



# Micro-plate chemiluminescence enzyme immunoassay for aflatoxin B1 in agricultural products

Luqiu Fang<sup>a,b</sup>, Hui Chen<sup>a</sup>, Xitang Ying<sup>c</sup>, Jin-Ming Lin<sup>a,\*</sup>

<sup>a</sup> The Key Laboratory of Bioorganic Phosphorus Chemistry & Chemical Biology, Department of Chemistry, Tsinghua University, Beijing 100084, China

<sup>b</sup> Department of Chemical Engineering and Chemistry, Yangtze Normal University, Fuling 408100, China

<sup>c</sup> Beijing Chemclin Biotech Co., Ltd., Beijing 100094, China

## ARTICLE INFO

### Article history:

Received 15 November 2010

Received in revised form

27 December 2010

Accepted 11 January 2011

Available online 15 January 2011

### Keywords:

Chemiluminescence enzyme immunoassay

Horseradish peroxidase (HRP)

Aflatoxin B1

Agricultural products

## ABSTRACT

In this work, a micro-plate chemiluminescence enzyme immunoassay by antibody-coated for the determination of aflatoxin B1 (AFB1) in agricultural products has been established. Aflatoxin B1 antibody (AFB1-Ab) was adsorbed physically on polystyrene micro-plate hole as solid phase antibody, which took place immunity-reaction between antigen and antibody with AFB1 standard solution or samples by direct competition. Luminol–hydrogen peroxide chemiluminescence system catalyzed by horseradish peroxidase (HRP) with *p*-iodophenol enhancement was used as signal detecting system. The effects of several factors, including composition and pH of coating solution, dilution ratio and amount of antibody and enzyme labeled antigen, time of antibody-coating, incubation and chemiluminescence reaction, and other relevant variables upon the immunoassay were studied and optimized. The linear range of proposed method for AFB1 was 0.05–10.0 ng g<sup>-1</sup> with a correlative coefficient of –0.9997. The sensitivity of the proposed method was 0.01 ng g<sup>-1</sup>. The RSDs of intra- and inter-assay were less than 12.2% and 10.0%, respectively. This method has been successfully applied to the evaluation of AFB1 in agricultural products with recoveries of 79.8%, 101.9% and 115.4% for low, middle and high concentration samples, respectively. It shows a good correlation with the commercial available ELISA kit for AFB1 with correlative coefficient of 0.9098 indicating that the established CLEIA method can be used to determine AFB1 in real samples.

© 2011 Elsevier B.V. All rights reserved.

## 1. Introduction

Aflatoxins (AF) are toxic compounds which are produced as secondary metabolites by the fungi of *Aspergillus flavus* and *Aspergillus parasiticus* growing on a variety of agricultural products [1]. It was first discovered and confirmed in the study of Britain “X” disease about died turkeys in 1969 [2]. Aflatoxins is a group of compounds containing molecular structure of a coumarin and a double-furan ring. These toxins are known to be carcinogenic, mutagenic, teratogenic and immunosuppressive. Among the 18 identified aflatoxins, the major ones are AFB1, AFB2, AFG1 and AFG2. In 1993, IARC of WHO classified AFB1 as Group 1 (carcinogenic to humans) [1,3]. AFB1 can cause chromosome aberration and DNA damage to animal and plant [4]. AFB1 exists widely in agricultural products, such as peanuts, corn, soybean, and rice, which can enter animal or human food chain and result in food safety problems. Therefore, developing a detection method for AFB1 in agricultural products with specificity and sensitivity, high

throughput, rapidity and simpleness has great practical significance.

Because of its wide existence, high toxicity and serious harmness, people have established various methods for the analysis of AFB1 including mainly chromatography, chromatography–mass spectrometry, fluorescence spectrometry and immunochemistry methods. In chromatography method, there are thin-layer chromatography (TLC) [5,6], high performance liquid chromatography (HPLC) [7,8], solid phase micro-extraction combined with high performance liquid chromatography (SPME–HPLC) [9], and ion mobility chromatography (IMS) [10]. Methods of chromatography tandem mass spectrometry contain liquid chromatography–mass spectrometry (LC/MS/MS) [11,12] and ultra high performance liquid chromatography tandem mass spectrometry (UHPLC/MS/MS) [13]. Fluorescence spectroscopy (FS) was also used to detect AFB1 [14,15]. Immunochemical analysis includes enzyme-linked immunosorbent assay (ELISA) [16,17], electrochemical immunosensor method (ECIS) [1,18,19], array biosensor method (ABS) [20], time-resolved fluorescence immunoassay (TRFIA) [21] and so on. TLC is time consuming in sample preparation and easy to be interfered in purification. HPLC has high sensitivity and short analysis time for AFB1. The result is accurate.

\* Corresponding author. Tel.: +86 010 62792343; fax: +86 010 62792343.  
E-mail address: [jmlin@mails.tsinghua.edu.cn](mailto:jmlin@mails.tsinghua.edu.cn) (J.-M. Lin).

Chromatography–mass spectrometry can simultaneously detect multi-component with high sensitivity. But it requires expensive equipment and skillful operation technique. Fluorescence analysis is sensitive and rapid, but unstable and is difficult to distinguish AFB1 from other aflatoxins. ELISA method has characteristics of specific, sensitive and low cost, but the enzymatic activity affect on the color reaction by composition of medium and operating conditions. The chromogenic substrate is harmful to operators' health. Immunoaffinity column and its associated usage (IAC) is used for enrichment through binding between anti-AFB1 and AFB1 with high specificity [22–25]. But it cannot be used widely for the high cost of testing. Sensor method is affected by response time and unable to conduct rapid determination of AFB1 in bulk volume [26–28]. Chemiluminescence enzyme immunoassay (CLEIA) combines advantages of the specificity of immunoassay and the sensitivity of chemiluminescence with characteristics of simplicity and rapidity, high throughput and low cost instrument [29]. Over the last decade, CLEIA has been widely used in clinical diagnostic testing and environmental monitoring. However, CLEIA for the determination of AFB1 in agricultural products and food products has not been reported.

AFB1 is a small molecule without immunogenicity, and only has antigenicity that is immunoreactivity between antigen and antibody. In this paper, CLEIA based upon antibody coated format with low detection limit was designed for the determination of AFB1 in agricultural products. The proposed method used the antibody coating micro-plate as solid phase, and a horseradish peroxidase (HRP)–luminol– $\text{H}_2\text{O}_2$  system with high sensitivity was introduced in the assay. Effects of several physicochemical parameters were investigated. The proposed method has been successfully applied to the determination of AFB1 in 15 agricultural samples and showed a good correlation compared with the commercially ELISA kit.

## 2. Materials and methods

### 2.1. Apparatus

BHP9504 micro-plate luminescence analyser was from Beijing Hamamatsu Technology Co., Ltd. (Beijing, China). DEM-3 automatic plate washer was purchased from Tuopu Analytical Instruments Co., Ltd. (Beijing, China). KJ-201C micro-oscillator was from Jiangsu Kangjian Medical Apparatus Co., Ltd. (Jiangsu, China). Sample injector bought from Dragon Medical Co., Ltd. was used in all experiments (Shanghai, China).

### 2.2. Reagents

The aflatoxin B1 (AFB1) standard sample was purchased from Sigma–Aldrich, Co. (039k4047, USA). The monoclonal anti-aflatoxin B1 (AFB1–Ab) was made by Sigma–Aldrich, Co. (098k4794, titer is 1:20,000, determination by ELISA method, USA). HRP–AFB1–BSA complex was prepared by Beijing Chemclin Biotech Co., Ltd. using the improved race infiltration labeling method. Aflatoxin B1–BSA (AFB1–BSA) was obtained from Sigma–Aldrich, Co. (109k4062, USA). AFB1 ELISA kit (96-well plate) was from BIOO Scientific Corp. (USA). Methanol (reagent for HPLC) was purchased from Tianjin 4 Friends of the Biomedical Technology Co., Ltd. (Tianjin, China). 1-Ethyl-(3-dimethylaminopropyl)-carbodiimide (EDC) was obtained from Beijing Chemical Reagent Co. (Beijing, China).

### 2.3. Buffers

Normal saline (physiological saline, NS, 0.9% sodium chloride solution) was used to wash the coated micro-plate. The 50 mmol  $\text{L}^{-1}$  carbonate buffer (CB, pH 9.6), 50 mmol  $\text{L}^{-1}$  phosphate buffer (PB, pH 7.4 and 8.0) and 46 mmol  $\text{L}^{-1}$  citric acid–citrate buffer

(pH 4.8) were used as AFB1–Ab coating solution. Blocking buffer was 50 mmol  $\text{L}^{-1}$  phosphate solution (PBS, pH 7.4) containing 1% BSA and 0.1% proclin-300. 50 mmol  $\text{L}^{-1}$  PBS (pH 7.4) with 2% BSA, 0.5% hydrolyzed glutin and 0.1% proclin-300 was used as the standard solution matrix. Washing solution was 10 mmol  $\text{L}^{-1}$  PBS (pH 7.4) with 0.05% Tween-20 (PBST). The chemiluminescence substrate was luminol, hydrogen peroxide and *p*-iodophenol solution.

### 2.4. Preparation of calibrators

5.0 mg AFB1 standard was dissolved in methanol to prepare 2.5 mg  $\text{g}^{-1}$  AFB1 solution. Serial dilutions of the stored solution with standard solution matrix were prepared for calibrators with concentrations of 0.05, 0.15, 0.5, 2.5, 7.5 and 10.0 ng  $\text{g}^{-1}$ , respectively. The prepared calibrators were stored at 4 °C.

### 2.5. Coating of AFB1–Ab on the micro-plate

Coating involves interaction between surface of solid phase and reagents in immunoassay. First, each well of the micro-plates was coated with 100  $\mu\text{L}$  (1:800) or 125  $\mu\text{L}$  (1:1000) of AFB1 antibody diluted with phosphate buffer solution (pH 8.0). The plate was allowed to stand sealed at 4 °C overnight. Then, the plate was washed by NS solution twice and was gently tapped against tissue paper to remove all fluid. After that, 350  $\mu\text{L}$  of BSA solution was added into each well and the plate was put at room temperature for 2–2.5 h in order to block the active sites. Subsequently, the solution in the well was aspirated and the plate was made dry. Finally, the plate was vacuumed and stored at 4 °C for further use.

### 2.6. Immunoassay procedure

50  $\mu\text{L}$  AFB1 calibrator or agricultural samples were added into each well of the coated micro-plates. Then 50  $\mu\text{L}$  diluted HRP–AFB1–BSA complex was added. After incubation with gently shaking at 37 °C for 1 h for the competition reaction, the micro-plate was washed five times with PBST washing solution. Finally, 100  $\mu\text{L}$  CL substrate was added into each well. The mixture was incubated for 5 min at room temperature in the dark, and the emitted photons were measured (expressed as relative light unit, RLU). Standard curves were obtained by plotting CL intensity against the logarithm of analyte concentration and fitted to the equation of  $\log Y - \log X$ . Principle diagram for the determination of AFB1 with CLEIA was illustrated in Fig. 1.

### 2.7. Sample preparation

The 2.0 g crushed agricultural products were extracted with 4 mL methanol–water, 40/60, for 2 h at 40 °C with shaking at speed of 150 rpm. Then the extracts were centrifuged at 4000 rpm for 10 min. The supernatant solution diluted with standard solution matrix by ratio of 1:5 was used for analysis.

## 3. Results and discussion

### 3.1. Optimization of coating solution

The composition and concentration of coating solution were important factors influencing the sensitivity of CLEIA. AFB1–Ab was diluted to 1:8000 with 46 mmol  $\text{L}^{-1}$  citrate buffer (pH 4.8), 50 mmol  $\text{L}^{-1}$  phosphate buffer (pH 7.4 and 8.0) and 50 mmol  $\text{L}^{-1}$  carbonate buffer (pH 9.6), respectively. The RLU was affected by different pH (shown in Fig. 2). It increased with increasing pH in the range of 4.8–8.0. The highest RLU was obtained when reaction time was 5 min and the HRP–AFB1–BSA was diluted with 50 mmol  $\text{L}^{-1}$  phosphate buffer (pH 8.0) by the ratio of 1:4000. RLU

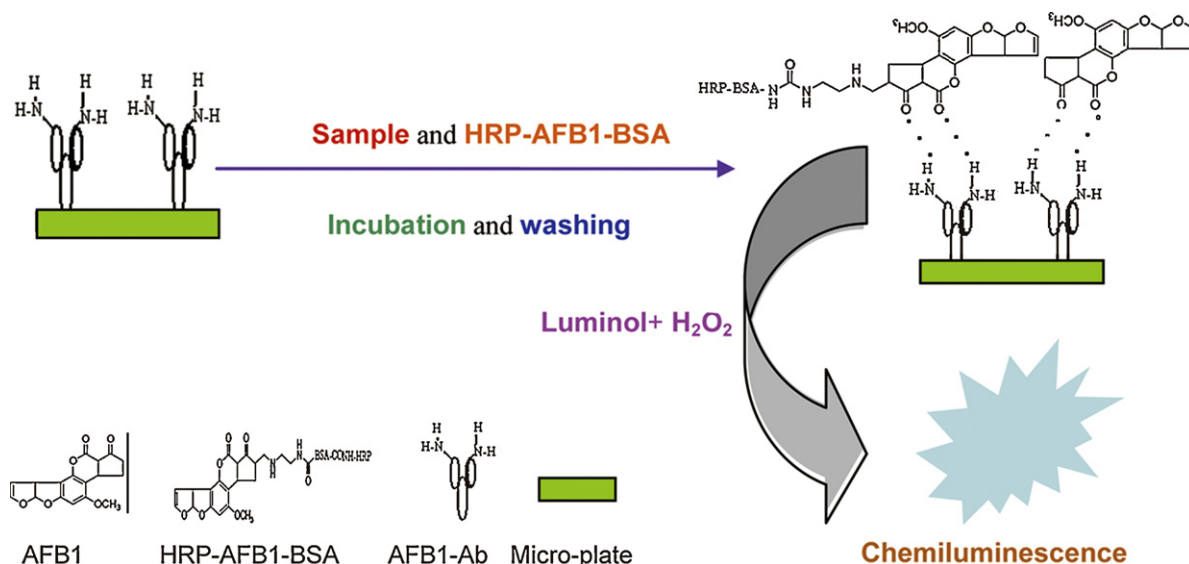


Fig. 1. Principle diagram for the determination of AFB1 with CLEIA method.

Table 1  
Effects of pH on standard curve.

pH	RLU <sub>50</sub>	RLU <sub>50.1/50</sub>	RLU <sub>510/50</sub>	Correlation coefficient (r)	Linear equation
4.8	379,722	0.9423	0.0761	−0.9931	Logit(Y) = 0.1076 − 2.5811Log(X)
7.4	413,084	0.9244	0.0481	−0.9984	Logit(Y) = −0.1698 − 2.6836Log(X)
8.0	433,734	0.8598	0.0403	−0.9941	Logit(Y) = −0.4595 − 2.4619Log(X)
9.6	107,879	0.7038	0.0402	−0.9582	Logit(Y) = −1.4999 − 2.2298Log(X)

decreased with the increasing reaction time. However, the lowest RLU was observed when 50 mmol L<sup>−1</sup> carbonate buffer (pH 9.6) was used as dilution buffer. It indicated that pH of coating solution was important for the reaction activity of AFB1-Ab. Dose-response curve drawn at different pH with the dilution ratio of 1:8000 for AFB1-Ab also showed that the highest linear correlation coefficient with RLU<sub>50.1/50</sub> < 90%, and RLU<sub>510/50</sub> < 15% was obtained when 50 mmol L<sup>−1</sup> phosphate buffer (pH 8.0) was used as coating solution (see Table 1). Therefore, 50 mmol L<sup>−1</sup> phosphate buffer (pH 8.0) was chosen as coating solution in the experiment.

### 3.2. Effects of dilution ratios of AFB1-Ab and HRP-AFB1-BSA

Chessboard titration was applied to optimize the dilution ratios of AFB1-Ab and HRP-AFB1-BSA. The RLU increased with the increasing concentration of AFB1-Ab (see Fig. 3a), and decreased with the increasing dilution ratio of HRP-AFB1-BSA (see Fig. 3b). Consideration of the sensitivity, reliability, kinetic range of assay, and volume of HRP-AFB1-BSA, the suitable dilution ratio of AFB1-Ab is 1:8000 and that of HRP-AFB1-BSA is 1:4000.

### 3.3. Optimization of the AFB1-Ab and HRP-AFB1-BSA volume

The volume of coating antibody and enzyme labeled antigen affected RLU and sensitivity of the CLEIA. Fig. 4a shows that RLU increased with the increasing volume of AFB1-Ab in the range of 25–125 μL, and decreased in the range of 125–200 μL. Effects of the volume of HRP-AFB1-BSA from 25 to 125 μL on RLU were tested. Results show that RLU increased with the increasing volume of HRP-AFB1-BSA (see Fig. 4b). In order to save reagents, 100 μL AFB1-Ab and 50 μL HRP-AFB1-BSA were chosen in the experiment.

### 3.4. Effect of coating and blocking time

Coating time will affect the number of antibody on the micro-plate. Blocking solution with BSA can reduce the non-specific binding sites. Fig. 5a shows that RLU increased with prolonging coating time in range of 20–22 h, and RLU began to decrease after 22 h. The excessive antibody caused increasing steric hindrance and reducing the binding chance of antigen and antibody. RLU increased with the blocking time of BSA in the range of 30–150 min (see Fig. 5b). However, RLU reduced when the blocking time was more than 150 min. The thick blocking layer increased steric effect. Con-

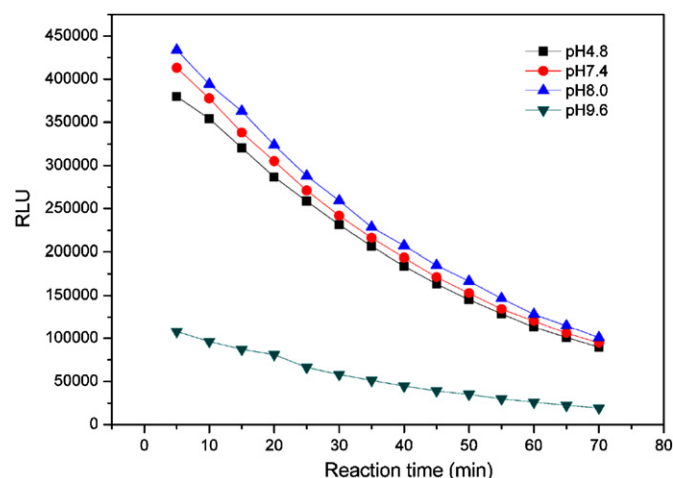


Fig. 2. Effects of coating buffer. The four curves correspond to different pH (i.e., citrate buffer 4.8, PB 7.4, 8.0, and CB 9.6).

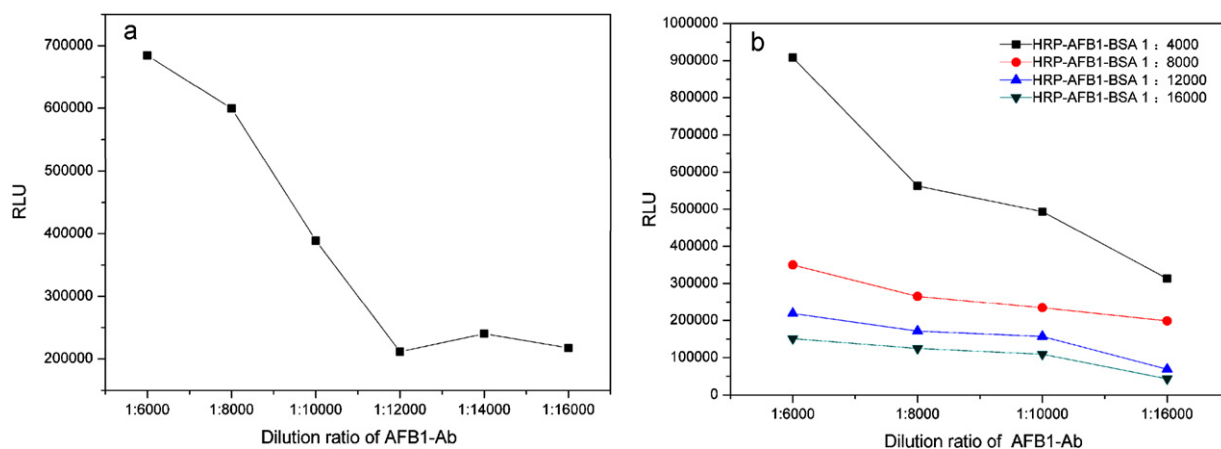


Fig. 3. Effects of dilution ratios of AFB1-Ab (HRP-AFB1-BSA is 1:4000, a) and HRP-AFB1-BSA (b) on CLEIA.

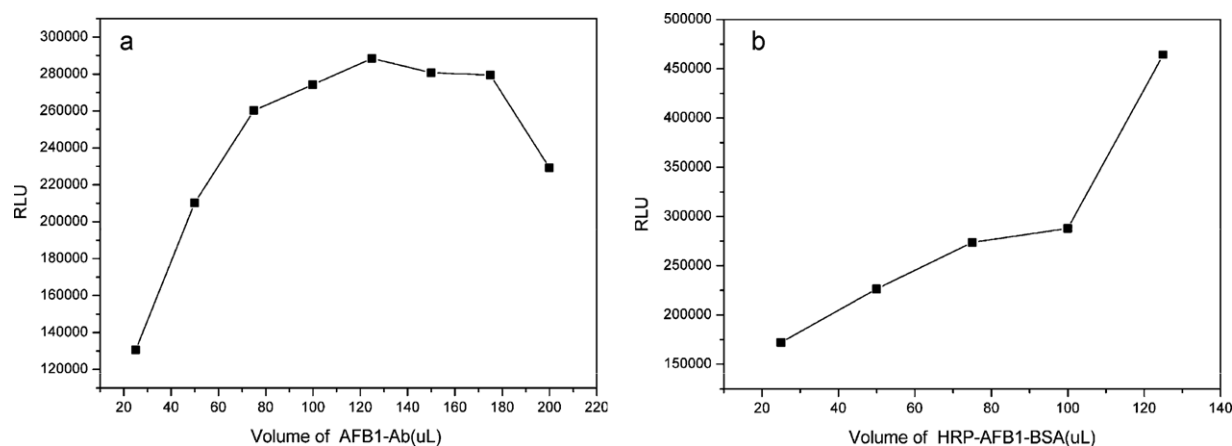


Fig. 4. Effects of the volume of AFB1-Ab (a) and HRP-AFB1-BSA (b) on CLEIA. The dilution ratios of AFB1-Ab and HRP-AFB1-BSA were 1:8000 and 1:4000, respectively.

sidering sensitivity and time-saving, the coating time was selected as 20–22 h, and blocking time was 120–150 min.

### 3.5. Optimization of incubation time

AFB1-Ab (1:8000) and HRP-AFB1-BSA (1:4000) diluted with 50 mmol L<sup>-1</sup> phosphate buffer (pH 8.0) were used to study the effect of incubation time on RLU at 37 °C. The results were shown

in Fig. 6 indicating that RLU increased linearly with the increasing incubation time. Effect of incubation time on AFB1 standard curve was shown in Table 2. RLU<sub>50</sub> increased with the increasing incubation time accompanying with the change of standard curve. Good correlation coefficient and high sensitivity were obtained when the incubation time was 60 min or 90 min. In order to save detection time and improve efficiency, 60 min was selected as the incubation time.

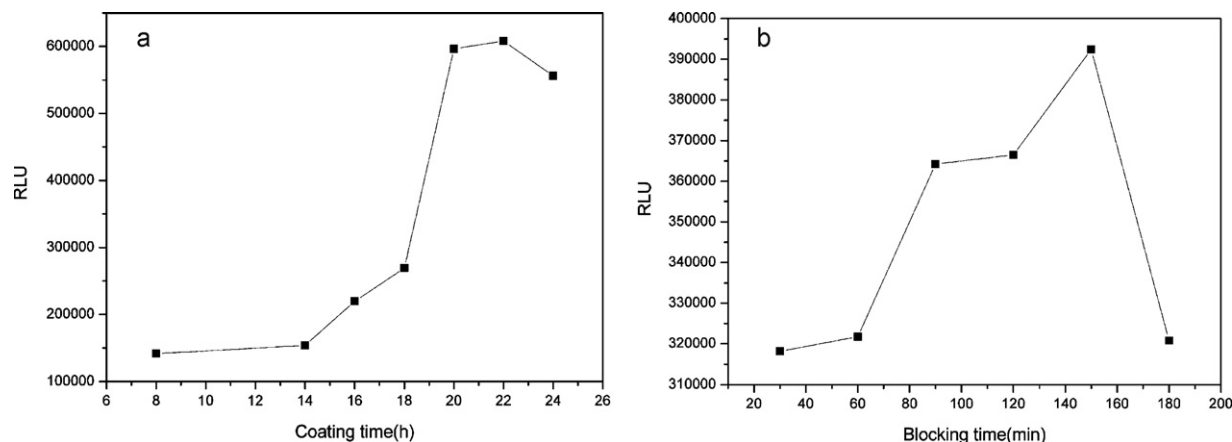


Fig. 5. Effects of coating time (a) and blocking time (b).

**Table 2**  
Effect of incubation time on standard curve.

Incubation time (min)	RLU <sub>50</sub>	RLU <sub>50.05/50</sub>	RLU <sub>510/50</sub>	r	Linear equation
15	38,103	0.9107	0.1304	−0.9761	Logit(Y) = 0.0428 − 1.8876Log(X)
30	128,017	0.9094	0.0805	−0.9624	Logit(Y) = −0.1166 − 1.9428Log(X)
60	364,861	0.8602	0.1247	−0.9833	Logit(Y) = −0.0320 − 1.6701Log(X)
90	492,601	0.9196	0.2001	−0.9892	Logit(Y) = 0.4349 − 1.7061Log(X)
120	728,984	0.9884	0.2455	−0.9826	Logit(Y) = 0.9141 − 2.2527Log(X)
150	816,671	0.8888	0.2304	−0.9817	Logit(Y) = 0.4998 − 1.4521Log(X)

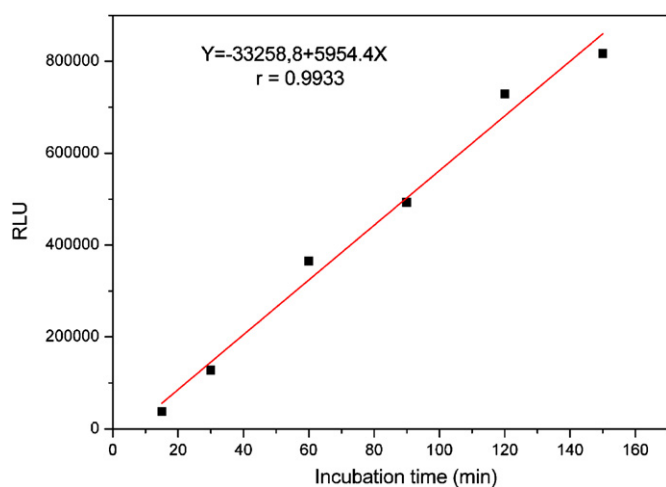


Fig. 6. Effect of incubation time.

### 3.6. Optimization of CL reaction time

In CLEIA, RLU changed with the CL reaction time, which will affect the detection sensitivity, linear correlation and range. HRP–AFB1–BSA with dilution of 1:4000 and AFB1–Ab with dilution of 1:8000 were used to study the effects of CL reaction time on RLU. The results shown in Table 3 indicated that RLU decreased with the increasing reaction time. When reaction time was in the range of 0–30 min, the linear range, sensitivity and correlation were suitable. Therefore, reaction time of 30 min was selected in the experiment.

### 3.7. Methodology evaluation

#### 3.7.1. Standard curve and sensitivity

Under the optimal conditions, dose–response curve for AFB1 was developed with the linear range of 0.05–10.0 ng g<sup>−1</sup>. The linear equation was Logit(Y) = 0.3451 − 2.3821 Log(X),  $r = -0.9997$ . The detection limit, defined as the minimal dose that can be distinguished from zero, the minimum detected concentration (mean − 2S.D. of zero standard, 10 replicates) of AFB1 was 0.01 ng g<sup>−1</sup>, which was lower than that of ELISA (kit of BIOO Sci-

entific Corp. with sensitivity of 0.05 ng g<sup>−1</sup> and linear range of 0.05–0.8 ng g<sup>−1</sup>).

#### 3.7.2. Precision

The intra- and inter-assay precisions, calculated by measuring AFB1 concentration in three different samples, were performed. Good precisions were obtained. The intra-assay coefficients of variation (CV) varied from 7.4% to 12.2% ( $n = 10$ ). The inter-assay coefficients of variation varied from 8.2% to 9.7%, were all less than 10.0% ( $n = 8$ ).

#### 3.7.3. Recovery

The proposed method was used to detect AFB1 in agricultural products and the accuracy was studied by recovery experiment. Different amounts of AFB1 standard was mixed with the agricultural samples before extraction procedure. The recovery experiment was repeated for five times and the average recoveries of low, middle and high concentration samples were 79.8%, 101.9% and 115.4%, respectively.

#### 3.7.4. Specificity

Specificity of immunoreaction should be one of the most important factors to evaluate the immunoassay and was usually represented by cross-reactivity. The lower the cross-reactivity, the more specific the antibody is, consequently the immunoassay becomes more reliable and acceptable. Five mycotoxins were selected for cross-reactive experiments to evaluate the specificity of AFB1–Ab by performing competitive assays, including vomitoxin (DON, deoxynivalenol), ochratoxin A (OTA), zearalenone (ZEA), AFB2 and AFG2. The AFB1 concentration causing 50% inhibition was used to calculate the cross-reactivity according to the equation:

$$\text{Cross-reactivity (\%)} = \frac{\text{concentration of AFB1 at IC}_{50}}{\text{concentration of cross-reactant at IC}_{50}} \times 100\%$$

The cross-reactive rates for DON, OTA, ZEA, AFB2 and AFG2 were 0.66%, 0.98%, 0.80%, 0.72% and 0.81%, respectively. They were all less than 1%, which were absolutely acceptable in the analysis.

#### 3.7.5. Stability

Several additives, such as BSA, hydrolyzed gluten and proclin-300, were used in the blocking buffer and standard solution matrix

**Table 3**  
Effect of CL reaction time on standard curve with 1:8000 dilution ratio of AFB1–Ab.

Time (min)	RLU <sub>50</sub>	RLU <sub>50.05/50</sub>	RLU <sub>57.5/50</sub>	r	Linear equation	Sensitivity (ng g <sup>−1</sup> )
5	506,400	0.9697	0.1365	−0.9974	Logit(Y) = 0.2710 − 2.4929Log(X)	0.2
10	462,092	0.9899	0.1353	−0.9899	Logit(Y) = 0.4014 − 2.8836Log(X)	0.28
20	372,562	0.9932	0.1301	−0.9836	Logit(Y) = 0.4266 − 3.0292Log(X)	0.28
30	291,883	0.9150	0.1230	−0.9951	Logit(Y) = −0.0442 − 2.0088Log(X)	0.1
40	233,877	0.9078	0.1177	−0.9908	Logit(Y) = −0.0488 − 2.0401Log(X)	0.06
50	179,036	0.9065	0.1073	−0.9921	Logit(Y) = −0.1248 − 2.0403Log(X)	0.09
60	142,825	0.9061	0.1044	−0.9891	Logit(Y) = −0.1053 − 2.0946Log(X)	0.06



**Table 4**

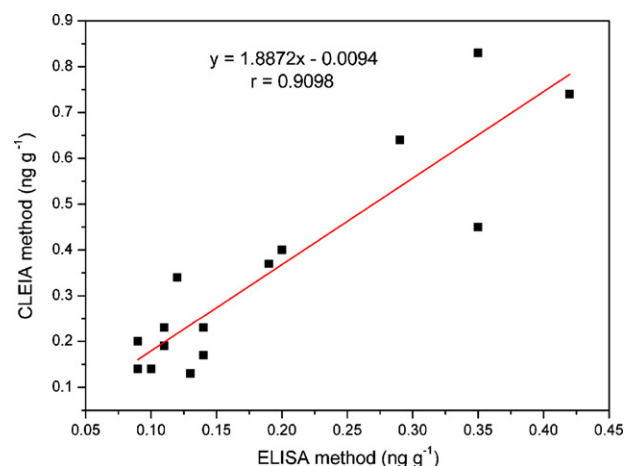
Stability test of the micro-plate, AFB1 standard solution and HRP antigen stored at 4 °C and 37 °C for different days.

Days	Temperature	RLU <sub>S0</sub>	RLU <sub>S0.05/S0</sub>	RLU <sub>S7.5/S0</sub>	<i>r</i>	Linear equation	AFB1 (ng g <sup>-1</sup> )
1	4 °C	359,174	0.8782	0.0930	−0.9942	Logit(Y) = −0.3977 − 1.9110Log(X)	0.98
	37 °C	146,965	0.8271	0.0464	−0.9917	Logit(Y) = −1.0916 − 1.9774Log(X)	0.47
3	4 °C	276,906	0.9698	0.1719	−0.9835	Logit(Y) = 0.4646 − 2.1794Log(X)	1.02
	37 °C	51,749	0.9123	0.0603	−0.9819	Logit(Y) = −0.318 − 2.4566Log(X)	0.32
5	4 °C	257,266	0.8972	0.0836	−0.9890	Logit(Y) = −0.42 − 1.9839Log(X)	0.67
	37 °C	27,935	0.8290	0.0856	−0.9922	Logit(Y) = −0.5938 − 1.8748Log(X)	0.77
7	4 °C	284,632	0.9133	0.1135	−0.9953	Logit(Y) = −0.2159 − 1.9269Log(X)	0.90
	37 °C	24,133	0.9662	0.1165	−0.9846	Logit(Y) = −0.1037 − 2.3051Log(X)	0.64
9	4 °C	235,151	0.9060	0.0985	−0.9856	Logit(Y) = −0.0976 − 2.0091Log(X)	0.96
	37 °C	19,784	0.8095	0.1663	−0.9902	Logit(Y) = −0.2721 − 1.3433Log(X)	0.28

for stabilizing purpose in the immunoreaction. Accelerated thermal degradation method was performed for the stability investigation, and the components of a kit, including solid phase coated antibody, HRP–AFB1–BSA complex, AFB1 standard solution and chemiluminescent substrate solution, were stored at 4 °C and 37 °C for 1, 3, 5, 7 and 9 days, respectively. Results in Table 4 showed that a little variation occurred at 4 °C, but greater changes took place at 37 °C for 9 days. According to the temperature experiment about kit stability, 1 day at 37 °C is equal to 60 days at 4 °C for stability [30], the components can be stored for more than one and a half years at 4 °C with good practicability.

### 3.7.6. Validity

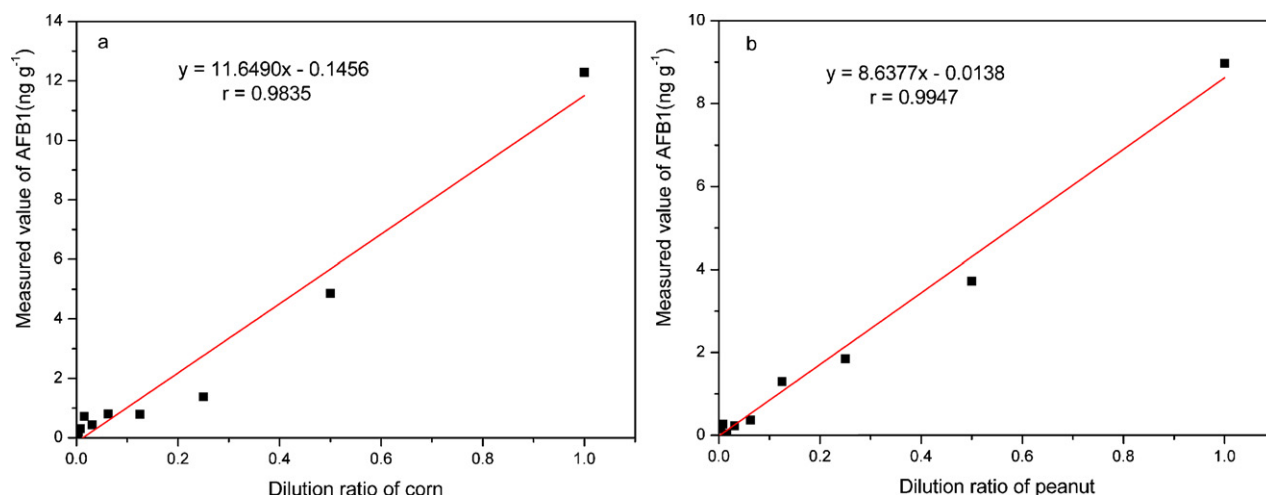
Dilution test was used to verify whether the calibrators had the same matrix effect with these samples to evaluate the reliability of the proposed assay. Extraction solution of corn and peanut samples were diluted with the matrix of AFB1 standard solution by ratio of 1:5, and AFB1 standard solution was added to obtain a solution containing 20 ng g<sup>-1</sup> of AFB1. A serial dilution of this solution was prepared in matrix of AFB1 standard solution by dilution ratio of 1/1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 and 1/512, respectively. Then the samples were analyzed using the proposed CLEIA. X axis represents the dilution ratios, and Y axis represents the measured concentration of AFB1. The linear equation for corn was  $Y = 11.6490 X - 0.1456$ ,  $r = 0.9835$ , and for peanuts was  $Y = 8.6377 X - 0.0138$ ,  $r = 0.9947$  (shown in Fig. 7a and b). The good linear correlation suggested that there was no matrix effect between the sample and the standard solution in the case of high concentration of AFB1. It shows that results obtained by this CLEIA were reliable.



**Fig. 8.** Correlation between results measured by the proposed CLEIA and ELISA. The equation for the regression line is:  $Y = 1.8872 X - 0.0094$  ( $r = 0.9098$ ,  $n = 15$ ).

### 3.8. Sample analysis

The proposed CLEIA was applied to evaluate AFB1 in 15 agricultural products, including wheat, corn, peanuts, soybeans, millet and others. Samples were prepared as described in Section 2.7. The results were compared with those obtained by a commercial ELISA kit. There was a good correlation between the proposed method and ELISA with a satisfied coefficient of 0.9098 (shown in Fig. 8), indicating that the proposed CLEIA was comparable and acceptable, and could be satisfactorily employed in real sample detection.



**Fig. 7.** Dilution experiments of corn (a) and peanut samples (b).

#### 4. Conclusion

A sensitive, specific, accurate, stable and rapid CLEIA was developed for the determination of AFB1 in agricultural products, using *p*-iodophenol enhanced HRP–luminol–H<sub>2</sub>O<sub>2</sub> CL system. AFB1 and HRP–AFB1 competitively react with AFB1–Ab coated on microplates. The determination range was 0.05–10.0 ng g<sup>−1</sup>, the cross reaction rates of DON, OTA, ZEA, AFB2 and AFG2 were less than 1%, the average recovery was 79.8–115.4%, and the sensitivity was 0.01 ng g<sup>−1</sup>, which was one-fifth of ELISA. It has been successfully applied for the determination of AFB1 in 15 agricultural products and the results correlated well with the accredited ELISA method. In conclusion, this proposed assay provided apparent advantages over ELISA, and facilitated the development of high-throughput screening and automated operation systems in the market.

#### Acknowledgement

This work was supported by National Nature Science Foundation of China (No. 90813015).

#### References

- [1] N.H.S. Ammida, L. Micheli, G. Palleschi, *Anal. Chim. Acta* 520 (2004) 159–164.
- [2] E. Papp, K. H-Otta, G. Záray, E. Mincsovcis, *Microchem. J.* 73 (2002) 39–46.
- [3] P. Li, Q. Zhang, W. Zhang, *Trends Anal. Chem.* 28 (2009) 1115–1126.
- [4] X. Sun, Z. Wang, Y. Fang, P. Chen, Z. Li, *Chin. J. Anal. Chem.* 38 (2010) 245–248.
- [5] G. Li, J. Liu, *Chin. J. Health Lab. Technol.* 5 (1995) 67–69.
- [6] I. Var, B. Kabak, F. Gök, *Food Control* 18 (2007) 59–62.
- [7] S.M. Herzallah, *Food Chem.* 114 (2009) 1141–1146.
- [8] Z. Fu, X. Huang, S. Min, *J. Chromatogr. A* 1209 (2008) 271–274.
- [9] E. Calleri, G. Marrubini, G. Brusotti, G. Massolini, G. Caccialanza, *J. Pharm. Biomed. Anal.* 44 (2007) 396–403.
- [10] A. Sheibani, M. Tabrizchi, H.S. Ghaziaskar, *Talanta* 75 (2008) 233–238.
- [11] M. Ventura, A. Gómez, I. Anaya, J. Díaz, F. Broto, M. Agut, L. Comellas, *J. Chromatogr. A* 1048 (2004) 25–29.
- [12] A. Bacaloni, C. Cavaliere, F. Cucci, P. Foglia, R. Samperi, A. Laganá, *J. Chromatogr. A* 1179 (2008) 182–189.
- [13] B. Huang, Z. Han, Z. Cai, Y. Wu, Y. Ren, *Anal. Chim. Acta* 662 (2010) 62–68.
- [14] M. Aghamohammadi, J. Hashemi, G.A. Kram, N. Alizadeh, *Anal. Chim. Acta* 582 (2007) 288–294.
- [15] J. Hashemi, G.A. Kram, N. Alizadeh, *Talanta* 75 (2008) 1075–1081.
- [16] A. Aydin, M.E. Erkan, R. Baokaya, G. Ciftcioglu, *Food Control* 18 (2007) 1015–1018.
- [17] M. Pesavento, S. Domagala, E. Baldini, L. Cucca, *Talanta* 45 (1997) 91–104.
- [18] Y. Tan, X. Chu, G. Shen, R. Yu, *Anal. Biochem.* 387 (2009) 82–86.
- [19] N. Gan, D. Xie, R. Li, T. Li, W. Wen, *J. Chin. Cereals Oils Assoc.* 24 (2009) 124–128.
- [20] K.E. Sapsford, C.R. Taitt, S. Fertig, M.H. Moore, M.E. Lassman, C.M. Maragos, L.C. Shriver, *Biosens. Bioelectron.* 21 (2006) 2298–2305.
- [21] B. Huang, J. Zhang, Z. Ma, Y. Zhang, L. Zhu, J. Jin, M. Xie, *J. Hygiene Res.* 38 (2009) 385–388.
- [22] Y.I. Goryacheva, S.D. Saeger, B. Delmulle, M. Lobeau, S.A. Eremin, I. Barna-Vetró, C.V. Peteghem, *Anal. Chim. Acta* 590 (2007) 118–124.
- [23] M. Ardic, Y. Karakaya, M. Atasever, H. Durmaz, *Food Chem. Toxicol.* 46 (2008) 1596–1599.
- [24] W.S. Khayoon, B. Saad, C.B. Yan, N.H. Hashim, A.S.M. Ali, M.I. Salleh, B. Salleh, *Food Chem.* 118 (2010) 882–886.
- [25] S. Ip, C. Che, *J. Chromatogr. A* 1135 (2006) 241–244.
- [26] F. Zhao, Y. Jiao, B. Lian, J. Liu, *Guizhou Agric. Sci.* 34 (2006) 123–126.
- [27] J. Tang, G. Pang, *Food Sci.* 30 (2009) 326–329.
- [28] C. Zhang, T. Yue, Z. Gao, J. Wang, *Acad. Period. Farm Prod. Process.* 142 (2008) 18–26.
- [29] L.J. Kricka, *Anal. Chim. Acta* 500 (2003) 279–286.
- [30] L.G. Xu, *J. Radioimmunol.* 19 (2006) 230–234.