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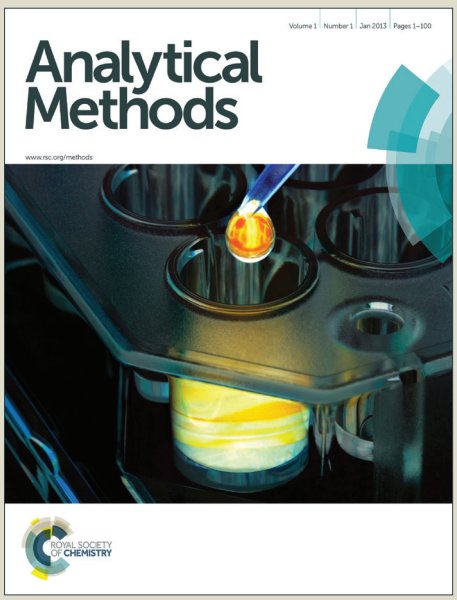

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Towards the development of a sensitive electrochemical sensor for the determination of chloramphenicol residues in milk

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Abstract

A simple and sensitive bioelectrochemical immunoassay method has been developed to detect chloramphenicol (CAP) residues in milk. Monoclonal anti-CAP antibodies and conjugates of ovalbumin-CAP (OVA-CAP) were used to establish the indirect competitive enzyme-linked immunosorbent reaction, in which the CAP in standard solutions or samples competed with the OVA-CAP immobilized on 96-well polystyrene reaction plate for the limited binding sites on the CAP monoclonal antibodies. After the competitive immunoreaction, the plate was rinsed and HRP-labeled goat anti-mouse IgG was added into the testing wells. *O*-phenylenediamine (*o*-PD)-hydrogen peroxide was applied as the hydrogen donor. The reaction solutions were transferred into the testing cells of a voltammetric analyzer and the electrochemical signals (oxidation peak currents of *o*-PD) were recorded by Differential Pulse Voltammetry (DPV) after the HRP-catalyzed reaction on the plate had been stopped by 1 M H_2SO_4. The results showed that the sensitivity of electrochemical immunoassay was higher than that of c-ELISA (competitive ELISA) in detecting chloramphenicol residue in milk. This method also demonstrated a linear response of oxidation peak current to chloramphenicol concentration over the 0.1-300 ng mL^{-1} range with a detection limit of 0.03 ng mL^{-1} CAP. The average recovery rate reached 87.7% based on the milk samples. Furthermore, the immuno-voltammetric apparatus is portable and can be used on site for detecting chloramphenicol residue in milk.

Keywords: chloramphenicol; Differential Pulse Voltammetry; immunovoltammetry; Enzyme-linked immunosorbent reaction

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Chloramphenicol (CAP) has been banned for use in food producing animals in many countries including the European Union, Switzerland, USA, and China owing to its serious toxic effects in humans in the form of bone marrow depression, fatal aplastic anemia, and other blood disorders [1-4]. The Ministry of Agriculture of the People's Republic of China declared formally in 2001 that a zero tolerance level had been established for CAP and other kinds of CAP derivate residue in both domestic and imported animal products [5]. However, CAP is still illegally used in cows to control mastitis and other animal diseases because of its low cost and availability [6]. Thus sensitive, accurate and robust analytical methods are very necessary for monitoring and controlling the compliance of the zero-tolerance level of CAP. Up to now, various analytical methods have been developed for the determination of CAP in different animal food including milk, chicken, egg, pork, honey and shrimp. These methods are mainly based on gas chromatography (GC) [7, 8], liquid chromatography (LC) [9], GC/mass spectrometry (MS) [10-12], LC/MS [13, 14], LC-MS/MS [15-18], and capillary zone electrophoresis [19]. Although these methods show their advantages of high sensitivity and accuracy, the complicated analytical procedures and requirement of expensive chromatographic instruments limit their extensive use. Electrochemical CAP detection is simple and fast [20], but its low sensitivity (> 15 ng mL^{-1}) and specificity are still big problems in practical use. Immunoassays are commonly employed as rapid and low-cost methods for screening the banned drugs in a large number of samples. Some enzyme-linked immunosorbent assays (ELISA) and chemiluminescent ELISA have been reported for detecting CAP in animal tissues [21-26]. These methods allowed the average detection limit of 0.3 ng mL^{-1}. However, the sensitivity of these methods was drawn back by the fundamental limitation of the spectrophotometric detection used in ELISA. In recent years, the sensors based on the molecularly imprinted polymers (MIP) were developed as new methodologies for the determination of antibiotics [27-29]. Unfortunately, the selectivity and sensitivity of these techniques were still not satisfactory and it is hard to see the analytic instruments based on the MIP in the market. Electrochemical immunoassay, proposed as early in 1951 by Breyer and Radcliff [30], is a methodology with almost unrivaled sensitivity, wide dynamic range, low detection limit and adoption to automation [31]. Few literatures have been reported on the determination of CAP by electrochemical immunoassay.

In this study, we developed a simple and highly sensitive bioelectrochemical immunoassay

method to detect CAP residue in milk samples using a portable potentiostat to measure the oxidation peak currents of the remained *o*-PD after the competitive Enzyme-Linked immuno-reaction. This method allows for a detection limit as low as to 0.03 ng mL^{-1} with a linear range of 0.1-300 ng mL^{-1} CAP. Furthermore, this technique showed high sensitivity, specificity and easy operation.

EXPERIMENTAL

Apparatus and chemicals

Voltammetric experiments were carried out on a CHI1230 Electrochemical Workstation (Chenhua Instrument Company, Shanghai, China) with a three-electrode system including a glass-carbon working electrode (6mm in diameter and 10 cm in length, Tianjin Lanbiao Electronic Technology Company (Tianjin, China), a platinum wire counter electrode and an Ag/AgCl reference electrode (Leici Branch of Shanghai Precise Instrument Company, Shanghai, China). The electrochemical data were collected and analyzed by ZF-10B Data Capturer (Shanghai Fangzheng Electronic Apparatus Company, Shanghai, China).

The following chemicals were purchased from Sigma (Sigma-Aldrich Chemical Co., St. Louis, MO, USA): Bovine serum albumin (BSA), Ovalbumin, Complete and Incomplete Freund adjuvant, o-phenylenediamine, Horse Radish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody, CAP, and CAP sodium succinate (HS-CAP). Other antimicrobial agents were obtained from Tongren Pharmacy Company (Shanghai, China) as follows: thiamphenicol, florfenicol, ampicillin, cephaloridine, furozolidone, gentamycin, lincomycin, tylosin, erythromycin and sulfamethoxazole. 1-ethyl-3- (3-dimethylaminopropyl) cardodiimide was obtained from Fluka (Buchs, Switzerland). RPMI-1640 medium, ready-to-use solution of Hypoxanthine-Thymidine (HT), Hypoxanthine-Aminopterin-Thymidine (HAT) was obtained from Gibco (Gibco Life Technologies Co., Grand Isle, NY, USA). New-born calf serum was purchased from Shanghai Amin Biotechnology Company. Mouse myeloma cells (SP2/0) were provided kindly as a present from the Animal Research Institute of Shanghai of Academia Sinica (Shanghai, China). All other chemicals such as isobutylchloroformate, citric acid, acetic ether, phosphoric acid, N-hexane and glycine were analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). 25 fresh milk samples were collected from a farm

of Changying Diary Company (Shanghai, China). 40 blank milk samples sterilized by instant autoclaving were kindly provided by Shanghai Institute of Food Quality Inspection and Technical Research.

Experimental method

CAP antigen synthesis and preparation of monoclonal antibody

A CAP-BSA-conjugated antigen for the immunization was prepared using a mixed anhydride reaction described by Campbell et al [22]. The reaction mixture was chormatographed on a Sephadex G-25 column (Pharmacia) and the first peak major, which represented the CAP-BSA complex, was collected. The purified CAP-BSA was sterilized through a 0.22-μm disk membrane filter. A CAP-OVA-conjugated antigen for use in the assay procedure was synthesized by a classical carbodiimide reaction in our laboratory. The preparation of the monoclonal antibodies against CAP-BSA were carried out according to the procedures reported by Shen et al [32].

Competitive Enzyme-linked immunosorbent reaction

96-well polystyrene reaction plates were used for the competitive enzyme-linked immunosorbent reaction. 100 μL of OVA-CAP antigens at a concentration of 0.2 μg mL^{-1} in bicarbonate buffers (0.1 M, pH 9.5) were added into the wells of the plates and allowed to be passively absorbed at 37 ^{o}C for 2 h. After the removal of the antigen solution by aspiration, the testing wells were washed with PBS-T (pH 7.2, containing 0.05% Tween 80). Then a 200 μL blocking solution prepared with PBS (pH 7.2) containing 3% skimmed milk powder was used in each well and allowed to stay at 37 ^{o}C for 1 h. The blocking solution was removed and washed twice with PBS-T. 50 μL of diluted CAP monoclonal antibodies (1 : 8000) and 50 μL samples of CAP standard concentrations (test samples or control solutions) were added into the wells of the plates and incubated at 37 ^{o}C for 1h. After all the wells were aspirated and washed three times with PBS-T, 100 μL of peroxidase-conjugated goat anti-mouse IgG diluted by 1 : 5000 with PBS was added and incubated at 37 ^{o}C for 1 hour. Then 100 μL of substrate solution prepared with 0.16 mM Na_2HPO_4-citric acid buffer (pH 5.0) consisting of 3.69 mM *o*-PD and 13.2 mM H_2O_2 was added and incubated in a dark place for 15 min at room temperature after triplicate washings with PBS-T. Finally, 50 μL of 1 mol H_2SO_4 was used in each micro-well to stop the enzymatic reaction.

Voltammetric detection

The solution in each well of the plates from the above step was transferred respectively into the electrochemical cell containing 3 mL of 0.2 mM Britton-Robinson buffer (BR buffer, 11.5 mL acetic acid, 13.5 mL phosphoric acid and 12.44 g boric acid per liter, pH 3.0). DPV measurements were performed on a CHI1230 Electrochemical Workstation.

Determination of CAP in field samples

All the fresh milk samples were pre-treated according to the methods described by Xiao F et al [20]. In brief, 5 mL milk sample is transferred into a 10 mL centrifuge tube and stirred for 15 min using a vibratory stirrer. The CAP is extracted with 3 mL ethyl acetate, and the organic phase is collected and evaporated at 40 ^{o}C under 240 mbar. Then 5 mL acenitrile is added in it and the sample is shaken vigorously and vortexed for 20 s. Some of the extracted solution is transferred in a cell containing 0.10 M PBS for determination. Electrochemical immunoassays and HPLC were performed respectively under optimized conditions to determine the CAP residues in these samples. Gas chromatography in combination with mass spectrometry detector (GC-MS) was used to assess the agreement between the above two methods. The GC-MS analysis was conducted according to the previous literature on an Agilent 6890 N gas chromatography equipped with an Agilent 7683 automatic liquid sampler coupled to an Agilent 5973 N mass selective capture detector [12]. HP-5MS (30 m × 0.25 mm i.d., 0.25 μm) capillary column (5% diphenyl, 95% dimethylpolysiloxane) with helium as carrier gas was applied for capillary GC analysis.

RESULTS AND DISCUSSION

Identification of conjugated complete antigens and determination of antibody valence

Figure 1A and figure 1B shows the UV scanning curves of BSA-CAP (0.1 mg mL^{-1}) and OVA-CAP (0.1 mg mL^{-1}) respectively. The incorporation ratio of CAP into BSA and OVA was 33 : 1 and 6 : 1 respectively.

Five Balb/c (SP2/0) mice were immunized using BSA-CAP as an immunogen. Two cell strains (named 20130723-1D11 and 20130723-4D2) secreting specific antibodies against CAP were obtained by cell fusion techniques. ELISA proved that the valence and specificity of

20130723-1D11 was better than that of 20130723-4D2, so the purified antibody secreted by 20130723-1D11 was applied for all the experiments. The antibody valence of the medium supernatant of 20130723-1D11 was 1 : 200 and that of peritoneal fluid induced by the hybridoma from this strain was 1 : 12000. The avidity was equal to 1.36×10^{8} LM^{-1} and the antibody concentration was 2.05 mg mL^{-1} based on the binding capacity. Further study proved that there was no cross reaction of the monoclonal antibody with BSA or OVA.

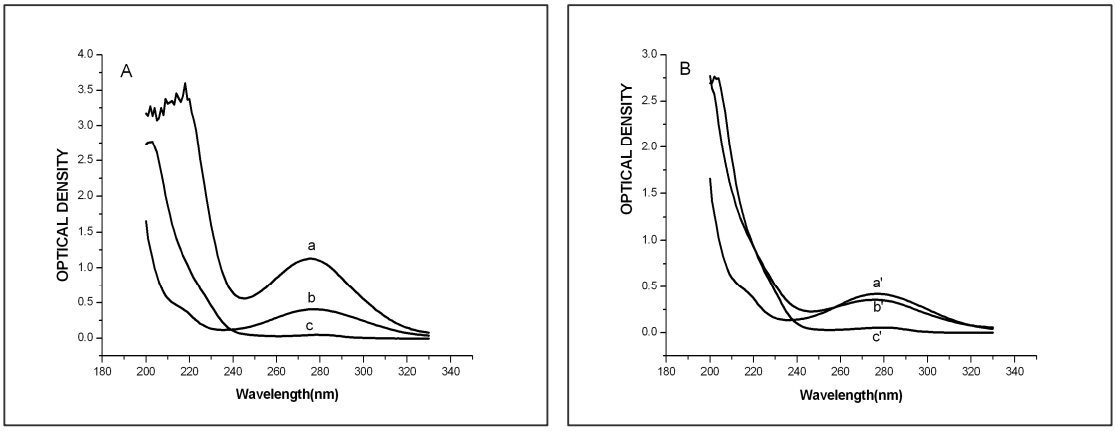

Fig.1. Ultraviolet Scanning spectra of conjugates and related substances. Fig 1A shows the ultraviolet spectrum of BSA-CAP conjugate and related substances. Curve a, b, c represents BSA-CAP, CAP and BSA respectively. Fig 1B shows the ultraviolet spectrum of OVA-CAP conjugate and related substances. Curve a', b', c' represents OVA-CAP, CAP and OVA respectively.

Electrochemical studies of *o*-PD and enzymatic product at GCE

In this study, *o*-PD-H_2O_2 (3.69 mM *o-PD* and 13.2 mM H_2O_2) was used as hydrogen donor for enzymatic reaction and GCE was applied as transducer to obtain electrochemical response of *o*-PD and enzymatic product. The voltammetric characteristics of *o*-PD-H_2O_2 and enzymatic product on GCE were discussed in previous literatures [33]. Our experiment also demonstrated that cyclic voltammograms of *o*-PD-H_2O_2 possess a couple of redox peaks (Epa 0.75 V, -0.75 V) in the potential range of –0.8 to 0.8 V with a potential scan rate of 100 mV s^{-1} in BR buffer and the oxidation peak current is higher than that of the reduction peak.

Although the enzymatic product of *o*-PD has a distinct voltammetric peak at -0.75 V, the linear response of the peak currents at -0.75 V to the concentrations of HRP is confined in a narrow range (2.3×10^{-6} - 2.5×10^{-2} unit mL^{-1}) when different concentrations of free HRP were used to catalyze the oxidation reaction of *o*-PD by H_2O_2. The enzymatic product of *o*-PD will precipitate on the bottom of the testing wells when the concentration of HRP is higher than 3.0×10^{-2} unit mL^{-1}. Up to now, several publications have introduced voltammetric enzyme-linked immunoassay systems for detecting trace biochemical substance [34, 35]. Their experiments were mainly focused on the investigation of the electrochemical characteristics of enzymatic product from the *o*-PD -H_2O_2-HRP reaction system and optimization of electrochemical conditions for the detection of the trace substance. However, after we tried to use different hydrogen donors (3, 3'-diaminobenzidine, *p*-phenylenediame, ortho-tolidine and tetramethyl benzidine) for the electrochemical immunoassay system and repeated the experiments according to their reported procedures, the results indicated that electrochemical immunoassays based on the measurement of the enzymatic product by voltammetry have relatively poor sensitivity and reproducibility due to the limited solubility of the stable enzymatic products.

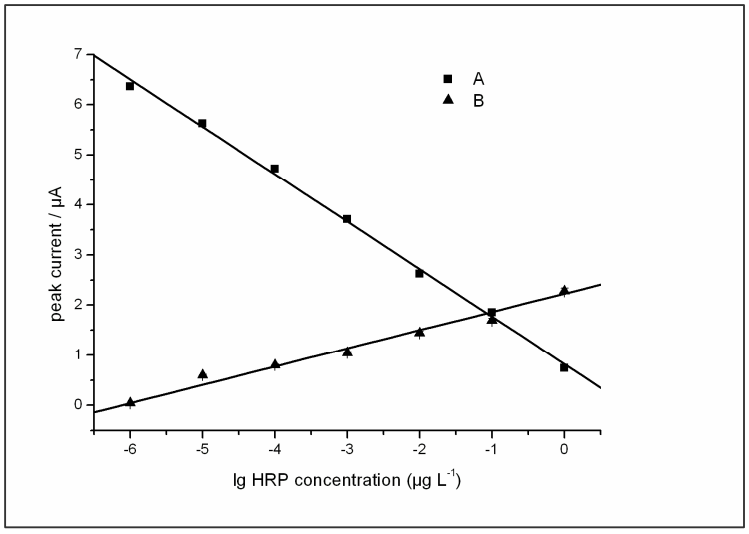

Figure 2. Linear responses of oxidation peak currents of the remained *o*-PD (A) and reduction peak currents of enzymatic products of *o*-PD (B) to the concentrations of HRP. The linear regression equation for (A) is Y = 0.821 - 0.948 lg C_{HRP} (R = -0.998, S.D.= 0.111, N = 7, P < 0.001) and for (B) is Y = 2.230 + 0.364 lg C_{HRP} (R = -0.988, S.D.= 0.275, N = 7, P < 0.001). The correlation coefficient (R^2) between the two linear graphies is 0.997 (P < 0.001).

In our study, owing to the advantage of the good solubility of *o*-PD and its sensitive oxidation peak at 0.75 V, the voltammetric peak currents of the remained *o*-PD was recorded after the competitive enzyme-linked immunosorbent reaction. Compared with the voltammetric determination of the enzymatic product of *o*-PD, a much better linear response of the decreased oxidation peak currents of *o*-PD to the concentrations of HRP in a wider range (1.5×10^{-7} - 1.0×10^{-2} unit mL^{-1}) was obtained by voltammetric measurement of the decreased oxidation peak currents of *o*-PD at 0.75 V after the competitive enzyme-linked immunosorbent reaction (Figure 2). Thus, our experimental data were based on the calculation of remained *o*-PD. In addition, solid-phase enzyme-Linked immunosorbent reaction was simply conducted in 96-well polystyrene reaction plates and electrochemical assays were performed on a portable electrochemical instrument (size: 22 cm × 11 cm × 3 cm) using a small cheap glass-carbon working electrode. So this detection system can be easily used on site.

Selection of the suitable assay conditions

The amount of CAP antibody and coating antigen (OVA-CAP) was chosen according to the principle of typical competitive immunoassays. It is required that the coating antigen should bind the antibody completely. During the solid phase enzyme-linked immunoabsorbant reaction, the immobilized OVA-CAP in the testing wells of polystyrene plates and the target analyte in standard solutions or samples bind competitively with the limited amount of CAP monoclonal antibodies. So a fixed amount of antibodies and coating antigens was applied respectively to estimate the suitable conditions for the immunoassays. Inhibitory rate (B/B_0) was used as the detection signal for the electrochemical immunoassay, where B_0 represents the subtraction of the peak current produced in sample without CAP from the i_{max} (oxidation peak current produced in 3.69 $mmoll^{-1}$ of *o*-PD) and B represents the subtraction of the peak current produced in each sample containing a certain concentration of CAP from the i_{max}. In this study, best inhibitory rate was obtained using 100 μL of OVA-CAP antigens at a concentration of 0.2 μg mL^{-1} and 50 μL of diluted CAP monoclonal antibodies (1 : 8000), so both of them were chosen at their adequate concentrations for all the electrochemical immunoassays.

We also investigated the effect of the immunoreaction time on the electrochemical response

signals. As it is known, the competitive binding of target analyte and OVA-CAP with antibody takes considerable time to reach a dynamic equilibrium. Experiments were performed by incubating 50 μL of diluted CAP monoclonal antibodies (1 : 8000) with 50 μL of CAP standard concentrations (0.30 ng mL^{-1}) for different periods. The maximum inhibitory signal caused by CAP standard solution trends to be stable after 60 min, which was used as the adequate incubation time for most experiments. The effect of pH of enzymatic reaction solution on the detection signal was checked. The highest voltammetric response is produced when the pH of Na_2HPO_4-citric acid buffer is 5.0, which was used as the suitable pH for enzyme-catalyzed reaction. Furthermore, the effect of pH value on the DPV peak of *o*-PD with a concentration of 3.69 mmol L^{-1} was investigated. Results showed that the peak potential shifted negatively with the increased pH. The peak current reached the maximum value at pH 3.0, which is selected as the suitable pH value for the electrochemical detection. The optimized instrumental conditions for the detection were selected as follows: scanning range: 0.1 ~ 0.9 V; pulse amplitude: 50 mV; pulse duration: 20 s; pulse period: 200 s; and scanning speed: 50 $mV.s^{-1}$.

Calibration and linearity

The immunochemical immunoassay of CAP was performed under optimized assay conditions. CAP stock solutions (5 μg mL^{-1}) were diluted to desired concentrations to establish calibration curves. Figure 3 shows the DPV scanning curves of *o*-PD according to different concentrations of CAP and Figure 4 illustrates the standard curve for determination of CAP by electrochemical immunoassay under optimized conditions. The curve was non-linear over the entire range of CAP concentrations analyzed and the voltammetric response was sigmoidally decreased according to the increase of CAP concentration. However, it was found that the current response (B/B_0 ratio) presents a good linear relationship with the logarithmic value of CAP concentrations in the range of 0.1-300 ng mL^{-1}. The equation of linear regression is $B/B_0 = 54.919 - 18.579 \log C$ ($\gamma = 0.99872$, S.D. = 6.376, N = 6, $p < 0.001$). To determine the limit of detection (LOD), defined as a signal higher than three-fold of standard deviation of base line drift, repeated analysis (n = 5) was performed using CAP-free milk samples. The LOD was 0.03 ng mL^{-1}. For comparison, spectrophotometric detection of c-ELISA was also performed in parallel using Bio-TEK ELX-800 spectrophotometer for the measurement of optical absorbance at 450 nm. The

results showed that the lowest detectable limit (LDL) of CAP was 0.27 ng mL^{-1} and the detecting linear range was 0.9 ng mL^{-1}~100 ng mL^{-1} by c-ELISA. These results indicated that the sensitivity of the immuno-voltammetric technique is better than that of c-ELISA. The limited solubility of the enzymatic product may affect the resolution power of c-ELISA. Moreover, electrochemical analysis of decreased *o*-PD is more sensitive than spectrophotometric methods for detecting the enzymatic product. In conclusion, electrochemical immunoassay suppass c-ELlSA both in sensitivity and detecting range.

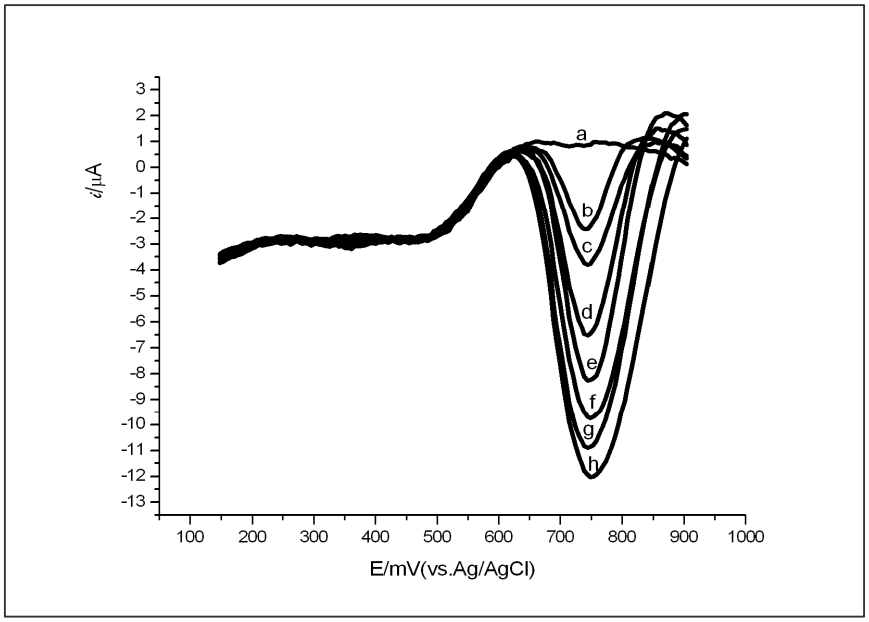

Figure 3. Variation of peak current with the related CAP concentration. CAP concentration: 0.10 ng mL^{-1}, 0.30 ng mL^{-1}, 0.90 ng mL^{-1}, 2.70 ng mL^{-1}, 8.10 ng mL^{-1} and 24.3 ng mL^{-1} (from b to g). The curve (a) reresents the voltammetric signal of the blank solution and the curve (h) represents the peak current produced from 3.69 ng mL^{-1} of *o*-PD (i_{max}).

Selectivity, reproducibility and accuracy

Representatives of antimicrobial agents, based on their availability for general use, structural similarity to the CAP molecule, and high probability of concurrent administration with CAP, were chosen to challenge the specificity of the immuno-voltammetric method. CR_{50}, the value of 100 × nanograms of CAP displacing 50% of antibody/nanograms of compound displacing 50% of antibody, was used to analyze cross-reactivity. The results showed that all the values of CR_{50} for

ampicillin, cephaloridine, furozolidone, gentamycin, lincomycin, tylosin, erythromycin and sulfamethoxazole were less than 0.01 while that for thiamphenicol and florfenicol reached 0.68 and 3.2 respectively, indicating measurable cross-reactivity. However, CAP sodium succinate was found to have a high cross-reactivity value with CR_{50} up to 639.0, showing an affinity of the antibody for this compound which was 6.4 times greater compared with CAP. Since the use of CAP and CAP sodium succinate were both banned in edible animals, the electrochemical immunoassay detection method established in this study is still of great value. The increased sensitivity for the determination of CAP sodium succinate is mainly caused by the higher dissolvability of this substance in aqueous solution compared to that of chloramphenicol. Therefore, it is necessary to establish the calibration curves for the determination of CAP and CAP sodium succinate respectively.

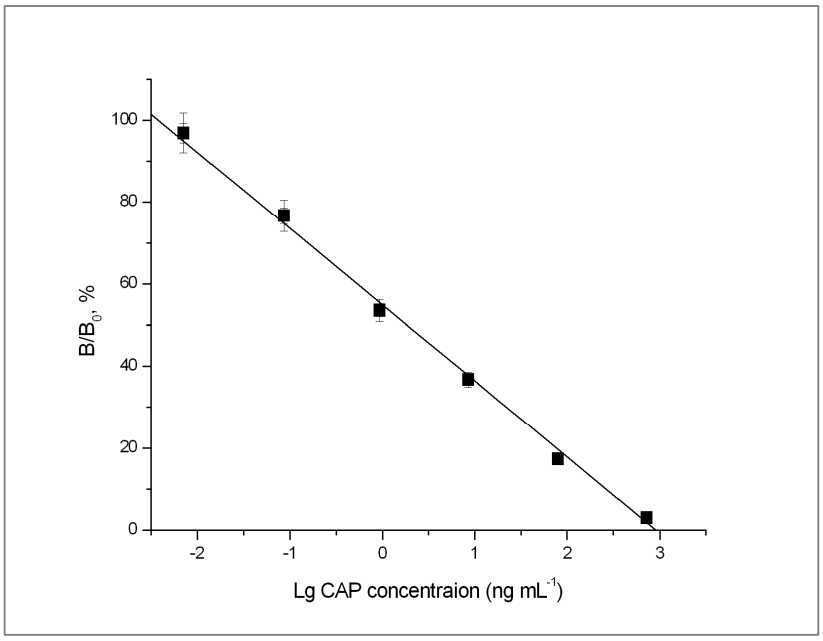

Figure 4. Calibration curve for the immunovoltammetric determination of CAP.

Repeatability and precision was calculated for the electrochemical immunoassay from the analysis of six blank milk samples spiked with CAP at three levels (0.1, 0.5 and 1.0 ng mL^{-1}). c-ELISA was also conducted for comparison. The extraction recovery for CAP and precision (within day) for spiked milk samples are summarized in Table 1. The electrochemical

immunoassay provides better accuracy and reproducibility than c-ELISA.

Detection of CAP in Field samples

To further demonstrate reliability of the electrochemical immunoassay for field detection, 25 fresh milk samples randomly collected from the farm of Changying Diary Company were analyzed using electrochemical immunoassay and HPLC. CAP was found in 4 out of the 25 milk samples by electrochemical immunoassay, but the data from HPLC methods indicated that CAP was found only in 3 out the same 25 milk samples (Table 2). Confirmatory GC-MS showed good agreement with the electrochemical immunoassay established in this study. However, HPLC method presented false-negative result in determination of CAP residue in milk due to its limited sensitivity.

Table 1 Repeatability and intermediate reproducibility of recovery rates for milk samples, spiked at three levels (six independent replicates for each level)

Spiked levels	Electrochemical immunoassay		c-ELISA	
(ng mL^{-1})	Recovery (%) ± SD (n = 6)	Precision	Recovery (%) ± SD (n = 6)	Precision
0.1	89.3 ± 4.6	7.1	104.7 ± 11.3	9.7
0.1	86.4 ± 3.7	6.8	93.2 ± 8.2	9.2
0.5	79.3 ± 5.3	5.3	81.4 ± 7.7	7.6
0.5	82.5 ± 3.9	5.5	89.1 ± 8.2	7.9
1.0	92.6 ± 6.5	4.2	87.5 ± 7.2	6.5
1.0	93.7 ± 8.1	3.7	84.9 ± 8.6	5.7

Table 2 CAP concentrations in milk samples from a farm of Changying Diary Company (n = 5)

Samples	CAP (ng mL^{-1}, mean ± S.D.)		
	Electrochemical immunoassay	HPLC	GC-MS
1	0.097 ± 0.03	-	0.103 ± 0.06
2	20.29 ± 3.24	18.62 ± 1.63	21.46 ± 5.19
3	43.16 ± 5.52	44.85 ± 7.38	42.37 ± 6.83
4	132.5 ± 12.74	129.33 ± 11.54	130.59 ± 13.62

CONCLUSION

In this study, we established a voltammetric immunoassay system to detect the residue of CAP in milk samples. Based on the combination of Enzyme-Linked immunoabsorbant process along with voltammetry, this detection technique showed an advantage of high sensitivity and specificity. A lower detection limit is also obtained compared with traditional c-ELISA. Thus it can be used as an effective and practical tool for screening CAP residue in animal products.

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