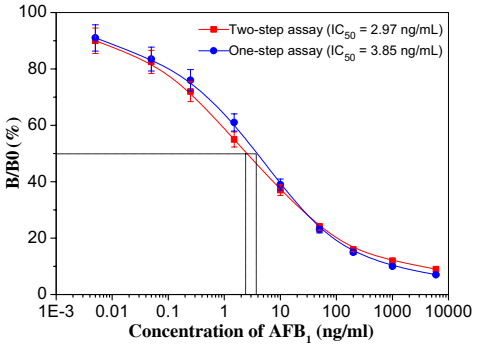

Figure 2. Optimized standard icELISA inhibition curves for AFB_1. Data were obtained in triplicate.

saved 40 min by omitting one procedure and shortening the antigen/antibody incubation time, thus the analysis was reduced to 90 min or so. With this tremendous saving in time, the rapid one-step ELISA provided higher analysis efficiency, therefore it was further evaluated.

Specificity: The cross-reactivity values for all compounds tested are presented in Fig. 3. Among the compounds, the main cross-reacting chemical was AFG_1, which dimensional structure is closely related to the specificity of AFB_1. It can be seen that antibody specificity depends on the choice of coupling sites between hapten and carrier protein, and cross-reactions usually occur in compounds similar to the original semi-antigen. When AFB_1-BSA as immunogen, because of the shielding effect of carrier proteins, molecular shape, hydrogen geometry, and double bond interactions, pAbs get higher cross-reactivity to G family (61.3% to AFG_1 and 25.5% to AFG_2) than to M family (18.6% to AFM_1 and 3.4% to AFM_2). The results pointed out that the carrier-protein-coupled sites should be as far away as possible from the characteristic structure of the hapten, so that the immune system can be better recognized. Because of the characteristics of generic specificity, this immunoassay has the potential to be incorporated into a multiresidue programme for detecting aflatoxin residues in animal-producing foodstuffs and breeding grass.

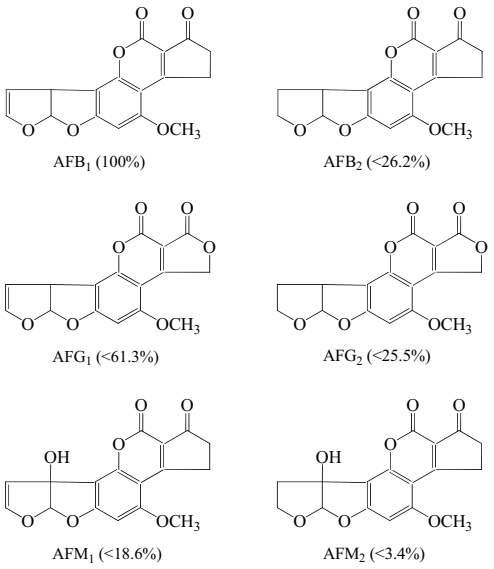

Figure 3. Cross-reactivities of related aflatoxins using one-step icELISA.

Matrix effects and assay parameters: In the present study, tea samples were simply extracted with acetonitrile and no cleanup step was employed, a comparison between calibration plots for AFB_1 prepared in PBS and those prepared in sample extracts are displayed in Fig. 4. As the dilution increased from 1:2 to 1:20, the sensitivity gradually increased to that of the PBS buffer. The average B_0 (antibody binding with no competitor) values for dilutions at 1:2, 1:4, 1:6, 1:10 and 1:20 had absorbencies of 1.265, 1.418, 1.670, 1.863, and 1.894, respectively, as compared to 1.902 in PBS. As Fig. 4 shows, the curve of 20-fold dilution had higher goodness-of-fit with standard curve than that of other dilution folds. However, a large dilution factor may reduce

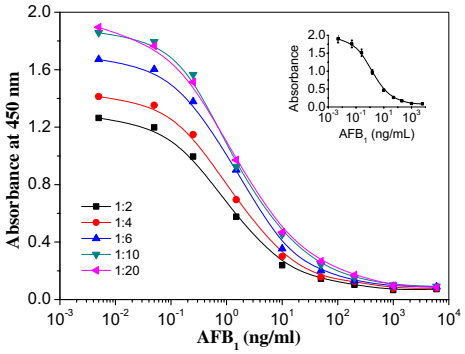

Figure 4. AFB_1 standard curves in the diluted tea sample extracts. Insets indicate the one-step icELISA standard curve in PBS.

sensitivity of the assay to some extent, even causing AFB_1 concentration to move out of the quantitative working range of the developed immunoassay. It can be seen that the two curves of 1:10 and 1:20 dilutions are superimposable, which suggested that the matrix effect actually can be ignored at a dilution of 1:10, and the pretreatment procedure was simple enough to be applied in practical analysis. Taking into account that AFB_1 concentration is recommend to dilute ten times during sample preparation, the LOD value of one-step icELISA may be corrected as 0.6 ng/ml (corresponded to 0.6 µg/kg), still far from the negative cut-off point defined by the European Union [6], which suggested an action range of 2-8 µg/kg for AFB_1 residues in foodstuffs.

Conclusions

In the current study, two specific indirect competitive ELISA standard curves were established, in which the one-step icELISA has been shown to be capable of detecting six aflatoxins in tea matrices simultaneously. As immunoassays provide only preliminary screening results, any suspected samples would then be submitted for further investigations, using more accurate and confirmatory methods such as LC-MS/MS. Since the percentage of negative samples is usually high, the combination of two analytical methods presents a practical advantage. Future studies will investigate the performance of multi-residue standard curves in tea matrix, as well as the applicability of these assays to the analysis of aflatoxins in fortified or real samples.

Acknowledgements

This project was supported by Program for Science & Technology Innovation Talents in Universities of Henan Province ([2011]1019), the Key Scientific & Technological Project in Henan Province of China (102102310328) and the Natural Science Research Program of Education Department in Henan Province of China (2010A230004).

References

[1]Devi, K. T., Mayo, M. A., Reddy, K. L., Delfosse, P., Reddy, G., Reddy, S. V. and Reddy, D. V. 1999. Production and characterization of monoclonal antibodies for aflatoxin B_1. Lett. Appl. Microbiol. **29**(5):284-288.

[2]Groopman, J. D. and Kensler, T. W. 2005. Role of metabolism and viruses in aflatoxin-induced liver cancer. Toxicol. Appl. Pharmacol. **206**(2):131-137.

[3]Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Luber, G. and Kieszak, S. 2005. Aflatoxin contamination of commercial maize products

during an outbreak of acute aflatoxicosis in eastern and central Kenya. Environ. Health Perspect. **113**(12):1763-1767.

[4]Liu, B. H., Yu, F. Y., Chan, M. H. and Yang, Y. L. 2002. The effects of mycotoxins, fumonisin B1 and aflatoxin B1, on primary swine alveolar macrophages. Toxicol. Appl. Pharmacol. **180**(3):197-204.

[5]Ghosh, R. C., Chauhan, H. V. and Jha, G. J. 1991. Suppression of cell-mediated immunity by purified aflatoxin B1 in broiler chicks. Vet. Immunol. Immunopathol. **28**(2):165-172.

[6]European Commission. Regulation 98/53/EC of 16 July 1998, laying down the sampling methods and the methods of analysis for the official control of the levels for certain contaminants in foodstuffs. Official Journal of the European Communities **L201**:93.

[7]Zhang, D., Li, P., Yang, Y., Zhang, Q., Zhang, W., Xiao, Z. and Ding, X. 2011. A high selective immunochromatographic assay for rapid detection of aflatoxin B1. Talanta **85**(1):736-742.

[8]Stroka, J. and Anklam, E. 2000. Development of a simplified densitometer for the determination of aflatoxins by thin-layer chromatography. J. Chromatogr. A. **904**(2):263-268.

[9]Gómez-Catalán, J., Piqué, E., Falcó, G., Borrego, N., Rodamilans, M. and Llobet, J. M. 2005. Determination of aflatoxins in medicinal herbs by HPLC. An efficient method for routine analysis. Phytochem. Anal. **16**(3): 196-204.

[10]Zöllner, P. and Mayer-Helm, B. 2006. Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography-atmospheric pressure ionisation mass spectrometry. J. Chromatogr. A. **1136**(2):123-169.

[11]Kolosova, A. Y., Shim, W. B., Yang, Z. Y. and Eremin, S. A. 2006. Direct competitive ELISA based on a monoclonal antibody for detection of aflatoxin B_1. Stabilization of ELISA kit components and application to grain samples. Anal. Bioanal. Chem. **384**(1):286-294.

[12]Arduini, F., Errico, I., Amine, A., Micheli, L., Palleschi, G. and Moscone, D. 2007. Enzymatic spectrophotometric method for aflatoxin B detection based on acetylcholinesterase inhibition. Anal. Chem. **79**(9):3409-3415.

[13]Paknejad, M., Javad Rasaee, M., Mohammoejad, J., Pouramir, M., Rajabibazl, M. and Kakhki, M. 2008. Development and characterization of enzyme-linked immunosorbent assay for aflatoxin B1 measurement in urine sample using penicillinase as label. J. Toxicol. Sci. **33**(5):565-573.

[14]Zhang, L., Jiang, J. Q., Li, G. L., Wang, Z. L., Yang, X. F., Ma, J. Y., Zhang, H. T., Huang, H. G. and Liu, X. Y. 2011. Development and optimization of a monoclonal antibody-based indirect competitive ELISA for detecting fluoroquinolone residue in milk. J. Food Agric. Environ. **9**(2):113-120.

[15]Li, C. Y. and Jiang, J. Q. 2012. Preparation of a polyclonal antibody based heterologous indirect competitive ELISA for detecting ractopamine residue. J. Food Agric. Environ. **10**(2):1349-1352.

[16]Jiang, J., Wang, Z., Zhang, H., Zhang, X., Liu, X. and Wang, S. 2011. Monoclonal antibody-based ELISA and colloidal gold immunoassay for detecting NT residue in animal tissues. J. Agric. Food Chem. **59**(18):9763-9769.