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Determination of Chloramphenicol Residues in Milk by Enzyme-Linked Immunosorbent Assay: Improvement by Biotin−Streptavidin-Amplified System

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A sensitive biotin−streptavidin amplified enzyme-linked immunosorbent assay (BA-ELISA) method was developed for the determination of chloramphenicol residues in milk. The biotin−streptavidin system was applied to enhance the sensitivity. After optimization, the detection limit of the method was found to be 0.042 ± 0.006 ng mL^{-1}, which is 8-fold more sensitive than the traditional competitive ELISA using the same antibody and coating antigen. The amplification mechanism of the biotin−streptavidin system and the major factors affecting the sensitivity of detection are discussed. This method was successfully applied to determine the chloramphenicol residues in milk samples with a simple and rapid extraction procedure, and good recoveries (85.66−109.67%) were obtained. The result indicated that the biotin−streptavidin system may be a valuable tool to improve the specific detection of trace veterinary drug residues and could be widely used for routine monitoring of food samples.

KEYWORDS: Chloramphenicol; biotin−streptavidin-amplified system; ELISA; milk

INTRODUCTION

Chloramphenicol (CAP) is an effective broad-spectrum antibiotic widely used in veterinary practice for the prevention and treatment of many bacterial infections. However, research has shown that it can lead to serious adverse effects in humans such as aplastic anemia, which is often fatal. These potential hazards have led to a prohibition of its use in the United States and European Union in 1994 (*1*). For administration, a rapid and sensitive method for the determination of CAP at trace levels is urgently needed.

The existing analytical methods for CAP include enzyme-linked immunosorbent assay (ELISA) (*2*−*4*), biosensor assay (*5*), gas chromatography (GC) (*6*, *7*), liquid chromatography (LC) (*8*, *9*), GC−mass spectrometry (MS) (*10*), and LC-MS or LC-MS/MS (*11*−*13*). Among these methods, ELISA is well suited in detecting trace constituents in a complex matrix because of its specificity, sensitivity, and high throughput.

In recent years, conventional ELISA methods have been improved to achieve high sensitivity and shortened assay time. In general, the sensitivity of ELISA has been enhanced with a combination of fluorescence (*14*−*16*), chemiluminescence (*2*, *17*), and the biotin−streptavidin system (*18*−*25*). Especially, the biotin−streptavidin system, which is based on the high specificity and strong affinity (*26*) of streptavidin to biotin, is an efficacious technique and has been widely used for sensitivity enhancement. Biotin, a vitamin, is a relatively small molecule, and it can usually be conjugated to protein without significantly altering the biological activity of the protein. Therefore, the signal intensity can be increased because several biotins conjugate with one antibody molecule, and then the SA−HRP combines with the biotin with extremely high affinity in the following step. As a result, this format can enable more enzyme molecules catalyzing the substrate. Excellent results can be achieved through signal amplification (*22*).

The biotin−streptavidin system has long been used in immunoassays for the quantitation of a wide range of clinically important analytes, including papaverine (*20*), digoxin (*21*), thyroid-stimulating hormone (*22*), estradiol (*23*), and many others. Unfortunately, this sensitive technique, to our knowledge, has not yet been widely used in veterinary drug residue analysis in food-producing animals, although several studies involving related techniques have been reported recently. ELISA methods mediated by the biotin−streptavidin system were developed to detect tetracycline residues in honey (*18*) and milk (*19*), and the results presented distinct improvement on sensitivity of the assay. As for the detection of CAP, the biotin−streptavidin system was applied by van de Water et al. (*25*) in 1990 for the first time. However, the limit of detection reported was 10 $\mu g\ kg^{-1}$ in muscle tissue with tedious pretreatment, which was not sensitive and convenient enough to be applied in regular inspection.

The aim of this study was to apply the biotin−streptavidin amplification technique to the enzyme-linked immunosorbent assay for the determination of chloramphenicol residues in milk and compare the sensitivity and accuracy to a conventional ELISA using the same antibody and coating antigen.

MATERIALS AND METHODS

Reagents. CAP sodium succinate, keyhole limpet hemocyanin (KLH), ovalbumin (OVA), biotinyl-*N*-hydroxysuccinimide ester (BNHS),

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streptavidin−horseradish peroxidase (SA−HRP), *N*-hydroxysuccinimide (NHS), *N*,*N*-dicyclohexyl carbodiimide (DCC), 3,3′,5,5′-tetramethylbenzidine (TMB), and hydrogen peroxide were purchased from Sigma (St. Louis, MO). Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), and HPLC grade acetonitrile were obtained from Merck (Darmstadt, Germany). HRP conjugated goat anti-rabbit IgG was from Promega (Madison, WI). Protein A−Sepharose 4B was purchased from Amersham Biosciences (Uppsala, Sweden).

Materials and Instruments. The polystyrene 96-well plates were from Nunc (Roskilde, Denmark), and the microplate washer was from Bio-Rad (Hercules, CA). Immunoassay absorbance was read with a Multiskan Spectrum purchased from Thermo (Labsystems, Vantaa, Finland) in dual wavelength mode (450−650 nm).

Solutions. Phosphate-buffered saline (PBS; 10 mmol L^{-1} sodium phosphate and 137 mmol L^{-1} NaCl, pH 7.4), coating buffer (50 mmol L^{-1} sodium carbonate buffer, pH 9.6), PBST (PBS with 0.05% Tween 20), and TMB substrate solution [prepared by adding 3.3 mg of TMB in 250 μL of DMSO to 25 mL of phosphate−citrate buffer (0.1 mol L^{-1} citric acid + 0.2 mol L^{-1} Na_2HPO_4; pH 4.3) containing 3.25 μL of a 30% H_2O_2 solution] were used.

Hapten Synthesis and Antibody Production. The synthesis of CAP hapten was performed as previously described by Campbell et al. (*27*) with some modifications. Protein−CAP conjugates were synthesized using the activated ester method. Briefly, CAP sodium succinate (1.113 g, 2.63 mmol) was dissolved in distilled water (3 mL), and HCl (1 mol L^{-1}) was added dropwise until the pH of the mixture was adjusted to 2.0. The precipitate was washed several times with distilled water. Finally, lyophilization led to white powdered succinyl CAP (0.6 g, 57% yield). Then succinyl CAP (423.2 mg, 1 mmol) and NHS (126.7 mg, 1.1 mmol) were dissolved in dry THF (20 mL, 0 °C) with magnetic stirring for 0.5 h. Then DCC (227.0 mg, 1.1 mmol) in dry THF (5 mL) was dropwise added. The solution was slowly brought back to room temperature and left stirring overnight. The precipitate formed was filtered, and the solution was purified on a silica gel column eluting with EtOAC/petroleum ether (3:1, v/v) and recrystallized with chloroform and petroleum ether. The white solid of activated ester was obtained (235.4 mg, 45% yield): ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.689(t, J = 6.4 Hz, 2H, $OCOCH_2$), 2.831 (s, 4H, CH_2), 2.964 (t, J = 6.6 Hz, CH_2COON), 4.192−4.120 (m, 1H, CHN), 4.303−4.279 (m, 2H, CH_2OCO), 5.051 (s, 1H, ph-CH), 6.258 (br, 1H, OH), 6.461 (s, 1H, $CHCl_2$), 7.651 (d, J = 8.7 Hz, 2H, OCHAr), 8.198 (d, J = 8.4 Hz, 2H, m-CH-Ar), 8.588 (d, J = 8.7 Hz, 1H, NH).

The active ester obtained was coupled to KLH for the immunogen or OVA for the coating antigen, referring to the procedure of Wang et al. (*28*). The molar ratio (MR) of the active ester to KLH (OVA) was 13:1 or 40:1, respectively. The antiserum was produced as described by Wang et al. (*28*). After purified by protein A−Sepharose 4B affinity chromatography, the IgG fraction was dialyzed and used for the immunoassay described below.

Preparation of Biotinylated Antibody. Biotinylated antibody was prepared according to the procedure described by Zhao et al. (*24*) with some modifications as follows: 5.0 mg of purified antibody was dissolved in 0.5 mL of 0.1 mol L^{-1} sodium carbonate buffer (pH 9.6) and then mixed with 50 μL of 1.0 mg mL^{-1} BNHS in DMSO. This reaction mixture was stirred for 4 h at room temperature and then kept overnight at 4 °C. After the solution was dialyzed against PBS for 3 days, it was stored at 4 °C before use.

Optimization of the BA-ELISA Procedure. To develop a sensitive detection of CAP, several parameters were optimized. The concentrations of biotinylated antibody and immobilized antigen were optimized first. Dose−response curves were developed under different concentrations of coated CAP−OVA (0.5, 0.1, and 0.05 mg mL^{-1}) and biotinylated antibody (in 1:3000, 1:6000, and 1:9000 dilutions). The optimum conditions providing the best sensitivity were then chosen.

The blocking solution was selected to decrease the background value. The 0.5% skimmed milk powder in PBS, 1% OVA in PBS, and 1% BSA in PBS were used as the blocking buffer, and the background signals were compared.

The incubation time of the competition reaction was tested to improve the sensitivity. Times of 20, 30, 45, 60, and 80 min were investigated.

To determine the optimum pH of the assay buffer, the CAP standards were prepared in PBS buffers with different pH conditions between 6.5 and 8.5. The effect of pH was evaluated by the A_0/IC_{50} ratio. The pH condition with the maximal A_0/IC_{50} ratio was chosen (*29*).

The diluents of SA−HRP should also be selected to decrease the nonspecific binding, and several different diluents including PBS buffer, 0.5% BSA in PBS, and 0.5% Tween 20 in PBS were tested. The diluent was determined giving the lower background.

Procedure of BA-ELISA and Traditional Competitive ELISA. The coating antigen was diluted with coating buffer, and 100 μL was added to each well of a 96-well microplate and incubated at 37 °C for 3 h. After three washings with PBST, unbound active sites were blocked by incubation with 200 μL of 0.5% skimmed milk powder as blocking reagent for 1 h. After the plate had been washed four times, 50 μL of CAP standards or sample solution, together with 50 μL of biotinylated antibody (for BA-ELISA) or antibody (for traditional competitive ELISA) solution, was added to each well and incubated for 45 mim at room temperature. After four washes, a volume of 100 μL of SA−HRP conjugate (for BA-ELISA) or HRP-conjugated goat anti-rabbit IgG (for traditional competitive ELISA) was added to the plate and incubated for 30 min. After five washes, the HRP tracer activity was measured by adding 150 μL per well of TMB substrate solution. The enzymatic reaction was stopped after 20 min by adding 1.25 mol L^{-1} H_2SO_4 (50 μL per well), and the absorbance was read in dual-wavelength mode (450 nm as test and 650 nm as reference).

For data analysis, the inhibition (%) is an important parameter in the ELISA method and is calculated as

$$\text{inhibition (\%)} = (1 - A/A_0) \times 100$$

A is the absorbance reading at the related dose of standard, and A_0 is absorbance reading at 0 dose of standard. The nonspecific blanks were subtracted prior.

To evaluate the sensitivity of the ELISA method, a dose−response curve of inhibition versus the logarithm of CAP concentration was performed. The sensitivity of the method was evaluated by the IC_{50}, which is defined as the concentration of CAP giving a 50% inhibition (*28*). Also, the limit of detection (LOD) was evaluated by the IC_{15}, which is approximately the lowest part of the linear portion of the calibration curve.

Sample Preparation. For extraction of CAP from milk, 2 mL of milk sample was thoroughly mixed with 4 mL of ethyl acetate for 2 min. Then, 2 mL of the supernatant was transferred to a new tube and dried by nitrogen. The residue was dissolved in 1 mL of PBS. After filtering through a polytetrafluoroethylene (PTEF) filter membrane (0.22 μm), the sample solution could be used for determination.

Validation Study. The ELISA results were verified using the HPLC method. A C_{18} reversed-phase column (15 cm long × 4.6 mm inner diameter, 5 μm) was used. The mobile phase was acetonitrile/water (30:70, v/v) at a flow rate of 1.0 mL min^{-1}. The temperature of the column oven was 30 °C. For detection, 20 μL of standard solutions containing different concentrations of CAP was injected. The ultraviolet was detected with the detection wavelength of 278 nm.

RESULTS AND DISCUSSION

Suitable Concentrations of Biotinylated Antibody and Coated Antigen. Experimentally, the concentrations of biotinylated antibody and immobilized antigen are very important to enhance the sensitivity for immunoassay. To determine the optimum concentrations of biotinylated antibody and CAP−OVA providing the highest sensitivity, the absorbance value and IC_{50} were investigated simultaneously. Dose−response curves of absorbance value versus the logarithm of CAP concentration and dose−response curves of inhibition ratio versus the logarithm of CAP concentration were performed, using a 1:5000 dilution of SA−HRP. Results are shown in **Figures 1** and **2**, respectively.

The optimal reagent concentrations are determined as those that give the maximum absorbance (A_0) value ranging from 0.8 to 1.2 and have the lowest IC_{50}, which represents the sensitivity of the ELISA method. According to **Figures 1** and **2**, an increase of the concentrations of the biotinylated antibody and CAP−OVA enhanced the signal of the test, but with a decrease of the sensitivity of the method. The result indicates that a greater inhibition at the same amount of CAP can be obtained only if

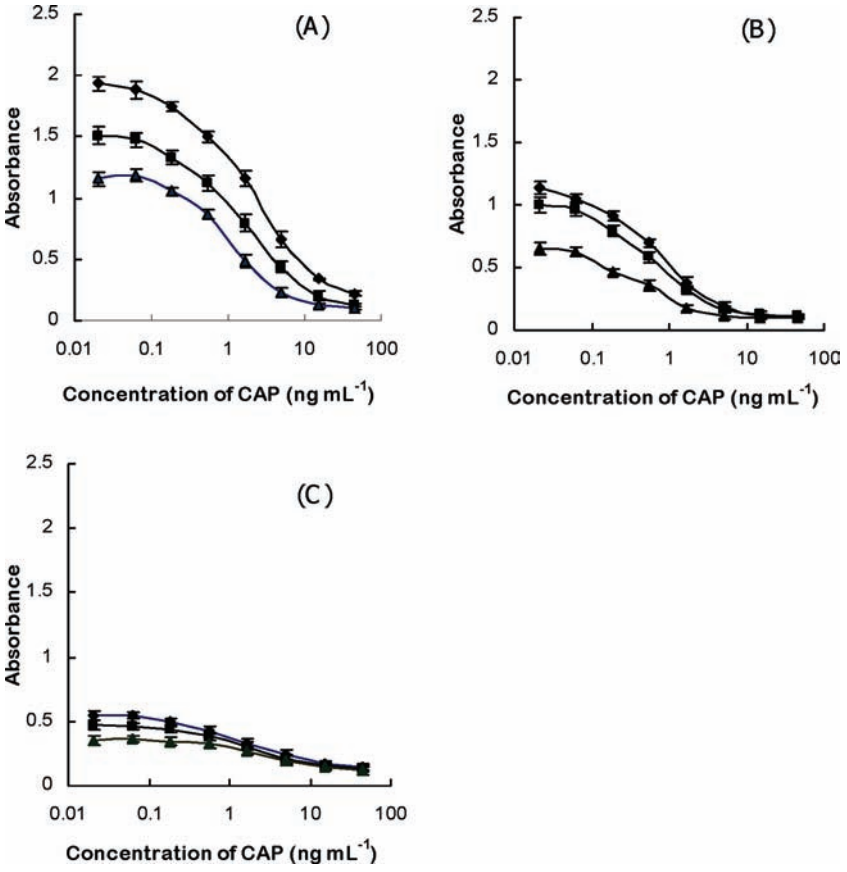

Figure 1. Optimization of concentrations of biotinylated antibody and CAP−OVA in terms of absorbance. The concentrations of CAP−OVA conjugate were (**A**) 0.5 mg mL^{-1}, (**B**) 0.1 mg mL^{-1}, and (**C**) 0.05 mg mL^{-1}, and the dilutions of biotinylated antibody were (◆) 1:3000, (■) 1:6000, and (▲) 1:9000.

lower concentrations of coating solution and antibody solution are used, which is in agreement with Vandewater's result (*25*). This can be explained by the principle of the competitive ELISA method. A competitive binding reaction for the binding sites on a limited amount of antibody is carried out between the CAP in the sample solution and the CAP−OVA immobilized on the plate, and lower concentrations of coating antigens can lead to a higher inhibition of the analyte in solution. However, inadequate reagents give low signals and may cause a less accurate result (**Figures 1C** and **2C**). Accordingly, optimal concentrations of the coated CAP−OVA and the biotinylated antibody were 0.1 mg mL^{-1} in 1:6000 dilution, respectively.

Optimization of BA-ELISA. The blocking step is important to avoid nonspecific absorption in the BA-ELISA. Otherwise, unoccupied sites of the plates may adsorb the components such as biotinylated antibody and SA−HRP during the subsequent steps, which may cause high background. As a result, the milk powder in PBS showed a better result because of the lower background value (0.05) than that of 1% OVA (0.15) or 1% BSA (0.11), so it was selected in the following experiments.

As the reaction of the antibody and the analyte is under a dynamic balance, the incubation time of competition reaction is an important factor for the work. The A_0 and the IC_{50} in different reaction times were tested. As a result, the time was selected to be 45 min because of the best sensitivity and the optimal absorbance, which is shown in **Figure 3**.

The binding of antigen and antibody is characterized by the weak intermolecular bonds and can be affected by the pH value. As for the optimum of the assay buffer, the parameters of A_0 and IC_{50} were considered together, and the maximal A_0/IC_{50} ratio was obtained at pH 7.4 (**Table 1**).

The nonspecific binding of SA−HRP was also a problem in this method. Results indicated that the addition of BSA could decrease the nonspecific binding (data not shown), so 0.5% BSA in PBS was chosen.

LOD and Working Range. Under the optimum conditions, the calibration curve of the method was performed as shown in **Figure 4**. The sensitivity and limit of detection (LOD) of the assay for CAP, which are represented by IC_{50} and IC_{15} values, were 0.43 ± 0.05 and 0.042 ± 0.006 ng mL^{-1}, respectively. The linear working range, which is determined as the concentrations causing 20−80% inhibition of color development (*28*), was 0.068−3.92 ng mL^{-1} for BA-ELISA. The intra-assay reproducibility and interassay reproducibility were determined to study the precision of the method, and the intra-assay and interassay coefficients of variation (CV) were below 7 and 16%, respectively. The relatively high interassay CV here is still acceptable for the ELISA method, because the colorimetric assays are prone to higher variation when the concentration of analyte is lowest (*30*).

Specificity. Assay specificity indicates the ability of antibody to generate a measurable response only for the target molecule. The specificity of the antibody is evaluated by cross-reactivity. The cross-reactivity percentage is calculated by the ratio of the IC_{50} of the analyte to the IC_{50} of the cross-reactant. Compounds structurally similar to CAP, such as flofenicol and thiamphenicol, had cross-reactivities of 0.05 and 0.008%, respectively. Veterinary drugs other than chloramphenicol such as ampicillin and sulfamethazine did not show any cross-reaction at 6 and 30 mg mL^{-1}. Therefore, the developed immunoassay can be used to the specific detection of chloramphenicol.

Comparison with Traditional Competitive ELISA. The traditional competitive ELISA method for CAP detection using the

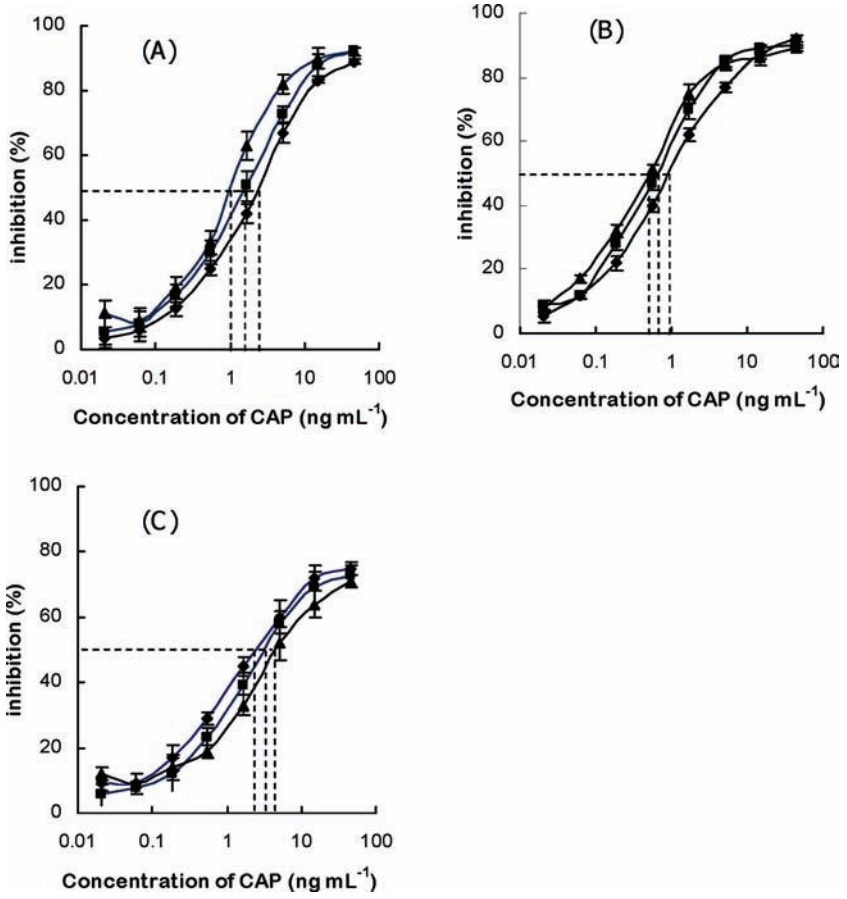

Figure 2. Optimization of concentrations of biotinylated antibody and CAP−OVA in terms of inhibition. The concentrations of CAP−OVA conjugate were (**A**) 0.5 mg mL^{-1}, (**B**) 0.1 mg mL^{-1}, and (**C**) 0.05 mg mL^{-1}, and the dilutions of biotinylated antibody were (◆) 1:3000, (■) 1:6000, and (▲) 1:9000.

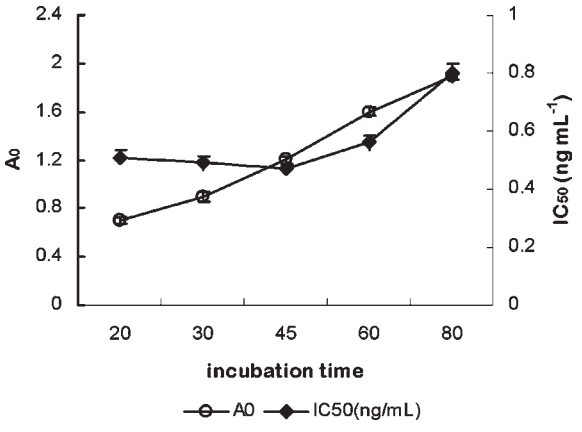

Figure 3. Effect of incubation time on the BA-ELISA. The BA-ELISAs were performed in different incubation times. In each incubation time, the A_0 value (○) and the IC_{50} (◆) were tested.

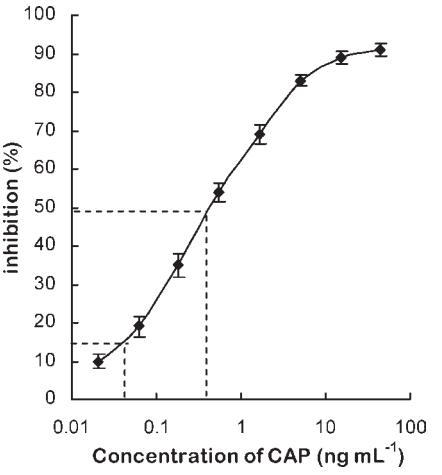

Figure 4. Calibration curve of BA-ELISA for CAP. The inhibition was calculated in different CAP concentrations.

Table 1. Effect of pH of the Assay Buffer on BA-ELISA

pH	A_0[a]	IC_{50}[b] (ng mL^{-1})	A_0/IC_{50}
6.5	0.95	0.65	1.46
7.0	1.03	0.51	2.01
7.4	1.16	0.45	2.57
8.0	1.02	0.53	1.92
8.5	0.97	0.67	1.45

[a] A_0 is the maximal absorbance reading at 0 dose of CAP. [b] IC_{50} is the concentration of CAP giving a 50% inhibition.

same antibody and coated antigen was also optimized, and the dose−response curve is shown in **Figure 5**. In the traditional ELISA, the LOD was 0.31 ± 0.04 ng mL^{-1} and the IC_{50} was 3.06 ± 0.33 ng mL^{-1}. Therefore, the BA-ELISA gives about an 8-fold increase in sensitivity over the traditional method. In addition, the BA-ELISA is more economical, requiring only one-fourth the amount of antibody that the traditional ELISA uses. The obvious improvement in sensitivity can be attributed to a signal amplification effect introduced by the biotin−streptavidin system.

Evaluation of Matrix Effect. Food matrix effect is quite common in immunoassays because some components in the samples, such as protein and fat, may affect the binding of antibody and antigen, reducing the sensitivity and reliability of the method. Thus, the matrix effect should be tested prior to the application of the method. The matrix effect can be evaluated by comparing the calibration curve of the analyte with that generated in the sample matrix. If the two curves are superimposable, the matrix effect is not significant and the samples can then be analyzed according to the calibration curve (*31*).

To gain information on the matrix effect, a calibration curve generated in PBS was compared with that obtained using milk matrices. The superimposition of the calibration curves suggested that there were no significant matrix effects from milk extracts (**Figure 6**), and the pretreatment procedure was feasible and simple enough to be applied in practical analysis.

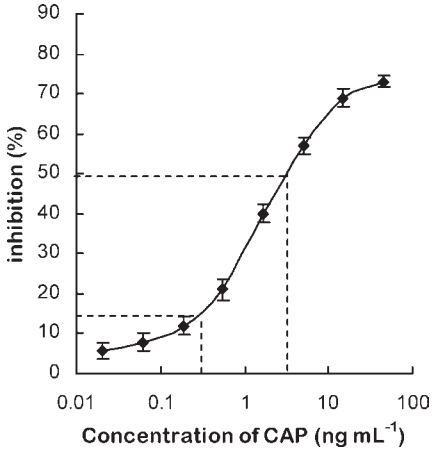

Figure 5. Calibration curve of traditional competitive ELISA for CAP. The inhibition was calculated in different CAP concentrations.

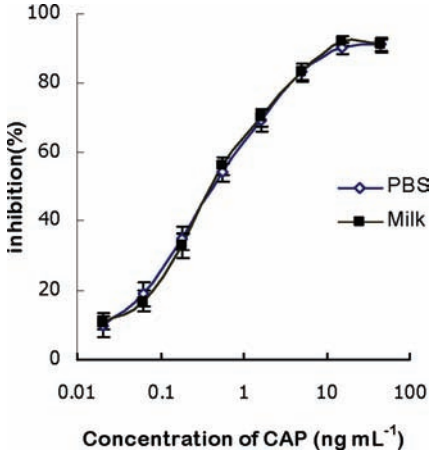

Figure 6. Calibration curves of CAP in PBS (◇) and in milk matrices (■).

Recovery. The analytical performance of ELISA is commonly assessed by the recovery of samples spiked with the target analyte. Blank samples (determined by HPLC) were spiked with different amounts of CAP, and the recoveries were measured. Each sample was evaluated three times in duplicate to verify the repeatability. For comparison, the spiked samples were measured simultaneously by the BA-ELISA as well as the traditional competitive ELISA. The recoveries were in the range of 85.66−109.67 and 89.33−113.33% for the BA-ELISA and traditional competitive ELISA, respectively (**Table 2**). The coefficients of variation (CVs) were all $<15\%$. In addition, the result indicated that when the low spiked levels were used, the CVs obtained from the traditional competitive ELISA were higher than that of the BA-ELISA. The explanation may be that the low concentrations of CAP are close to the margin of the detection range of the traditional competitive ELISA, which is comparatively less accurate and precise. Therefore, the BA-ELISA is preferable in terms of trace determination of CAP.

Correlation with HPLC Method. The classical HPLC method was used to verify the accuracy of BA-ELISA. In the HPLC assay, the retention time was 4.6 min. Samples were analyzed by BA-ELISA and HPLC method, and good correlation (r^2 = 0.97) was obtained (**Figure 7**). The result confirmed that the BA-ELISA showed good performance for the analysis of CAP residues.

In conclusion, this work provides an efficacious method for the rapid screening of CAP residues in milk with simplicity. The result demonstrates that the biotin−streptavidin amplification system, as a highly sensitive and practical method, shows great potential in detection work concerning extremely low concentrations of analyte. Thus, it would be a valuable technique to be used in high-throughput food inspection for poisonous constituents as well as related administration.

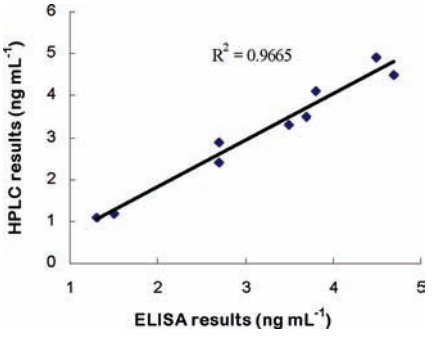

Figure 7. Correlations between ELISA and HPLC results for milk samples spiked with CAP.

Table 2. Recoveries of CAP from Spiked Milk Samples by BA-ELISA and Traditional Competitive ELISA

	BA-ELISA			traditional competitive ELISA		
spiked level (μg L^{-1})	mean ± SD (μg L^{-1})	recovery (%)	CV (%)	mean ± SD (μg L^{-1})	recovery (%)	CV (%)
0.1	0.085 ± 0.008	85.66	9.72	–[a]	–	–
0.5	0.49 ± 0.03	97.33	7.22	0.56 ± 0.07	113.33	12.52
1	1.06 ± 0.10	106.33	9.65	0.89 ± 0.12	89.33	13.07
5	5.48 ± 0.43	109.67	7.87	5.03 ± 0.46	105.27	8.68
10	8.57 ± 0.56	85.67	6.56	10.08 ± 1.00	100.83	9.95

[a] The concentration spiked was lower than the limit of detection and undetectable.

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