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ORIGINAL PAPER

A new sensitive method for the detection of chloramphenicol in food using time-resolved fluoroimmunoassay

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Abstract A time-resolved fluoroimmunoassay (TRFIA) technique was developed to detect chloramphenicol (CAP) contamination in food. By using CAP-ovalbumin, anti-CAP antibodies, and europium-labelled goat anti-rabbit antibodies, an indirect, competitive method for CAP-TRFIA was established. The sensitivity was high, with a detection limit of 0.008 μg/L (8 ppt) for indirect competitive TRFIA formats. Testing showed that the sensitivity of the technique was 2.67 μg/kg in honey, prawn, and chicken muscle tissues, and 0.32 μg/L in milk. The detection range was between 0.008 and 100 μg/L: within this, the intra- and inter-batch coefficients of variation of the CAP-TRFIA method were 6.8 and 13.5 %, respectively. The study suggested that CAP-TRFIA was a simple, sensitive, and cost-effective method of screening large quantities of samples and had good prospects for further application.

Keywords Chloramphenicol (CAP) · Veterinary drug residues · Time-resolved fluoroimmunoassay (TRFIA) · Food

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Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic that is effective against both gram-positive and gram-negative organisms, rickettsiae, chlamydiae, and mycoplasmas. CAP has been widely used to treat a number of infections in cattle, poultry, and swine. However, this had led to the possibility of CAP residues being found in human food. The side effects include severe or fatal bone marrow depression, aplastic anaemia, and a syndrome of cyanosis and cardiovascular collapse known as the 'grey syndrome' [1]. It is therefore banned in many countries, including the USA, Canada, Australia, all EU member states, and China for use in animals used as food [2–7]. Therefore, the development of sensitive and simple methods for the detection of CAP was important. Several methods were proposed for the determination of CAP residual concentration in various matrices, such as high-performance liquid chromatography (HPLC) [8–10], gas chromatography (GC) [11–13], high-performance liquid chromatography-mass spectroscopy (HPLC–MS) [14, 15], and enzyme-linked immunosorbent assay (ELISA) [16, 17]. The development of immunochemical approaches has produced more sensitive, and faster, tools for the detection and quantification of CAP in a variety of contaminated samples. For example, ELISA, based on the monoclonal and polyclonal antibodies of CAP, has been widely applied in recent years. In this paper, a new analytical method for the detection of CAP—time-resolved fluorescence immunoassay (TRFIA) [18]—is described. It was based on the use of a europium (Eu)-labelled antibody. The detection limit of CAP-TRFIA was significantly enhanced compared with that of commercially available ELISA kits (0.05 ng/mL). The ELISA kits returned high false-positive rates, and their stability was poor, because

enzyme activity was affected by temperature, pH, and other factors. It was shown that CAP-TRFIA with high stability and an optimal range was the most sensitive of the assays reported and will thus be useful to screen CAP residuals simply and economically.

Materials and methods

Reagents and instrumentation

CAP, chloramphenicol succinate, thiamphenicol, penicillin, and norfloxacin were obtained from the Jiangsu Institute of Microbiology (Wuxi, China). Affinity purified goat anti-rabbit antibody was bought from Huamei Biotechnology Co. (Wuhan, China). The europium-labelling kit was purchased from PE-Life-Sciences (Turku, Finland). Dimethyl sulphoxide (DMSO), diethylenetriaminepentaacetate (DTPA), 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC), bovine serum albumin (BSA), and Tris were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Polystyrene 96-well microtitre plates were obtained from Nunc International (Roskilde, Denmark). The PD-10 column and Sepharose CL-6B were supplied by Pharmacia (Piscataway, NJ, USA). The β-naphthoyltrifluoroacetone (β-NTA) was synthesised in our laboratory. Female 3-week-old rabbits from New Zealand were used for the immunisation trials: these were obtained from a local supermarket. Other reagents used were of analytical grade.

A model DU-650 spectrometer from Beckman Coulter (Fullerton, CA, USA) was used for the detection of antibodies labelled with Eu^{3+}. An AutoDELFIA 1235 fluorometer from PE-Life-Science was used to measure Eu^{3+} fluorescence. A model 450 micro-plate reader from BIO-RAD (Hercules, CA, USA) was used for ELISA.

The buffer solutions used in this research were: a coating buffer (50 mM carbonate–bicarbonate, containing 0.9 % NaCl, and 0.05 % NaN_3, pH 9.6), assaying buffer (50 mM Tris–HCl, containing 0.9 % NaCl, 0.2 % BSA, 0.01 % Tween-20, 20 μM DTPA, and 0.05 % NaN_3, pH 7.8), a washing buffer (50 mM Tris–HCl, containing Tween-20, and 0.05 % NaN_3, pH 7.8), and a blocking buffer (50 mM Tris–HCl, containing 0.9 % NaCl, 1 % BSA, and 0.05 % NaN_3, pH 7.8).

Preparation of enhancement solution

Each litre of enhancement solution contained 15 μmoL β-NTA, 50 μmoL trioctylphosphine oxide (TOPO), and 1 mL Triton X-100, at pH 3.2. The working procedures have been previously described in detail [19].

Labelling secondary antibody (goat anti-rabbit IgG) with Eu^{3+}

Goat anti-rabbit antibodies labelled with europium were prepared according to the instruction manual. In brief, 2 mL of goat anti-rabbit antibody (10 g/L dissolved in 50 mM of PBS at pH 7.0) was loaded into a PD-10 column. The eluant used was a carbonate buffer (Na_2CO_3–$NaHCO_3$, pH 8.5). Next, 2 mg/500 μL of goat anti-rabbit antibody with changed buffer conditions was mixed with 1 mg DTTA-Eu^{3+}, and the mixture incubated for 20 h at 25 °C. The labelled antibody was separated and purified by gel filtration on a Sepharose CL-6B column with an elution buffer (50 mM Tris–HCl, containing 0.9 %, NaCl and 0.05 % NaN_3, and pH 7.8). The concentration of the europium-labelled protein was determined by spectrometer. The labelled antibody was stable for several months when stored in an amber bottle at −20 °C.

Synthesis of protein-CAP conjugates

CAP was coupled with carrier proteins BSA and OVA to prepare the complete antigen CAP-BSA and CAP-OVA by mixed anhydride (MA) reaction (Fig. 1). A succinyl derivation of CAP was prepared by mixing the CAP hapten with succinic anhydride in anhydrous pyridine and acetone with heating at 60 °C. The pyridine was removed *in vacuo* on a rotary flash evaporator, and the resulting colourless glass compound was dissolved in acetoacetate, and 1 M HCl was added to yield a hygroscopic white solid compound. This compound was dissolved in 10 % $NaHCO_3$. The solution was extracted with acetoacetate and evaporated in a rotary flash evaporator to obtain chloramphenicol succinate (CAP-HS). The CAP-HS was identified by HPLC–MS. 15.33 mg of CAP-HS was dissolved in 5.0 mL of dimethyl formamide and cooled to 4 °C for 10 min. To the cooled solution, some 9.5 μL of tributylamine was added, and the solution mixed. Then, 5.24 μL of isobutyl chloroformate was added to the mixture and cooled for 20 min at 4 °C. Concurrently, BSA (50:1 ratio of CAP-BSA) was dissolved slowly in 10 mL of a 50 % dimethyl formamide solution, cooled to 4 °C, and mixed. The pH of the BSA solution was slowly increased to 8.0 with 1 M NaOH, while the temperature was maintained at 4 °C. The BSA solution was rapidly added to the CAP-HS solution, stirred at 4 °C for 4 h, and maintained at pH 8.0. The reaction mixture was dialysed in pH 7.4 PBS for 3 days. CAP-OVA were synthesised by the same method. The coupling ratio of CAP to BSA was determined by ultraviolet scanning.

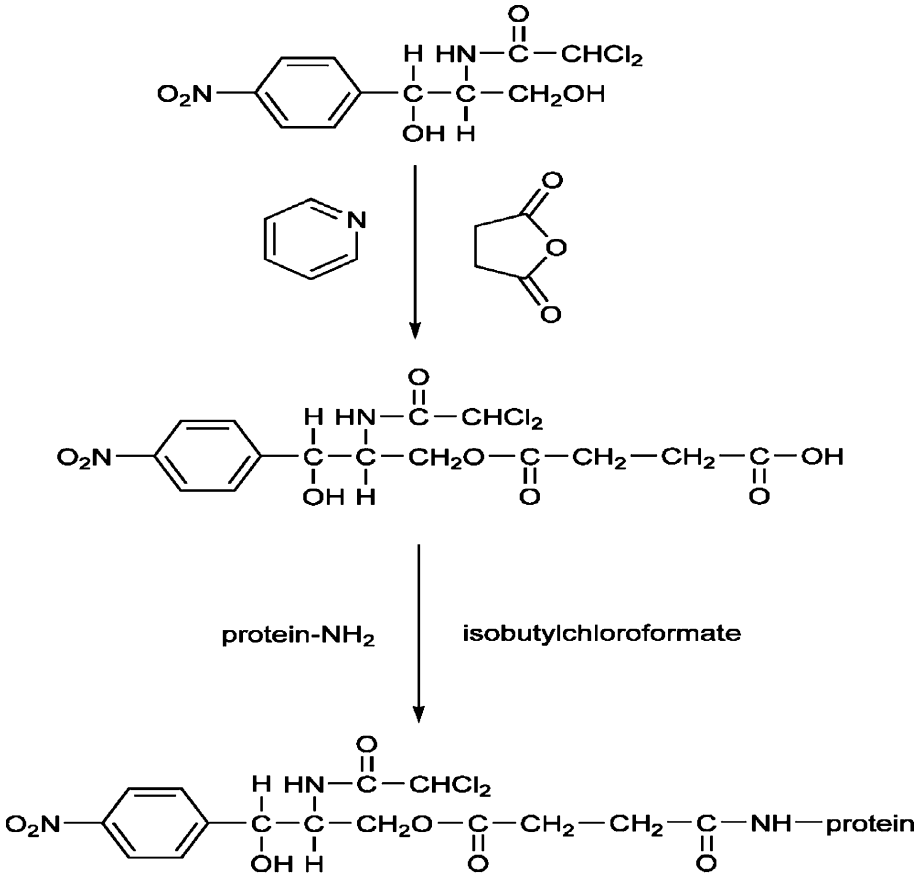

Fig. 1 Synthesis of mixed anhydride (MA) reaction

Preparation of antiserum

A series of four female New Zealand rabbits were immunised with the mixture of 1 mg CAP-BSA and the complete Freund's adjuvant *per* rabbit by intradermal multi-site injections. After 10 days, booster injections were given using the mixture of 1 mg CAP-BSA and incomplete Freund's adjuvant. After that, five additional boosters were given at regular intervals. After the third booster injection, 1 mL blood was collected some 3 days later to identify any antiserum dilution by CAP-ELISA. The blood was collected 7 days after the last booster and was allowed to clot at 37 °C for 2 h: the serum was collected by centrifugation.

Coating of the micro-plate wells

The micro-plate wells were coated overnight at 4 °C with 100 μL of a 2 μg/mL solution of CAP-OVA conjugate in the coating buffer. The plates were washed three times with washing buffer and then blocked with blocking buffer (150 μL/well) for 2 h in a plate shaker at 37 °C. After these steps, the blocking buffer was removed. The coated plates were preserved by rapid freezing and drying under a hard vacuum and stored at −20 °C until use.

Preparation of standard and buffer

A stock solution of CAP (1 mg/mL) was prepared in methanol. The stock solution was diluted to 100, 10, 1, 0.1, and 0.05 ng/mL. CAP buffer: 10 % (vol/vol) methanol and PBS (pH 7.4). Assay buffer: 8 mM NaCl, 0.1 % BSA, 50 μmol/L DTPA, 0.1 mL/L Tween-20, and 0.1 % NaN_3, pH 7.8 Tris–HCl. Washing buffer: 0.5 % Tween-20, 0.9 % NaCl, and 50 mM Tris–HCl (pH 7.8).

Preparation of samples

The samples of milk and honey were purchased from local stores. The milk and honey samples were pre-treated as follows [20]:

Milk: A sample (5 mL) was defatted by centrifugation for 15 min (2,000*g* at 4 °C). A 2.5 aliquot of defatted sample was transferred to a glass tube, and 5 ml of ethyl acetate was added, and the mixture agitated on a mini-shaker for 1 min. After a 10 min delay to allow phase separation, 4 mL of the upper layer (ethyl acetate) was evaporated to dryness at 50 °C under a nitrogen stream. The residue was dissolved in 200 μL of the dilution

buffer provided with the TRFIA kit, and 50 μL portions were used for subsequent tests.

Honey: A 3 g of sample was weighed into a polypropylene tube and solubilised in 3 mL of ultrapure water using a mini-shaker. A 6-mL volume of acetone/dichloromethane (1:1, v/v) was added. The mixture was then manually shaken for 2 min. After centrifugation (2,000*g* for 5 min), 4 mL of the upper layer was evaporated at 50 °C under a nitrogen stream. The residue was dissolved in 1 mL of the dilution buffer provided with the TRFIA kit, and 50 μL portions were used for subsequent tests.

Chicken and prawn: A 3-g sample was homogenised with a 6 mL volume of acetone/dichloromethane (1:1, v/v). The mixture was then manually shaken for 2 min. After centrifugation (2,000*g* for 5 min), 4 mL of the upper layer was evaporated at 50 °C under a nitrogen stream. The residue was dissolved in 1 mL of the dilution buffer provided with the TRFIA kit, and 50-μL portions were used for subsequent tests.

Indirect competitive TRFIA of CAP

The indirect competitive assay was performed as follows: 50 μL of standard or diluted sample extract (see above) and 50 μL of CAP antibody were added to the well. The immunoreactions were allowed to proceed with shaking for 1 h at 25 or 37 °C, and the plate was washed three times with washing buffer. Then, 100 μL Eu^{3+}-labelled goat anti-rabbit antibody was added to the well and shaken for a further 1 h. The plate was washed six times, and 100 μL enhancement solutions were added. The solution was then incubated for 5 min. The fluorescence was measured with a fluorometer (AutoDELFIA 1235). The concentration of CAP in the sample was determined from standard curves.

Some ingredients in the samples may have affected the reaction between the antigen and antibody. To reduce matrix interference, the optimal dilution ratio for the spiked samples was first sought.

Matrix effect (integrity)

The curves obtained from normal integrity experiments, in which there was no matrix interference, should overlap, or be parallel to, the standard curves. If there were any interfering substances present, then the experimental values would show disproportionate increases. Food samples containing certain amounts of CAP were prepared and serially diluted. The diluted samples were used for the CAP-TRFIA assay, and the measured results plotted for comparison with the standard curves.

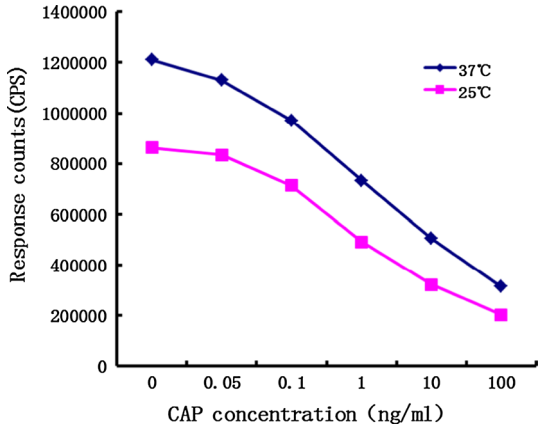

Fig. 2 Standard curve of CAP-TRFIA at different incubation temperatures

Results

Production of antibody

CAP is a molecule with a low molecular weight. Coupling of this happens to carrier proteins and is required to induce a better specific immune response. The conjugate BSA was produced and used to immunise the rabbits. Sera obtained from these rabbits were monitored with non-competitive ELISA for antibody production. Analysis conditions were optimised by means of a 'checkerboard' titration. After 12 weeks, antibodies were produced and antisera against BSA were collected from those rabbits. The polyclonal antibodies can be used at a dilution exceeding 1:6,000. As a result, good antigen titration was achieved.

Optimisation of CAP-TRFIA

Incubation temperature and time significantly influenced the reaction. For this research, a contrast test was carried out at 25 and 37 °C which are temperatures frequently used for incubation in other methods. The results are shown in Fig. 2. When the temperature is 37 °C, the reaction time was significantly reduced, but the slope of the standard assay curve showed no significant difference to that at 25 °C. Thus, 37 °C was selected as the incubation temperature.

Then, at 37 °C, different incubation times (15, 30, 45, and 60 min) were compared. These results are shown in Fig. 3. The results showed that long incubation times could enhance the sensitivity of the assay. However, when the incubation time exceeded 45 min, the fluorescence intensities of all of the standard points reached a dynamic equilibrium. Therefore, 45 min was selected as the first incubation time and 30 min as the second incubation time.

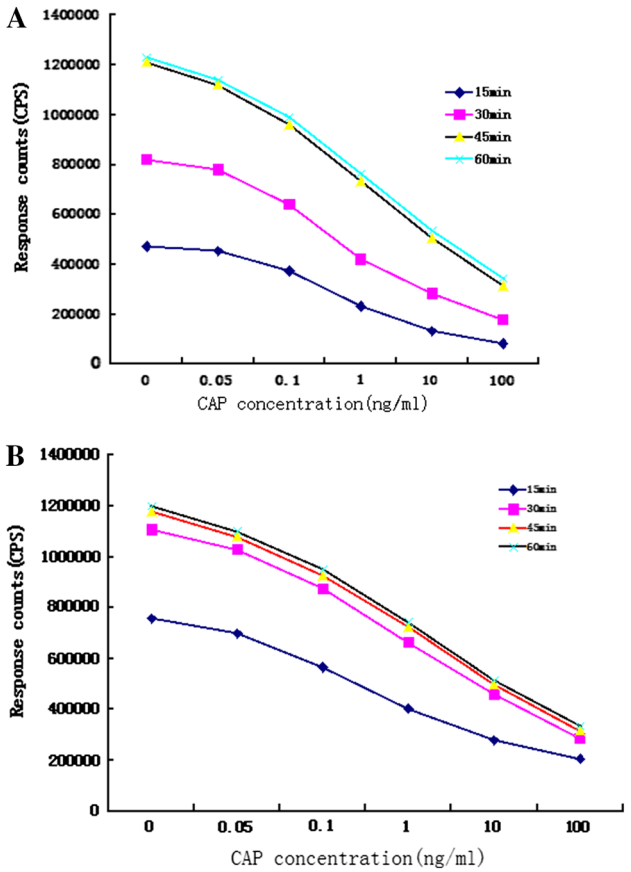

Fig. 3 Standard curve of CAP-TRFIA at different incubation times **a** the first incubation time, **b** the second incubation time

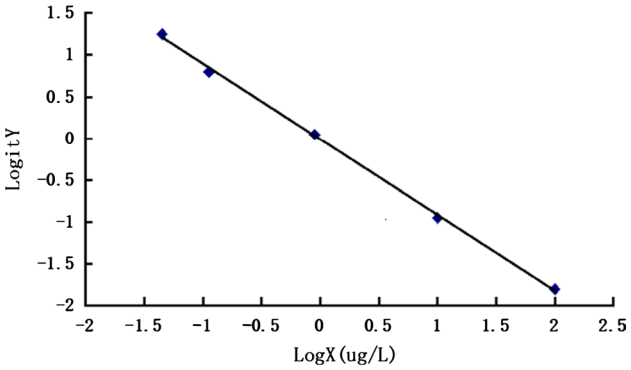

Fig. 4 Calibration curve of indirect competitive CAP-TRFIA

Table 1 Recovery of CAP from milk samples as determined by TRFIA

CAP added (μg/L)	CAP detected (μg/L)	Recovery (%)	RSD (%)
0	1.20	–	6.3
0.32	1.44	75.0	7.4
0.64	1.71	79.7	7.6
0.96	1.98	81.2	5.7

Table 2 Recovery of CAP from honey samples as determined by TRFIA

CAP added (μg/kg)	CAP detected (μg/kg)	Recovery (%)	RSD (%)
0	7.82	–	8.1
2.67	10.52	101.1	6.9
5.34	12.85	94.2	4.7
8.01	15.88	100.6	5.5

Evaluation of the CAP-TRFIA technique

The sensitivity, detection range, recovery rate, reproducibility, and stability of CAP-TRFIA were analysed. The results from indirect competitive CAP-TRFIA experiments were processed by plotting standard curves (Fig. 4) using the log–logit function, $\log it(Y) = \ln\left[Y/(1-Y)\right]$, where $Y = B/B_0$ and B_0 corresponded to the fluorescence count at maximum binding (or the fluorescence count at zero concentration).

On the standard curve, the concentration corresponding to the fluorescence count $X - 2SD$, where X represents the fluorescence count at zero concentration, was used to determine the sensitivity of the assay. For this study, the sensitivities were 0.008 μg/L for indirect competitive TRFIA formats with a detection range of 0.008–100 μg/L, 2.67 μg/kg in honey, prawn, and chicken muscle tissues, and 0.32 μg/L in milk.

The reproducibility of the CAP-RFIA was evaluated using the mean coefficient of variation (CV). Within the detection range, the intra- and inter-batch CVs of the CAP-TRFIA were 6.8 and 13.5 %, respectively.

Recoveries were obtained using the same method and different food samples. Four kinds of food samples were spiked with CAP at different concentrations and analysed by the aforementioned indirect competitive TRFIA method. The results are summarised in Table 1, 2, 3 and 4. The recovery from milk was found to be a little low, and it may have been that the matrix of the sample interfered with the reaction.

The cross-reactivity of the CAP-TRFIA with chloramphenicol succinate was 19.28 %, while that with thiamphenicol was <0.1 %, and that with penicillin and norfloxacin was <0.01 %.

The concentrations, causing 80, 50, and 20 % inhibition (ED80, ED50, and ED20) of CAP-TRFIA experiments, were (25.75 ± 0.442) ng/ml, (0.917 ± 0.081) ng/ml, and (0.033 ± 0.0016) ng/ml, respectively. The inter-batch CVs

Table 3 Recovery of CAP from prawn samples as determined by TRFIA

CAP added (μg/kg)	CAP detected (μg/kg)	Recovery (%)	RSD (%)
0	3.89	–	7.5
2.67	6.29	89.9	6.7
5.34	8.99	95.5	5.5
8.01	11.42	94.0	4.9

Table 4 Recovery of CAP from chicken samples as determined by TRFIA

CAP added (μg/kg)	CAP detected (μg/kg)	Recovery (%)	RSD (%)
0	4.41	–	7.4
2.67	7.15	102.6	6.9
5.34	9.62	97.6	4.4
8.01	12.33	98.9	5.7

determined for these three effective dose points were all below 10 %, indicating a small drift in the dose–response curves and, therefore, good assay stability. After storing the assay reagent at 37 °C for 7 days, the binding ratio at each concentration decreased by an average of 13.25 %. This indicated that the shelf life of a commercial kit would meet the requirements for practical application.

Analysis of matrix interference

The diluted extract from dry samples and diluted food (negative confirmed by LC-MC) were spiked with CAP (100 ng/mL). To evaluate and correct for matrix interference, samples were diluted to different standard points. Diluted samples were then tested by the CAP-TRFIA method established above, and the results were compared with the standard curve. The results are shown in Fig. 5.

If there was little, or no, matrix interference in a sample, the curve from the dilution experiment should be coincident with, or parallel to, the standard curve of CAP-TRFIA. The results showed that the interference in the milk, honey, prawn, and chicken matrices appeared to be negligible.

Discussion

A new indirect competitive TRFIA to detect CAP in foods was established. First, the reaction conditions were optimised. It was found that a high temperature can reduce the reaction time, and a long incubation time can be used to increase sensitivity. Subsequently, 37 °C was selected as the incubation temperature and 45 min as the first incubation time and 30 min as the second incubation time.

For the CAP-TRFIA established by the present work, the sensitivity was high, with a detection limit of 0.008 μg/L. Its detection range was 0.008–100 μg/L. Tests showed that the sensitivity was 2.67 μg/kg in honey, prawn, and chicken, and 0.32 μg/L in milk. Within the detection range, the intra- and inter-batch CVs of the CAP-TRFIA were 6.8 and 13.5 %, respectively.

Four kinds of food samples spiked with CAP were analysed by the aforementioned CAP-TRFIA procedure: the recoveries from milk were found to be a little low, perhaps because the matrix of the sample interfered with the reaction. The tests also showed that this method had good

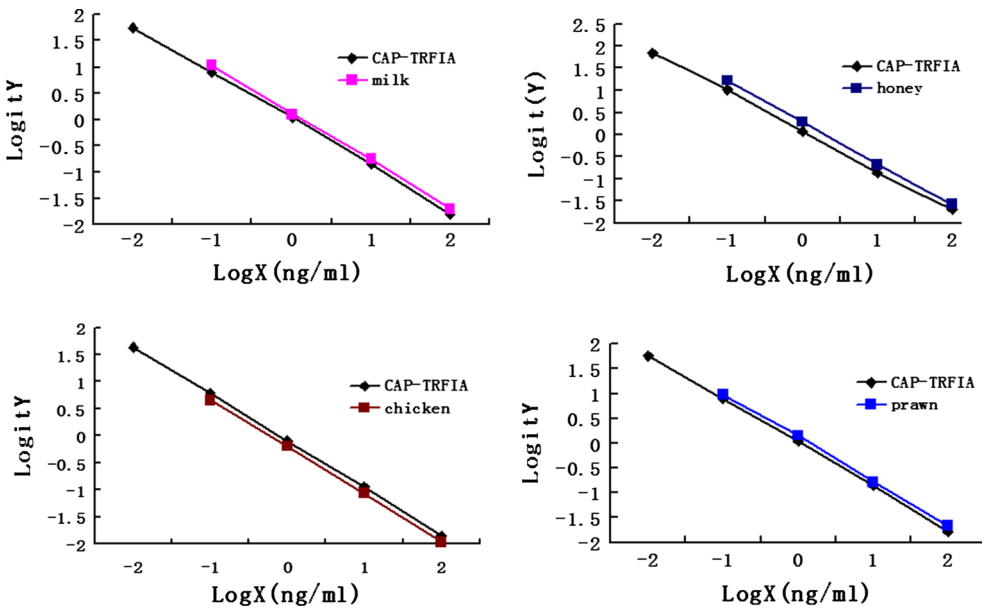

Fig. 5 Dilution curves for milk, honey, chicken, and prawn by indirect competitive CAP-TRFIA

reproducibility and stability. Furthermore, the assay did not cross-react with thiamphenicol, penicillin, or norfloxacin. Also, it was found that the interference of the milk, honey, prawn, and chicken matrices appeared to have been negligible. The results reported in this study suggested that a simple and sensitive method for the analysis of CAP had been established. In terms of practical use, the method could be applied to the rapid detection and estimation of CAP quantities in food samples containing very small amounts of CAP. The detailed results of such an application of this method will be reported in the future.

With the increased demand for food safety, the standard limit for CAP has been revised. In general, the concentration limit of CAP is stipulated as being approximately 2.67–33,375 μg/kg in honey, prawn, and chicken, and 0.32–4,000 μg/L in milk. An ideal detection method should be applicable to both food and feed. Due to their methodological limitations, ELISA kits are not sufficiently sensitive, or do not have the necessary scope, to completely meet practical demands. In addition, CAP requires different detection reagents. Considering the aforementioned shortcomings, the CAP-TRFIA technique developed in this study has many advantages including high sensitivity and a wide measurement range.

Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human subjects. All institutional and national guidelines for the care and use of laboratory animals were followed.

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