Talanta 82 (2010) 1113–1121

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A sensitive immunoassay based on direct hapten coated format and biotin–streptavidin system for the detection of chloramphenicol

Na Sai[a,1], Yiping Chen[b,1], Nan Liu[c], Guanggui Yu[d], Pu Su[c], Yi Feng[b], Zhijiang Zhou[a], Xiaoyu Liu[b], Huanying Zhou[c], Zhixian Gao[b,c,*], Bao an Ning[c,**]

[a] School of Chemical Engineering and Technology, Tian Jin University, Tianjin 300072, China
[b] College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, China
[c] Tianjin Key Laboratory of Risk Assessment and Control Technology for Environment & Food Safety; Institute of Hygiene and Environmental Medicine, Tianjin 300050, China
[d] College of Bioscience and Biotechnology, YangZhou University, JiangSu 225009, China

ARTICLE INFO

Article history:
Received 4 February 2010
Received in revised form 5 June 2010
Accepted 10 June 2010
Available online 23 June 2010

Keywords:
Polymerized glutaraldehyde
Biotin–streptavidin system
Immunoassay
Chloramphenicol

ABSTRACT

A novel enzyme liked immunosorbent assay (ELISA) was developed for the detection of chloramphenicol (CAP). In this assay, the small molecular hapten (Hap) was directly coated on the surface of microtiter plates and biotin–streptavidin system (BSAS) was employed to improve the sensitivity of immunoassay (BSAS-direct Hap coated ELISA). The surface of microtiter plates was treated with glutaraldehyde (GA) polymer network to introduce aldehyde group, which was used to cross-link with amino group of CAP. Compared with conventional ELISA (the plates were coated with Hap–carrier protein conjugates), the modified plates presented significantly high antibody and antigen (Ab–Ag) affinity and showed excellent stability. And then the biotinylated monoclonal antibody (mAb) and HRP-labeled streptavidin were employed in this assay for amplification of signals. The sensitivity of BSAS-direct Hap coated ELISA was increased by approximately 20-folds and the stability was also improved greatly compared to conventional ELISA. Its 50% inhibition concentration (IC_{50}) for CAP was 10.5 ng mL^{-1} and the limit of detection (LOD) was 0.2 ng mL^{-1} after optimization of reaction conditions. To our knowledge, this was one of the most sensitive immunoassay for CAP yet reported. In sample analysis, the results of CAP detected by this assay were in accordance with which obtained by conventional ELISA and high performance liquid chromatography (HPLC). Therefore, it is an attractive alternative compared to conventional immunoassays in routine supervision for residue detection in food and environment.

1. Introduction

CAP is an effective broad-spectrum antibiotic which was widely used in both human and poultry medicine. However, many countries and organizations have prohibited the use of CAP for the treatment of food-producing animals due to its toxic and allergic reactions such as bone marrow suppression, "gray baby syndrome", aplastic anemia and acute leukemia [1–3]. The maximum residue levels (MRLs) have also been legislated as zero tolerance [4,5]. Thus, it is critical to monitor CAP levels in patients' blood and food products. There have been several methods employed for the detection of CAP including microbiological [6,7], chromatographic [8,9], enzymatic [10], immunological methods [11,12], biosensors and microarray technologies [13]. Especially, enzyme linked immunosorbent assay (ELISA) is recognized as a valuable tool in veterinary residual analysis and a complements conventional analytical method due to its rapid, sensitive, cheap and selective features [14,15].

The format of competitive ELISA was widely employed to detect small molecular hapten (Hap) in food and environment samples. In this format, the low-molecular-weight Hap do not have multi-epitopes, they should be conjugated with carrier protein when immobilized on the surface of microtiter plates [16,17]. However, this format has many disadvantages: firstly, the ratio of Hap and carrier protein is always inconsistent and non-reproducible during the preparation of the conjugate, thus it is unfavorable to make assay standardization and evaluate Hap–protein stoichiometry [18]. Secondly, immobilization of Hap–protein conjugate on the surface depends on hydrophobic interactions between Hap–carrier protein conjugate and the surface of plates. This inter-

* Corresponding author at: College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, China. Tel.: +86 022 84655191; fax: +86 022 84655191.
** Corresponding author. Tel.: +86 022 84655403; fax: +86 022 84655191.
E-mail addresses: gaozhx@163.com (Z. Gao), ningba@163.com (B.a. Ning).
[1] These authors contributed equally to this work.

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.06.018

action often involves significant conformational changes which lead carrier protein easily to screen small molecular Hap, thus causing unsuitable presentation and orirentation of the Hap [19].

In order to avoid these drawbacks, the low-molecular-weight Hap would be attached directly to surface of microtiter plates. The surface of microtiter plates can be pre-activated and modified with different functional groups such as hydroxyl, amino, carbonyl, and carboxlyl, these functional groups could link Hap through covalent linkage. The polystyrene surface has been successfully modified by 3-aminopropyltriethoxysilane and bisphenol A was directly linked to the surface in our previous work [20]. There have also been some reports that macromolecules and antibodies were linked to microtiter plates by GA network [21,22].

The biotin–streptavidin system (BSAS), a signal amplification system, has been widely applied in immunohistochemistry and immunoassay for its high specificity and strong affinity [23]. Streptavidin (SA) contains four binding sites with an excessively strong affinity for the small molecule biotin. Moreover, biotin which enables a solid binding between the proteins and SA can be easily covalently coupled to proteins [24].

In this paper, we have developed a highly sensitive BSAS-direct Hap coated ELISA for CAP. In this assay GA network was applied to introduce reactive aldehyde groups binding amino-CAP on the surface of microtiter plates and BSAS was utilized to enhance the efficiency of Ab–Ag reaction. Furthermore, the detected amounts of CAP were also confirmed by the conventional ELISA and HPLC. As far as we concerned, it has not been reported that the immunoassay combining direct Hap coated format with BSAS was applied to detect CAP or other relevant veterinary drug residues.

2. Materials and experimental

2.1. Reagents

The monoclonal antibody against CAP (mAb) was purchased from Abcam, UK. *N*,*N*-dimethylformamide (DMF), CAP, chloramphenicol succinate (CAP succinate), streptomycin, penicillin, tetracycline, dicyclohexylcarbodiimide (DCC), bovine serum albumin (BSA) and ε-amino-*n*-caproic-acid (Ac) were purchased from Sigma (St. Louis, MO, USA). HRP–streptavidin (HRP–SA) conjugate was supplied by Bioworld Technology Inc., Beijing. Biotinyl-*N*-hydroxysuccinimideester (BNHS) and N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) were obtained from Pierce, USA. Common solvents (including GA) and salts were purchased from Tianjin Regent Corp. (Tianjin, China). The water used was prepared by Milli-Q system (Millipore, Bedford, MA). Coating buffer was 0.05 M carbonate buffer (pH 9.6). Washing buffer PBST was 0.01 M phosphate-buffered saline (PBS) with 0.05% Tween 20 (pH 7.4).

2.2. Apparatus

The 96 wells ELISA microtiter plates (low affinity) were obtained from Wanger Biotech Co. (Beijing, China). A Multiskan MK3 ELISA reader (Thermo, USA) was applied for the determination of absorbance. High performance liquid chromatography (HLPC) system (2695, Waters, USA) was employed for detection of shrimp samples. CAP–protein conjugate was determined by DU® 530 spectrophotometer (Beckman, USA) and Reflex III MALDI-TOF-Mass Spectrum (Bruker Inc., Germany). 6930 centrifuge (KUBOTA Inc., Japan) and MS3 digital orbital shaker (IKA Inc., Germany) were used for the preparation of CAP–protein conjugate and amino-CAP. Biotinylated monoclonal antibody (biotinylated-mAb) was separated and purified by YM-10 columns (Millipore, USA).

2.3. Preparation of CAP–BSA conjugate, amino-CAP and biotinylated-mAb

2.3.1. CAP–BSA conjugate preparation

Preparation of CAP–BSA conjugate as coating protein was carried out by previous method [25] with some modifications. Due to the absence of carboxyl groups in CAP, CAP succinate was selected to prepare the CAP–BSA conjugate. The conjugating mechanism of CAP succinate and BSA was shown in Fig. 1.

Briefly, CAP succinate (17.8 mg) together with sulfo-NHS (4.6 mg) and DCC (8.24 mg) were dissolved in 2 mL of DMF at 25 °C and strirred for 24 h at room temperature (RT), and then centrifuged at 12,000 r/min to remove precipitate. The obtained Hap solution was added dropwise to BSA solution (BSA: 100 mg in 0.05 M phosphate buffer saline at pH 8) by BSA:CAP succinate molar ratio equaled to 1:40. The mixture was stirred at 4 °C overnight and then dialysed (the pore size of membrane was 8000 Da) against PBS (0.01 M, pH 7.4, 0.9% NaCl) for 4 days. After preparation, CAP–BSA conjugates were primary identified by UV–vis spectrophotometer, and then the molar ratio of CAP/BSA in conjugate was determined by mass spectrum (MS).

Assuming that the molar absorptivity of Hap was the same for the free and conjugated forms, the Hap desities i.e. coupling ratios (the number of Hap molecules per molecule of protein) of the conjugate could be approximately estimated directly by the mole absorbance ε [26] shown in Eq. (1).

$$\text{Coupling ratio} = \frac{\varepsilon_{\text{conjugate}} - \varepsilon_{\text{BSA}}}{\varepsilon_{\text{Hap}}} \quad (1)$$

At the same time, the coupling ratios of the conjugate also could be measured by MS shown in Eq. (2).

$$\text{Coupling ratio} = \frac{\text{MW}_{\text{conjugate}} - \text{MW}_{\text{BSA}}}{\text{MW}_{\text{Hap}}} \quad (2)$$

2.3.2. Synthesis of amino-CAP

CAP (64.6 mg) was dissolved in absolute ethyl alcohol (700 μL) and zinc powder (29.2 mg) was added. The solution was heated at 80 °C for 40 min and then centrifuged at 12,000 r/min to extract the yellow supernatant. The obtained solution was purified by liquid extraction. The result was monitored by thin-layer chromatography (TLC). Furthermore, NH_2 group from amino-CAP can react with COOH group of BSA. In this study, amino-CAP was linked to BSA to confirm the successful reduction of NO_2 to NH_2 and the amino-CAP–BSA conjugate was characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Amino-CAP–BSA conjugate was formed according to previous report with some modifications [27].

2.3.3. Preparation of biotinylated-mAb

BNHS was diluted to 1 mg mL^{-1} by DMF and mAb was diluted to 1 mg mL^{-1} by 0.1 g L^{-1} $NaHCO_3$, pH 9.6. The two solutions were mixed with the ratio 1:10 by volume and stirred for 3–4 h for biotinylation of mAb. After labeling, excessive unreacted biotin and ions in aqueous solution were removed by YM-10 column, and the biotin-labeled mAb was diluted with 10 mM PBS, pH 7.2. And then, it was aliquot and stored in −20 °C until use.

2.4. The process of BSAS-direct Hap coated ELISA

Microtiter plates were coated with Hap molecules directly following the previous report [28,29] with some modifications. As shown in Fig. 2, microtiter plates were pretreated with a dilution of GA in carbonate buffer (0.05 M, pH 9.6) at 37 °C for 2 h. After washing plates 3 times with PBST buffer, an optimized dilution of amino-CAP in PBS (0.05 M, pH 8.0) was incubated at 37 °C for 2 h.

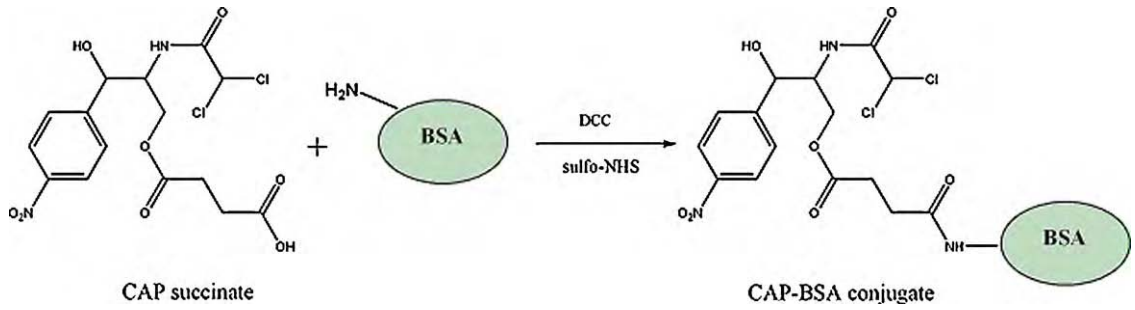

Fig. 1. The conjugating mechanism of CAP succinate and BSA.

The plates were then washed with PBST buffer thoroughly. Incubating a selected solution of blocking buffers in PBS buffer (pH 8.0) at 37 °C for 1 h, the remaining binding sites of the GA-network were blocked. The plates were then treated with 50 μL optimized specific biotinylated-mAb and 50 μL different concentration of free CAP (100, 50, 25, 10, 1, 0.1, 0.02 and 0 ng mL^{-1}) for 1.5 h at 37 °C. After incubation, the plates were washed thoroughly with PBST. Then 100 μL of HRP–SA in the dilution buffer (0.01 M PBS, pH 7.4, containing the optimized dilution of BSA) was added into each well of the plates. The plates were incubated for 30 min at 37 °C and washed with PBST for 6 times, and then 100 μL tetramethylbenzidine substrate system was added into each well and incubated for 10 min at 37 °C in dark. Absorbance at 450 nm was determined in an ELISA reader. The data analysis was performed by normalizing the absorbance with the following Eq. (3).

$$\frac{B}{B_0}(\%) = \frac{A - A_{ex}}{A_0 - A_{ex}} \times 100 \quad (3)$$

where A: absorbance of CAP at standard concentration, A_0: absorbance at zero CAP concentration and A_{ex}: aborbance at excess CAP concentration.

2.5. Conventional ELISA

Microtiter plates were also coated with CAP–BSA conjugate by adding 100 μL of the conjugate per well of microtiter plate and incubating at 4 °C overnight. The plates were then treated with 50 μL optimized specific mAb and 50 μL different concentration of free CAP (100, 50, 25, 10, 1, 0.1, 0.02 and 0 ng mL^{-1}) for 1.5 h at 37 °C. After incubation, the plates were washed with PBST thoroughly, 100 μL goat anti-mouse IgG-HRP conjugate at 1:3000 diluted in PBS was added into each well of the plates. The next step and date analysis were followed by Section 2.4.

2.6. Optimization of reaction parameters in immunoassay

2.6.1. Selection of proper concentration of GA, amino-CAP and species of blocking buffers

To optimize the conditions for coating "low affinity" ELISA plates, concentration of GA and amino-CAP were varied. Microtiter plates were pretreated with different concentration of GA (6%, 5.5%, 5% and 4.5%) in carbonate buffer (0.05 M, pH 9.6) for 2 h at 37 °C, and then coated by a series concentrations of amino-CAP (25–200 nmol mL^{-1}) for 2 h at 37 °C. The subsequent steps were as described in Section 2.4.

The suitability of BSA, skim milk power and Ac (0.1 mol L^{-1}in PBS, pH 8.0) were tested for blocking effectiveness.

2.6.2. Optimize the concentration of biotinylated-mAb and dilution level of HRP–SA conjugate

Chequerboard titration has been used for selecting optimum concentration of biotinylated-mAb and dilution level of HRP–SA conjugate in this assay. Based on Section 2.4, different concentration of biotinylated-mAb (10.4 ng mL^{-1}, 5.2 ng mL^{-1} and 2.6 ng mL^{-1}) and a serial of dilution level of HRP–SA conjugate (1:1500 and 1:3000) were observed.

2.7. Cross-reactivity

In our study, CR of mAb was evaluated by using of its analogues: CAP succinate, tetracycline, streptomycin and penicillin. The CR values were calculated according to Eq. (4).

$$\mathrm{CR}(\%) = \frac{\mathrm{IC}_{50}\ \text{of chloramphenicol}}{\mathrm{IC}_{50}\ \text{of analogues}} \times 100 \quad (4)$$

2.8. Shrimp sample preparation for analysis

CAP residue mainly exists in seafood [30]. In this assay, the shrimp samples were taken and analysed by immunoassay and HPLC methods. Shrimp samples A, B and C were collected from three local supermarkets in TianJin. They were taken with the following further treatment.

2.8.1. Sample preparation for immunoassay methods

Homogenized shrimp samples (2 g) was extracted with 4 mL ethyl acetate and shaken for 20 min at RT and centrifuged at 6000 r/min for 10 min. The supernatant was evaporated in a 40 °C water bath under a stream of nitrogen, and was dissolved with 1 mL isooctane–chloroform mixture (3:2, v/v) and 1 mL PBS (0.01 M, pH 7.4) buffer. Then the obtained mixture was shaken for 1 min and centrifuged at 3000 r/min for 10 min, the supernatant was extracted and collected for immunoassay analysis.

2.8.2. Sample preparation for HPLC method

Homogenized shrimp samples (2 g) was extracted with 4 mL of ethyl acetate and shaken for 20 min at RT and centrifuged at 6000 r/min for 10 min. The supernatant was evaporated in a 40 °C water bath under a stream of nitrogen, and was dissolved in 2 mL methanol for HPLC analysis.

2.8.3. Incurred samples for recovery and precise analysis

Shrimp samples which were free from CAP were added a series of amounts of CAP standard (5, 50 and 100 μg kg^{-1}), and then analyzed by both BSAS-direct Hap coated ELISA and conventional ELISA.

2.9. HPLC method

HPLC method was carried out by some reports [31,32]. The preceding extractive samples were prepared for analysis. HPLC instrument was used for the experiments and the chromatographic data were collected and processed by the empower software. The optimized chromatographic conditions were as follows:

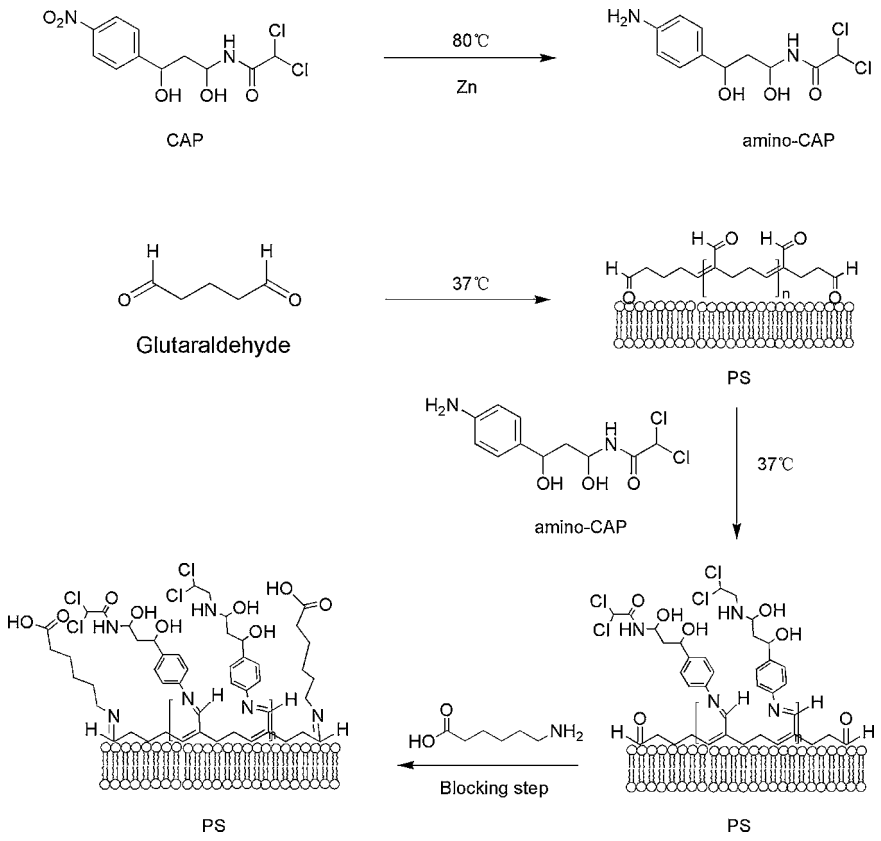

Fig. 2. Reaction mechanism of direct immobilization of amino-CAP on the surface modified with GA.

Column: C_{18} (250 mm × 4.6 mm, 5 μm)
Injection volume: 50 μL
Mobile phase:methanol–water (65:35, v/v)
Flow rate: 0.8 mL min^{-1} at RT
Detection wavelength: 278 nm.

3. Results and discussion

3.1. CAP–BSA conjugate and amino-CAP verification

As shown in Fig. 3(a), there were great differences in absorbance of UV spectra among CAP, BSA and CAP–BSA conjugate when three substances in the same concentration.

We could observe that CAP–BSA conjugate has maximum absorption peak wavelength in 276 nm which was similar with CAP. Furthermore, the result also indicated that absorption peak strength of CAP–BSA conjugate (1 mg mL^{-1}) in 276 nm was weaker than CAP (1 mg mL^{-1}) in the same wavelength. This was probably a consequence of different substances (CAP, CAP–BSA conjugate and BSA) have different absorbance ε, thus, different absorbance of UV spectra were obtained in the same concentration. So it could be primarily confirmed that the CAP–BSA conjugate were successfully prepared. Coupling ratio of CAP–BSA conjugate was 18:1 according to the Eq. (1). What is more, molecular weight (MWs) of CAP–BSA conjugate and BSA were 75,660 and 66,997 determined by MS as shown in Fig. 3(b) and the coupling ratio was 20:1 which was relatively close to the result of UV spectrum scanning.

The product of amino-CAP was verified by TLC. There was a new product generated as shown in Fig. 4(a), it could be primary proved that amino-CAP was successfully prepared. In the study, 12% separation gel was used and the markers were low molecular weight proteins ranging from 14 kDa to 97 kDa. As shown in Fig. 4(b), BSA moved further than amino-CAP–BSA conjugate because molecular weight of BSA was smaller than that of conjugate, it suggested that the conjugate was successfully prepared and CAP has been changed into amino-CAP.

3.2. Optimization of sensitivity of the assay

3.2.1. Effect of concentration of GA and amino-CAP

To optimize conditions for coating ELISA plates, dilutions of GA and amino-CAP concentration were modified. When the absorbance values A_0 and A_{ex} are highly close to the background value (A_{bg}), the values was from the plates which were not treated with GA (control experiment). It is indicated that the amino-CAP was not immobilized. As shown in Fig. 5, the maximum difference $A_0 - A_{ex}$ was achieved by use of a 6.0% dilution of GA, higher and lower dilutions of GA resulted in smaller $A_0 - A_{ex}$ difference. 6.0% GA was selected in the following experiments.

In Fig. 6(a), it appeared that OD_{450} value increased with increase of amino-CAP concentration, but run through a maximum value at approximately 1 μmol mL^{-1}.

It was indicated that amino-CAP was successfully prepared and immobilized on the surface modified by GA. However, it was noteworthy that A_0 signals showed a drop beyond 1 μmol mL^{-1} with increasing amino-CAP concentration. This extraordinary result may be explained by steric effects, which arising from the fact that each atom with a molecule occupies a certain amount of space (http://en.wikipedia.org/wiki/Steric_effects). If atoms are brought too close together, there is an associated cost in energy due to overlapping electron clouds, and this may affect the molecule preferred reactivity. In other words, the steric effects could influence reaction between the mAb and surface-immobilized hapten. When the amino-CAP concentration was beyond 1 μmol mL^{-1}, the mass of amino-CAP affected each other to generate conformational changes

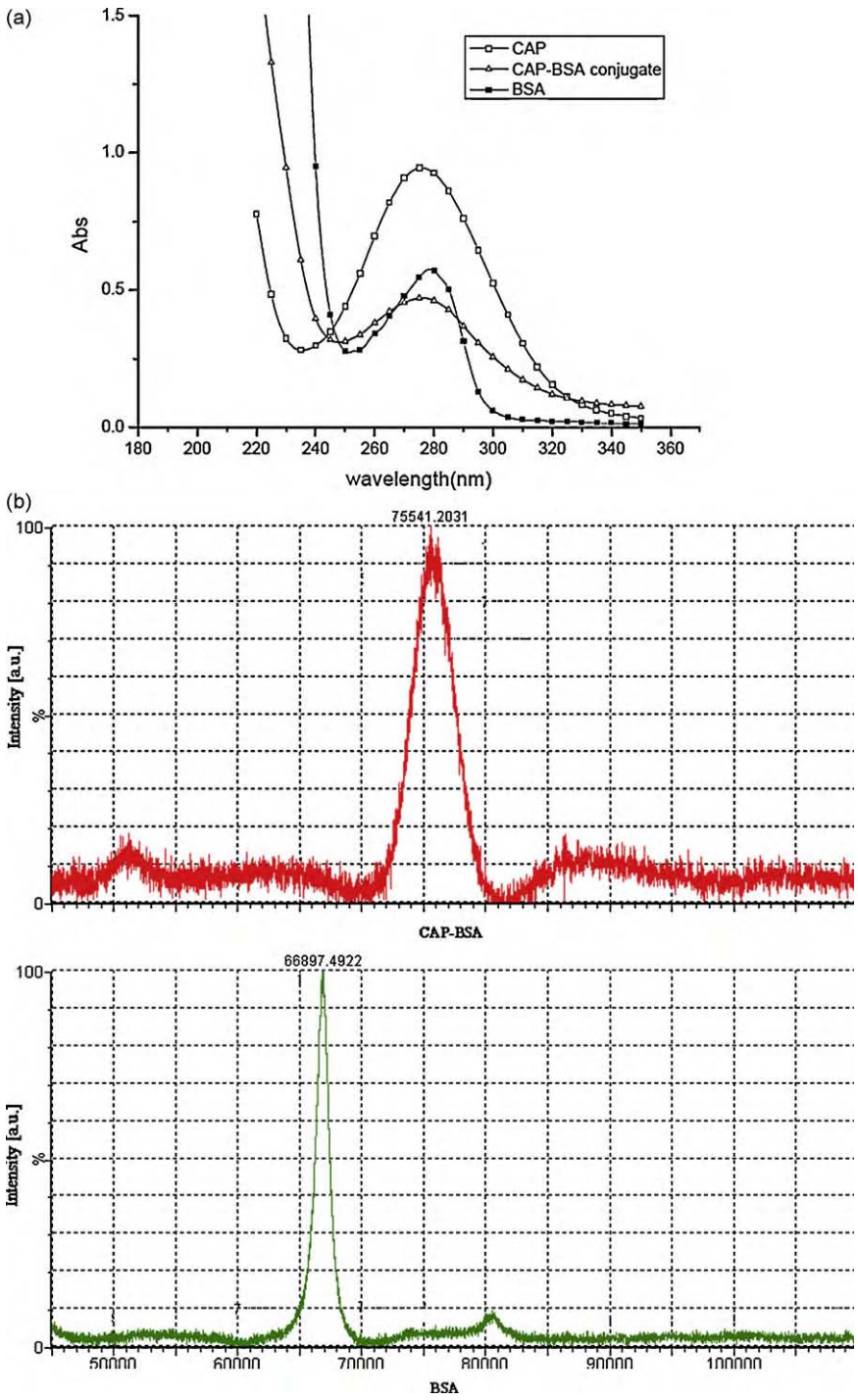

Fig. 3. (a) UV spectrograms of CAP, CAP–BSA conjugate and BSA. (b) Mass spectrograms of CAP and CAP–BSA conjugate.

which resulted in unfavorable recognization between mAb and CAP. On the other hand, the new binding mechanism from univalent to bivalent might also be a factor [20].

From 25 nmol mL^{-1} to 200 nmol mL^{-1} of amino-CAP concentration, there was higher $A_0 - A_{ex}$ difference (Fig. 6(a)) and when the concentration of amino-CAP was under 25 nmol mL^{-1}, the value of A_0 was very small. So we select this range of concentration for next step. Within this range, the competitive inhibition assay was performed afterwards to select the optimal amino-CAP concentration (Fig. 6(b)). Among all of curves, the sensitivity of assay was the highest when amino-CAP concentration was 25 nmol mL^{-1}.As a result, 25 nmol mL^{-1} of amino-CAP concentration was selected according to high $A_0 - A_{ex}$ and sensibility.

3.2.2. *Effect of biotinylated-mAb concentration and dilution level of HRP–SA conjugate*

The binding of HRP to mAb is through the interaction between streptavidin and biotin. So the relative molar ratio of the HRP–SA conjugate to biotinylated-mAb is of great importance. As showed in Fig. 7, the optimal concentration for biotinylated-mAb was 5.2 ng mL^{-1} and dilution level of HRP–streptavidin conjugate was 1:3000.

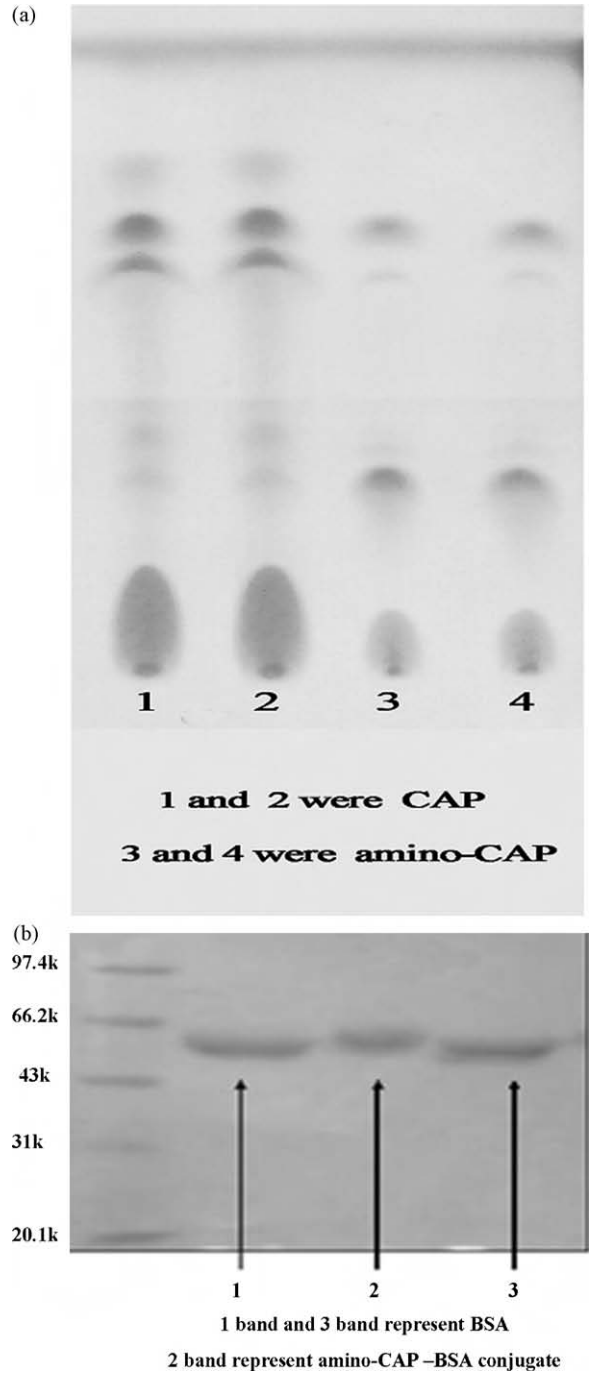

Fig. 4. (a) TLC of CAP and amino-CAP. (b) SDS-PAGE of amino-CAP–BSA conjugate and BSA.

3.2.3. *Effect of solid immobilization conditions*

Sensitivity of an assay depends on the solid phase condition to a great extent. Ideal solid phase should be consistent, stable and have low background signal. We selected special ELISA microtiter plates because it can largely prevent from non-specific absorption, as described in the product manual.

The mAb and HRP–SA were prevented from non-specific binding to the surface by blocking step. In this work, different blocking reagents were tested for their effectiveness-BSA, OVA, skimmed milk, ε-amino-*n*-caproic-acid (Ac). Ac was used for the first time before the incubation of mAb. As shown in Table 1, after using Ac, there was higher $A_0 - A_{ex}$ difference and high values of A_{bg} and A_{ex}. It indicated that the antibodies still bound non-specifically to the surface when just using Ac as a blocking reagent. Our study found that non-specific binding problem could be effectually solved while the mAb and the HRP–SA conjugate were diluted in PBS with BSA. With the increasing of BSA concentration in PBS, A_{bg} and A_{ex}

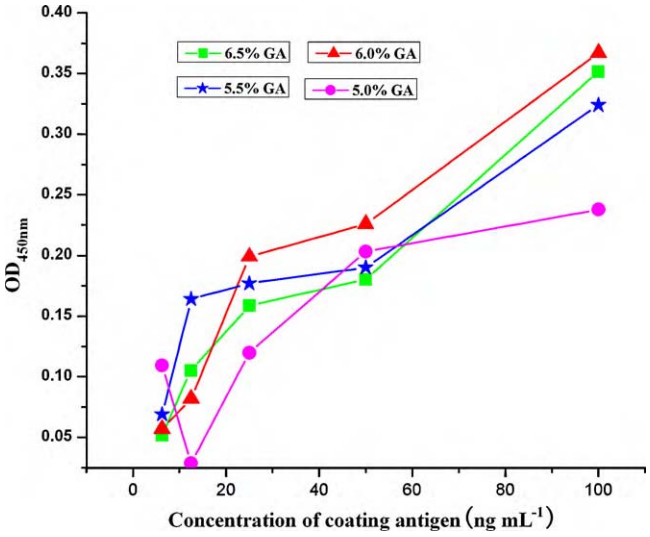

Fig. 5. Effect of the concentration of GA on $A_0 - A_{ex}$ difference.

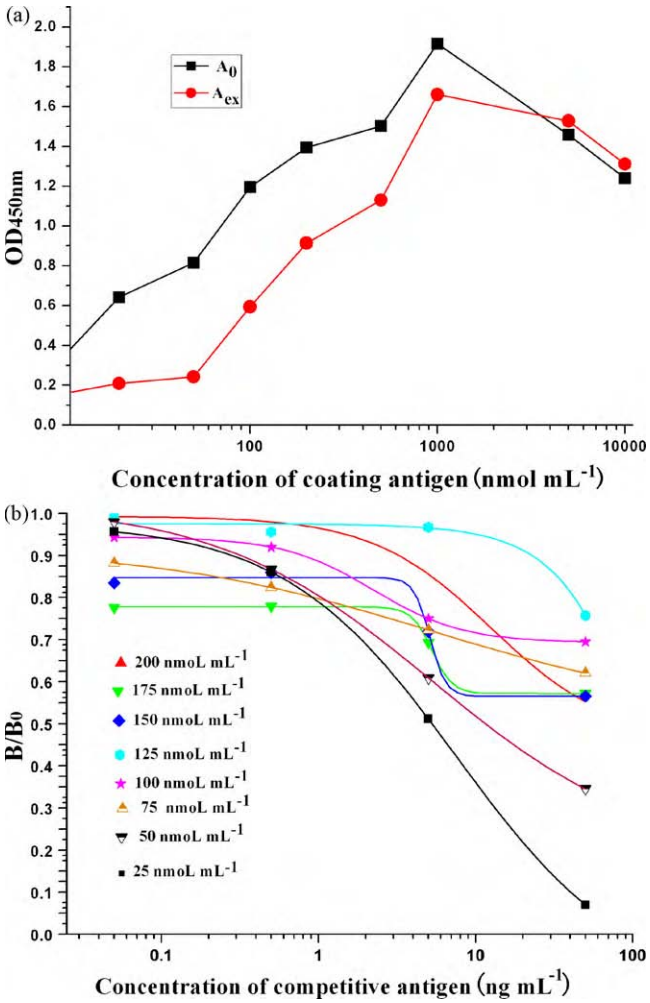

Fig. 6. (a) Effect of the concentration of the coating hapten CAP on $A_0 - A_{ex}$ difference. (b) Effect of the concentration of the amino-CAP on competitive inhibition.

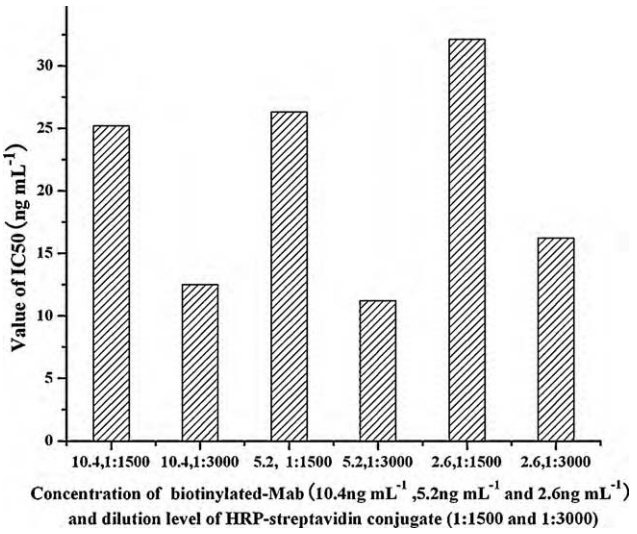

Fig. 7. Optimization of biotinylated-mAb concentration and dilution level of HRP–streptavidin conjugate.

Table 1
Effect of the blocking reagent on absorbance values, shown as mean values and standard deviations from three independent measurements.

Blocking reagent	$A_{blocking}$ [a]	A_{bg} [b]	A_0 [c]	A_{ex} [d]
BSA	0.08 ± 0.03	0.06 ± 0.02	0.33 ± 0.02	0.08 ± 0.03
Ac	0.27 ± 0.02	0.32 ± 0.04	0.98 ± 0.04	0.12 ± 0.05
Skim milk power	0.10 ± 0.05	0.04 ± 0.03	0.28 ± 0.08	0.06 ± 0.02

[a] Surface only coated with Ac, BSA or Skim milk power, respectively.
[b] Absorbance at negative group.
[c] Absorbance at zero hapten concentration.
[d] Absorbance at excess hapten concentration.

decreased gradually. However, a problem was appeared that the positive values (A_0) also decreased simultaneously. It was learned that although BSA prevented non-specific binding, it also restricted the specific binding of Ab–Ag reaction. Therefore, it was important to optimize the percent of BSA in PBS for diluting mAb and HRP–SA. Different concentrations of BSA in PBS (0.1%, 0.2%, 0.3% and 0.5%, m/v) were selected to observe the blocking effect. After careful optimization, 0.1 mol mL^{-1} Ac was firstly used for blocking the excess aldehyde groups of the surface modified with GA (Table 1) and the 0.1% BSA in PBS was selected to dilute the biotinylated-mAb and HRP–SA afterwards (Table 2). In this way, A_{bg} and A_{ex} were decreased greatly while positive value (A_0) was not impacted, so the non-specific binding in this assay was largely eliminated.

3.3. Method evaluation

3.3.1. Sensitivity

Fig. 8(a) and (b) showed standard curves for CAP obtained with conventional ELISA and BSAS-direct Hap coated ELISA. The IC_{50} values of conventional ELISA and BSAS-direct Hap coated ELISA were about 65.5 and 10.5 ng mL^{-1} respectively. The LOD value of conventional ELISA and BSAS-direct Hap coated ELISA were approximately

Table 2
Blocking effectiveness of BSA with different concentration, the absorbance values were shown as mean values ($n = 3$).

BSA in PBS (m/v, %)	A_0	A_{ex}	$A_0 - A_{ex}$
0.1	0.93	0.13	0.8
0.2	0.88	0.12	0.76
0.3	0.65	0.13	0.52
0.5	0.55	0.08	0.47

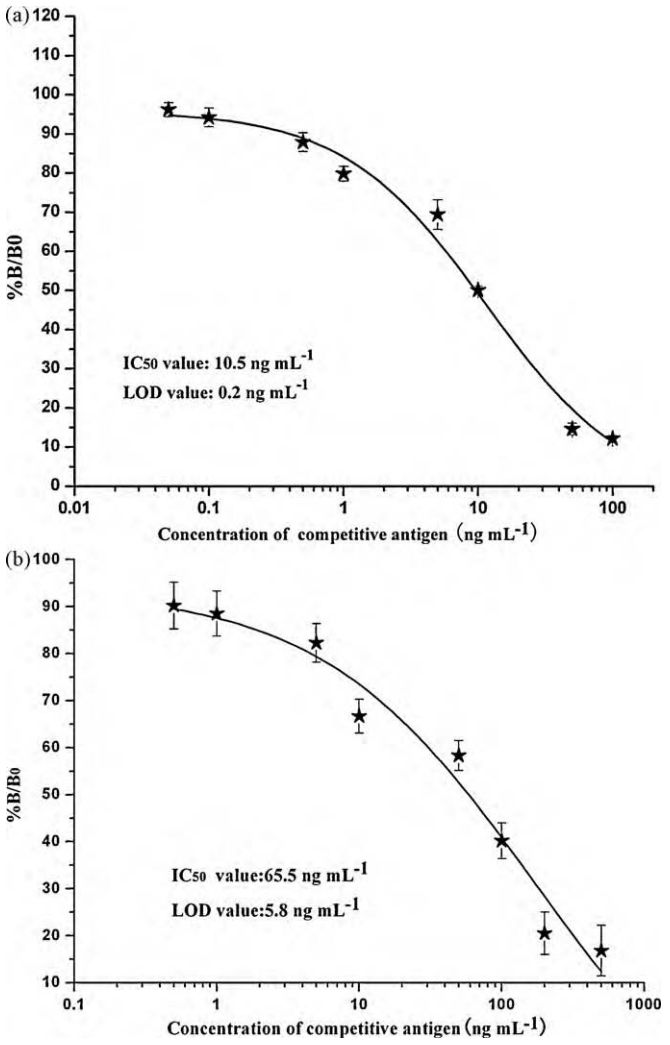

Fig. 8. (a) Standard inhibition curves of CAP in BSAS-direct Hap coated ELISA. (b) Standard inhibition curves of CAP in conventional ELISA.

5.8 and 0.2 ng mL^{-1}. In view of LOD, the sensitivity of BSAS-direct Hap coated ELISA was improved 20-folds compared with that of conventional ELISA.

This high sensitivity would be resulted from the following reasons. On the one hand, due to the very small MWs and the single reactive site of small molecular Hap, its reactive site was easily screened by carrier protein in preparation of Hap–carrier protein conjugate. The immobilization of Hap–protein conjugate on the surface of microtiter plates depends on hydrophobic interactions. It may involve significant conformational change which lead carrier protein easily to screen small molecular Hap during this immobilization. However, the Hap was directly conjugated to surface of microtiter plates in BSAS-direct Hap coated ELISA. This format not only avoids the screening effect by carrier protein but also gener-

Table 3
Cross-reactivities of the inhibition assay with selected compounds.

Compounds	Cross-reactivity	
	Conventional ELISA	BSAS-direct Hap coated ELISA
CAP	100%	100%
CAP succinate	28.8%	30.2%
Tetracycline	0.10%	0.30%
Streptomycin	0.20%	0.20%
Penicillin	0.30%	0.20%

Table 4
Accuracy and precision of chlorpyrifos in spiked sample by BSAS-direct hapten coated ELISA and conventional ELISA.

CAP added ($\mu g\,kg^{-1}$)	BSAS-direct hapten coated ELISA			Conventional ELISA		
	CAP detected, X ($\mu g\,kg^{-1}$)	Recovery (%)	CV (%)	CAP detected, X ($\mu g\,kg^{-1}$)	Recovery (%)	CV (%)
5	4.4	88	1.3	5.2	104	6.5
50	52.5	105	1.5	44.5	89	8.2
100	82	82	1.1	102	102	5.0

Table 5
Results from analysis of CAP in samples by BSAS-direct hapten coated ELISA, conventional ELISA and HPLC method ($n = 3$).

Samples	BSAS-direct hapten coated ELISA		Conventional ELISA		HPLC	
	Results ($ng\,g^{-1}$)	CV (%)	Results ($ng\,g^{-1}$)	CV (%)	Results ($ng\,g^{-1}$)	CV (%)
A	56.2	3.2	52.2	8.6	55.6	5.3
B	16.2	1.5	18.5	10.4	14.8	4.5
C	8.6	2.0	10.5	13.2	8.2	6.2

ates GA network on the surface to enlarge the distance between Hap and surface, so that more reactive sites of Hap are exposured abroad. Therefore, direct Hap coated format is profit for reaction of Hap and antibody. On the other hand, BSAS, a well-known signal amplification system, was applied in this assay. Thus it would be another reason to enhance the efficiency of Ab–Ag reaction and improve the sensitivity.

3.3.2. Specificity

Cross-reactivity (CR) is an important parameter to evaluate the specificity in immunoassay. The results were summarized in Table 3. The BSAS-direct Hap coated ELISA results showed hat the anti-CAP mAb had CR of 100% to CAP, 28.8% to CAP succinate, and negligible (<1%) to other analogs. The results were similar to what were observed in conventional ELISA. Therefore, the mAb was very specific against CAP.

3.3.3. Recovery and precision

In Table 4, the recoveries were ranged from 88% to 105% with CVs of 1.1–1.5% in BSAS-direct Hap coated ELISA. These values of recoveries were in accordance with those in conventional ELISA (Table 4). But the values of CVs were much lower than conventional ELISA. Therefore, the results suggested that BSAS-direct Hap coated ELISA performed higher stability and feasibility.

Hap–protein BSAS-direct Hap coated ELISA displayed higher stability, the possible reasons were as following. Firstly, covalent bond of Hap–protein may be dissociated during storage. Secondly, some of Hap could destruct activity and structure of carrier protein. Thirdly, in conventional ELISA, the ratio of Hap and carrier protein is always inconsistent and non-reproducible in the process of conjugate formation. On the contrast, BSAS-direct Hap coated ELISA refrained from complex preparation of Hap–protein conjugate and avoided the previous drawbacks [33].

3.4. Analysis of shrimp samples

To assess the applicability and validity of the BSAS-direct Hap coated ELISA, immunoassay and HPLC were employed to detect CAP in shrimp samples. From the results shown in Table 5, determination of CAP measured by three methods was similar, but the CVs in BSAS-direct Hap coated ELISA was lower than the other methods. It was demonstrated that the BSAS-direct Hap coated ELISA could be used for the determination of CAP in real samples.

4. Conclusion

We have developed a new strategy for the highly sensitive detection of CAP based on direct Hap coated format and BSAS. Compared with conventional ELISA, this method not only greatly improved sensitivity and stability but also refrained from complex process of conjugation of Hap–protein. Therefore, the immunoassay employing direct Hap coated format and BSAS together will be applied widely in detection of small molecules residue in food and environment. Further research should be focused on exploiting more means to link small molecules Hap with microtiter plates and applying some signal amplification systems simultaneously to improve the sensitivity and stability of immunoassay.

Acknowledgements

The authors gratefully acknowledge the financial supports by National High Technology R&D Program of China (no. 2007AA06Z419), Natural Science Foundation of China (no. 30771810) and Science and Technology Program of Tianjin (no. 09ZCKFSH02700).

References

[1] J.B. Maciej, H. Huda, A.E. Eid, I. Zuhour, A.T. Mohammed, J. Chromatogr. B 807 (2004) 343–356.
[2] X.Z. Shi, A.B. Wu, S.L. Zheng, R.X. Li, D.B. Zhang, J. Chromatogr. B 850 (2007) 24–30.
[3] K. Vivekanandan, S.M. Guru, S. Prasad, Rapid Commun. Mass Spectrom. 19 (2005) 2025–3030.
[4] I. Shalit, M.I. Marks, Drugs 28 (1984) 191–281.
[5] D. Zhang, P. Zuo, C.B. Ye, J. Agric. Food Chem. 56 (2008) 9862–9867.
[6] J.L. Thomas, P.T. Francis, G.B. John, L.G. Sherwood, Antimicrob. Agents Chemother. 9 (1976) 874–878.
[7] D.J. Louvois, J. Antimicrob. Chemother. 9 (1982) 253–265.
[8] D.J. Berry, J. Chromatogr. 385 (1987) 337–341.
[9] H.Y. Shen, H.L. Jiang, Anal. Chim. Acta 535 (2005) 33–41.
[10] P.S. Lietman, T.J. White, W.V. Shaw, Antimicrob. Agents Chemother. 10 (1976) 347–353.
[11] S.X. Zhang, Z. Zhang, W.M. Sui, A.E. Sergei, J.Z. Shen, J. Agric. Food Chem. 54 (2006) 5718–5722.
[12] A. Strasser, R. Dietrich, E. Usleber, E. Märtlbauer, Anal. Chim. Acta 495 (2003) 11–19.
[13] R. Levi, S. McNiven, S.A. Piletsky, S.H. Cheong, K. Yano, I. Karube, Anal. Chem. 69 (1997) 2017–2021.
[14] J.C. Hall, R.J.A. Deschamps, K.K. Krieg, J. Agric. Food Chem. 37 (1989) 981–984.
[15] J.S. Parnell, J.C. Hall, J. Agric. Food Chem. 46 (1998) 152–156.
[16] M.T. Muldoon, J.O. Nelson, Food Agric Immunol. 6 (1994) 357–370.
[17] K. Nishizawa, T. Konno, M. Takai, K. Ishihara, Biomacromolecules 9 (2008) 403–407.
[18] K. Fujiwara, T. Kitagawa, J. Biochem. 114 (1993) 708–712.
[19] M. Bocher, T. Giersch, R. Schmid, J. Immunol. Methods 151 (1992) 1–8.
[20] Y. Feng, B.A. Ning, S. Pu, H.Y. Wang, C.H. Wang, F.S. Chen, Z.X. Gao, Talanta 80 (2009) 803–808.
[21] P.J. Dobbins, H.R. Schroeder, J. Immunol. Methods 48 (1982) 251–260.
[22] A. Rembaum, S. Margel, J. Levy, J. Immunol. Methods 24 (1978) 239–250.
[23] J. Guesdon, T. Ternyck, S. Avrameas, J. Histochem, Cytochemistry 27 (1979) 1131–1139.
[24] Y.G. Alevy, C.M. Blyrm, J. Immunol. Methods 87 (1986) 273–281.
[25] M. Wortberg, M.H. Goodrow, S.J. Gee, B.D. Hammock, J. Agric. Food Chem. 44 (1996) 2219–2710.

[26] N. Liu, P. Su, Z.X. Gao, M.X. Zhu, Z.H. Yang, X.J. Pan, Y.J. Fang, F.H. Chao, Anal. Chim. Acta 632 (2009) 128–134.
[27] C. Van de Water, N. Haagsma, J. Assoc. Off. Anal. Chem. 73 (4) (1990) 534–540.
[28] J. Kaur, K.V. Singh, G.C. Raje Varshney, C.R. Suri, Anal. Chim. Acta 506 (2004) 133–135.
[29] J. Wu, C. Chang, W.P. Ding, D.P. He, J. Agric. Food Chem. 56 (2008) 8261–8267.
[30] S.Y. Ding, J.X. Chen, H.Y. Jiang, J.H. He, W.M. Shi, W.S. Zhao, J.Z. Shen, J. Agric. Food Chem. 54 (2006) 6139–6142.
[31] H.Y. Shen, H.L. Jiang, Anal. Chim. Acta 535 (2005) 1–2.
[32] H. Holthues, U. Pfeifer-Fukumura, I. Hartmann, W. Baumann, F. Senius, J. Anal. Chem. 371 (2001) 897–902.
[33] K. Jasdeep, C.B. Robin, W. Nishima, R.S.C. Kumar, S. Raman, Anal. Chim. Acta 607 (2008) 92–99.