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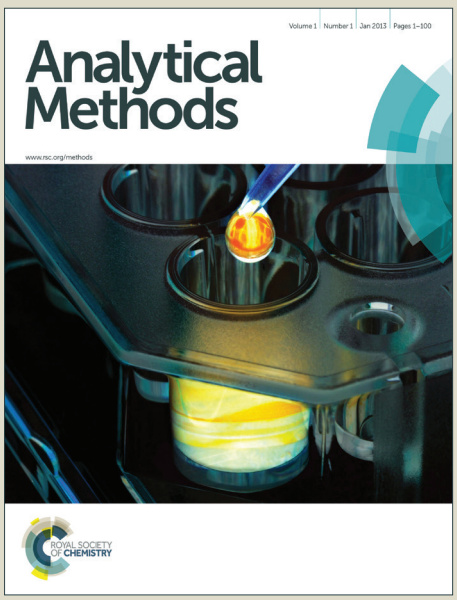

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Approaches for determination of florfenicol and thiamphenicol in pork using a chemiluminescent ELISA

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Abstract

A chemiluminescent competitive indirect enzyme-linked immunosorbent assay (CL-ciELISA) for detection of florfenicol (FF) and thiamphenicol (TAP) residues in pork has been first developed. The 50% binding inhibition (IC_{50}) value of the CL-ciELISA was 0.15 μg ŀkg^{-1} for FF with the cross-reactivity of 48.4% for TAP. FF and TAP were co-extracted from pork with ethyl acetate, obtaining recoveries of 80.0-93.3% (FF) and 80.0-88.3% (TAP) above the limit of detection (LOD). The LODs were 0.015 μg ŀkg^{-1} for FF and 0.030 μg ŀkg^{-1} for TAP, respectively. Moreover, 20 field pork samples were analyzed with the developed CL-ciELISA and the results correlated well with those obtained using traditional ELISA and a previously reported liquid chromatography–tandem mass spectrometry (LC-MS/MS), confirming the utility of CL-ciELISA for quantitation of FF and TAP in pork with a good accuracy and reliability. Moreover, the CL-ciELISA method has been first established for detection of FF and TAP by far.

Introduction

Florfenicol (FF), thiamphenicol (TAP) and chloramphenicol (CAP), broad spectrum antibiotics with similar structural formulas (Table 1), are widely applied in veterinary practice for prevention and treatment of many bacterial infections. However, CAP is a hemotoxic substance for humans and can cause bone-marrow depression, aplastic anaemia and acute leukaemia,[1] and consequently it has been banned from use in food-producing animals in China, USA and EU.[2,3,4] TAP, a methyl–sulfonyl analogue of CAP and less toxic, could induce haematological changes in the mouse and rat, paralleling the dose-dependent, reversible marrow depression reported in man.[5] Based on toxicological studies, a maximum residue limit (MRL) for TAP was set at 20.0-50.0 μg kg^{-1} for all food-producing species in the target tissues of muscle, fat, liver, kidney and milk in China, EU and Japan.[2, 6, 7] FF is a derivative of TAP and synthesized by substitution of a fluorine atom for the hydroxyl group at the 1, 3-propandiol moiety. However, the use of FF in animal husbandry has the potential to result in the presence of residues in tissues and the increased emergence of resistance of pathogenic bacteria that could have potential health risks to humans.[8] The MRLs for FF and/or its metabolite florfenicol amine (FFA) in various food-producing animals are fixed at 100.0-3000.0 μg kg^{-1} by many countries or organizations.[2, 6, 9] In particular, FF is used as the marker residue for pork in USA, Taiwan, Japan, and Canada.[10, 11] Hence, it is of great importance to develop sensitive, reliable and available methods for FF and TAP detection in animal-derived food samples for ensuring food safety.

Various instrumental methods have been described for determination of TAP and/or FF in foods of animal origin, including gas chromatography (GC), [12] gas chromatography–mass spectrometry (GC–MS), [13] liquid chromatography–mass spectrometry (LC–MS), [14] liquid chromatography tandem mass spectrometery (LC-MS/MS),[15,16,17,18,19] surface molecularly imprinted Sol–Gel polymer, [20,21] and copolymer of divinylbenzene and N-vinylpyrrolidone.[22] Several immunoassays for detection of FF have been recently reported. [23,24] However, there were only two reported methods for analysis both of FF and TAP in one immunoassay. [25,26] We have previously published two articles for determination of FF and its metabolite FFA in animal meat products, [27,28] in which the polyclonal antibodies can bind with FF and FFA with high cross-reactivity (CR), only with CR of 4.0% for TAP, then not suitable for determination of TAP in animal meat products. The objective of this work was to develop a rapid, sensitive, routine and selective method for the determination of both of residual FF and TAP in pork. The chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) offers the possibility of improving the sensitivity of immunoassay to at least 2-3 orders of magnitudes compared to conventional colorimetric detection. The light intensity of enhanced chemiluminescence reaches a maximum within 3 min, thus providing rapid detection of the analytical signal. These advantages of chemiluminescent techniques make them useful system for detecting trace residue of TAP and FF in animal products. In this study, in order to improve the sensitivity of immunoassays, a firstly reported chemiluminsecent competitive indirect ELISA (CL-ciELISA) for the determination of trace FF and TAP applicable in pork

was developed and optimized (Figure 1).

Materials and methods

Materials and Reagents

(a) Apparatus

Chemiluminescence was measured with Veritas Microplate Luminometer (Turner BioSystems, Sunny Vale, CA, USA). The colorimetric ELISA was measured by Sunrise microtiter plate reader (TECAN, Groedig, Austria). Transparent 96-well microtiter ELISA plates for colorimetric assay and 96-well chemiluminescent opaque high binding plates were purchased from Costar (Cambridge, MA, USA). UV-Vis spectrophotometer (Model 751GW) was from Shanghai Analytical Instrument (Shanghai, China). All buffers were prepared using Milli-Q H_2O system (18 MΩ/cm) (EMD Millipore Corporation, Belleria, MA, USA).

(b) Buffers

Coating buffer (CB, pH 9.6) was made with 1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$ in 1 L of purified water. A 0.01M phosphate-buffered saline (PBS, pH 7.4) was prepared by dissolving 8.0 g NaCl, 0.2 g KCl, 0.24 g KH_2PO_4, and 3.63 g $Na_2HPO_4 \cdot 12H_2O$ in 1 L of purified water. Blocking buffer was prepared by 0.01 M PBS and 1% BSA (pH 7.4). PBST was made with 0.01 M PBS and 0.05% Tween-20. A 0.02 M sodium phosphate (PB, pH 7.4) was prepared with 1.1 g $NaH_2PO_4 \cdot 2H_2O$ and 5.16 g $Na_2HPO_4 \cdot 12H_2O$ in 1 L of purified water.

(c) Standards

FF, TAP, CAP and FFA were puchased from Schering-Plough Corp. (Kenilworth, NJ, USA); clenbuterol (CLE), sulfadiazine (SUL), ciprofloxacin (CIP), penicillin (PEN) were purchased from Shanghai Caienfu Technology Co. Ltd. (Shanghai, China). The stock solution (2 mg·mL^{-1}) was stored at -20 ℃, and working standards in the 0.015–16.2 μg·L^{-1} range were prepared from the stock solution by serial dilution in 0.02 M PB. The CAP, FF and FFA, stock solutions were prepared in methanol; CLE and SUL were prepared in ethanol; CIP and PEN were prepared in purified water; TAP was prepared in dimethylfomamide.

(d) Analytical grade regents

N-hydroxysuccinimide (NHS, Catalog# 130672), N, N-dicyclohexylcarbodiimide (DCC, Catalog# 36550), N, N-dimethylformamide (DMF, Catalog# D4551), human serum albumin (HAS, Catalog# A6608), ovalbumin (OVA, Catalog# S7951), Freund's complete (Catalog# F5881) and incomplete adjuvants (Catalog# F5506), goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (Catalog# SAB3700972) and tetramethylbenzidine (TMB, Catalog# 860336) were purchased from Sigma (St. Louis, MO, USA). All other chemicals and organic solvents were of reagent grade and were from Beijing Chemical Co. (Beijing, China).

(e) The chmiluminescence substrate solution called Super Signal was purchased from Pierce (Rockford, IL, USA).

Preparation of immunogen and coating antigen

The haptens were synthesized by coupling FF with succinic anhydride (SH),[29] or maleic anhydride (MH).[30] Briefly, a solution of FF (3.57 g, 10 mmol) in dry pyridine (30 mL) was added to SH (3.0 g, 30 mmol) at room temperature. After stirring for 24 h, the mixture was concentrated with a rotary evaporator. The residue was dissolved with ethyl acetate (40 mL) and then was washed 3 times with hydrochloric acid (0.1 M, 40 mL) followed by purified water (40 mL) for 3 times. The solvent was evaporated and dried in vacuum for 48 h. The FF-SH was obtained as a pink solid. In the same way, FF-MH was obtained as a white solid.

The hapten FF-HS was coupled to HSA by the active eater method to prepare immunogen (FF-HS-HSA).[29] Briefly, the hapten FF-HS (50 mg, 0.11 mmol) dissolved in DMF (4 mL) was mixed with NHS (27 mg, 0.23 mmol) and DCC (49 mg, 0.24 mmol). The mixture was stirred for 2 h at room temperature. HSA (50 mg, 0.00073 mmol) was dissolved in 14 mL of PBS to which 2 mL of DMF was added. The solution of an active ester was added dropwise to the stirred protein solution. This solution was stirred overnight and dialyzed against PBS (0.01M) for 72 h at 4 ℃. Then the solution was centrifuged at 1,000 g for 5 min at 4 ℃ to discard sediment. The coupling ratio of FF-HS to HSA was approximately 10:1 and the concentration of FF-HS-HSA was 8.4 mg $\cdot$ mL^{-1}.

The coating antigens, FF-MH-OVA, were synthesized by mixed anhydride reaction.[31] These contents were dialyzed against PBS (0.01M) for 72 h at 4 ℃ and then centrifuged at 1,000 g for 5 min at 4 ℃ to discard sediment. The coupling ratio of

FF-MH to OVA was approximately 7:1 and the concentration of FF-HS-OVA was 9.0 mg mL^{-1}.

Production of polyclonal antibodies (PAb)

The procedures of antibody production and characterization of PAb were the same as that reported by Luo.[25] Especially, after five booster injection, the sera were collected and purified by ammonium sulfate precipitation. The concentrated PAb solution was then supplemented with an equal volume of glycerol and stored at -20 ℃ until testing. The concentration of the PAb was 17.6 mg mL^{-1}.

Procedure of CL-ciELISA and traditional ELISA

Opaque high binding plates were coated overnight at 4 ℃ with 100 μL of FF-MS-OVA dissolved in coating buffer (0.15 μg mL^{-1}). The plates were washed with 260 μL/well PBST manually three times and blocked with 200 μL/well of blocking buffer and the plates were incubated at 37 ℃ for 1 hour. After the plates were washed as described above, then 100 μL/well of standard in 0.02 M PB or sample solution, followed by 50 μL/well of anti-FF PAb at a dilution of 1/100,000 in 0.02 M PB were added, respectively. The competitive reaction was allowed to take place for 30 min at room temperature. After washing five times, a 100 μL/well peroxidase-labeled goat anti-rabbit immunoglobulins (1/5000 dilution of in PBST) was added, and plates were incubated at 37 ℃ for 30 min. After washing five times, the peroxidase activity was revealed by adding 100 μL/well of a freshly prepared substrate mixture of Super

Signal substrate solution. The intensity of light emission was measured at 425 nm with a chemiluminesence reader immediately after the addition of chemiluminescence substrate and results were expressed in relative light units (RLU).

The procedure of traditional ELISA was the same as CL-ciELISA procedure described above except the substrate addition and the measurement. The transparent 96-well microtiter ELISA plates were incubated for 15 minutes at room temperature after addition of 100 μL/well TMB substrate, and the absorbance was measure at 450 nm after stop-solution addition (50 μL/well 2.0 M H_2SO_4) .

Optimization of CL-ciELISA

Several physicochemical factors influencing immunoassay performance were investigated in CL-ciELISA. In order to assess the influence of buffer ionic strength, Tween-20, pH and competitive time and temperature, standard curves and PAb were prepared as follows: (1) PAb and standards were added to serial dilutions of Tween-20 (from 0 % to 0.1 %, v/v) in 0.02 M PB (pH 7.4); (2) Standards and a constant concentration of PAb was diluted by PB (pH 7.4) at different concentrations (0.01, 0.02, 0.05, 0.1, 0.2 mol/L); (3) Standards and a constant concentration of PAb in 0.02 M PB at different pH values (from 6.6 to 8.0). Meanwhile, the competitive reaction was taken place at room temperature or 37 ℃ for 30, 45, 60, 75 min, respectively.

Data analysis

Standards and samples were run in quadruplicate wells, and mean chemiluminescence intensity values were divided by RLU_{max} (chemiluminescence intensity in the absence of analyte). The ratio is defined as B/B_0. Standard curves were obtained by plotting B/B_0 against the logarithm of analyte concentration and fitted to a four-parameter logistic equation using Origin (version 8.0, Microcal, USA) software packages

$$y = \{(A-D)/[1+ (x/C)^B]\} +D$$

where A is the asymptotic maximum 1, B is the curve slope at the inflection point, C is the x value at the inflection point (corresponding to the analyte concentration that reduces RLU_{max} to 50%, corresponding that the value of B/B_0 is 0.5), and D is the asymptotic minimum ($RLU_{background\ signal}$/ RLU_{max}).

Cross-reactivity (CR)

The specificity of the PAb was assessed by evaluating the extent of CR with three compounds structurally related and four another structurally unrelated to FF in optimized CL-ciELISA and their IC_{50} values were compared to IC_{50} of FF. CR was calculated as follows:

$$CR\% = IC_{50,\ FF}/IC_{50,\ cross\text{-}reactant} \times 100\%$$

Sample preparation

A 3 g pork sample was homogenized and mixed with 3 mL of double distilled water in a 50 mL tube. After vortexing for 1 min, 6 mL of ethyl acetate was added and the

mixture was shaken for 10 minutes at room temperature, then centrifuged at 4000 g for 10 min, 4 mL of the organic supernatant was dried by nitrogen at 60 ℃. The residue was dissolved in 2 mL of 0.02 M PB and 1 mL of hexane. The mixture was vortexed gently for 1 min. After centrifugation for 5 min at 4000 g, the lower fraction was transferred to a new tube. Then 100 μL of the solution was added to opaque high binding plate microtiter wells for measurement.

Analysis of field pork samples

Twenty pork samples were collected from retail outlets in Chongqing. The samples were homogenized and stored at −20 ℃ until use. Each sample was divided into three portions; one was analyzed by the CL-ciELISA, one was analyzed by traditional ELISA and the third by LC-MS/MS. LC-MS/MS analysis of FF and TAP was adopted according to previous report.[32]

Results and discussion

Specificity of the PAb

The PAb from the rabbits showed high titer (1:100,000) in CL-ciELISA. The CR of some related compounds such as FFA, TAP and CAP were tested. There is no significant CR except for TAP with 48.4% and FFA with 0.3% (Table 1). The CR of other structurally unrelated drugs including CLE, SUL, CIP, PEN were also tested. No CR was observed. The PAb in our previous studies [27, 28] can bind with FF (CR of 100% in both studies) and FFA with high CR of 134.6% and 81.2, only with CR of

4.0% and 4.4% for TAP, respectively. The different specificities for determination of the FF, FFA and TAP could be explained by the different immunogens and coating antigens-FFA-formaldehyde–BSA (FFA-F-BSA) and FF-glutaric anhydride–ovalbumin (FF-G-OVA) in our previous studies,[27,28] FF-succinic anhydride-human serum albumin (FF-HS-HSA) and FF–maleic anhydride–OVA (FF-MH-OVA) in this study.

Physicochemical parameter optimization

The RLU_{max}/IC_{50} ratio had been shown to be a useful parameter to estimate the effect of a certain factor on the CL-ELISA performance. The highest ratio indicated highest sensitivity.[33] In this study, optimum parameters of the established CL-ciELISA were 0.15 μg mL^{-1} per well of coating antigen FF-MS-OVA, 30 minutes of competition time and the use of 0.02 M PB (pH 7.4) as a PAb (1:100,000 dilution) and standard analyte diluent buffer (data not shown). Under these conditions, higher RLU_{max} and lower IC_{50} values representing optimal assay conditions were obtained.

Assay sensitivity

The sensitivities of the developed CL-ciELISA for FF and TAP represented by IC_{50} values, were 0.15 μg L^{-1} and 0.31 μg L^{-1}, respectively. The linear working range for FF determined as the concentrations causing 20%—80% inhibition of chemiluminescence intensity was 0.028—4.77 μg L^{-1} (Figure 2). The sensitivity of the CL-ciELISA for FF was about 14 times greater compared to traditional ELISA method with colorimetric detector developed by our own study (IC_{50} =2.13 μg L^{-1})

(Figure 2), about 6.8 times more sensitive compared to the colorimetric ELISA (IC_{50} =1.02 μg Ł$^{-1}$) in previous report,[25] about 166.7 times more sensitive compared to the lowest IC_{50} (25.0 μg Ł$^{-1}$) in previous report.[26] Moreover, the IC_{50} values for FF in our previously published articles about determination of FF and its metabolite FFA in animal meat products, [27, 28] were 0.21 μg Ł$^{-1}$ and 0.24 μg Ł$^{-1}$, respectively.

Matrix effect elimination

To apply a new method in real sample analysis, a matrix effect is an important issue to be considered, especially in animal tissues due to the complicated matrix. The simplest way to overcome such a problem was diluting and/or masking the matrix effect with the same or similar matrix. In this study, interferences are quantified by comparing a standard inhibition curve of FF (0, 0.01, 0.05, 0.2, 0.6, 1.8, 5.4, 16.2 μg Ł$^{-1}$) with a standard curve of FF generated in the blank pork extract matrix (0, 0.01, 0.05, 0.2, 0.6, 1.8, 5.4, 16.2 μg Ł$^{-1}$). The IC_{50} values for CL-ciELISA and traditional ELISA were 0.15 and 0.16 μg Ł$^{-1}$, respectively. The linear working ranges for FF determined as the concentrations causing 20%—80% inhibition for both assays were 0.028—4.77 μg Ł$^{-1}$ and 0.029—4.84 μg Ł$^{-1}$, respectively. The superimposition of the calibration curves suggested that there was no significant matrix effect (Figure 3). Then, the pork samples can be analyzed using the standard inhibition curve instead of the matrix curve.

Limit of detection

The limit of detection (LOD) was calculated as the mean of the measured content of blank pork samples (n = 20) plus three standard deviations (mean + 3SD). The each of 20 blank pork samples was obtained by 20 different animals and analyzed according to the developed CL-ciELISA. The LOD for FF was 0.015 μg ŀkg^{-1} and the LOD for TAP was 0.03 μg ŀkg^{-1} (data not shown). The good performance of developed CL-ciELISA was good enough to screen the trace FF and TAP residues in pork.

Precision and recovery

To confirm that the developed CL-ciELISA performed well around the LOD, the blank pork samples were fortified at 0.0075 (1/2 LOD), 0.015 (LOD) and 0.03 (2 LOD) μg ŀkg^{-1} with FF, and 0.015 (1/2 LOD), 0.03 (LOD), and 0.06 (2 LOD) μg ŀkg^{-1} with TAP prior to analysis, respectively. All samples fortified at 0.015 and 0.030 μg ŀkg^{-1} for FF, and 0.030 and 0.060 μg ŀkg^{-1} for TAP resulted in positive readings. Each sample was evaluated 10 times in duplicate and on three consecutive days to verify the repeatability. The average intra-assay and inter-assay recoveries of FF and TAP in the pork fortified at concentrations greater than or equal to the LOD were at least 83.3 %, with coefficients of variation (CV) less than 15%. However, the recovery of FF and TAP from pork samples fortified at a concentration of 1/2 LOD was highly variable (percent recoveries ranged from 60.0% to 173.3% with the CV of 36.4%-100.0%) (Table 2). Hence, the developed CL-ciELISA could detect the presence of FF above the LOD (0.015 μg ŀkg^{-1}) and TAP above the LOD (0.030 μg ŀkg^{-1}) and will eliminate the possibility of false-positive and false-negative results.

Moreover, two more concentrations between the working range (around IC_{50} and 80% inhibition) for each of FF (0.15 μg kg^{-1} and 1.5 μg kg^{-1}) and TAP (0.3 μg kg^{-1} and 3.0 μg kg^{-1}) were selected to evaluate the recovery and precision. The intra-assay and inter-assay recoveries of FF and TAP were in the range of 80.0–86.7%, respectively. The CVs of intra- and inter-assay with FF and TAP ranged from 7.5% to 10.8% and from 8.0% to 12.5%, respectively (Table 2).

Analysis of FF and TAP in field pork samples

To evaluate determination capability of the developed CL-ciELISA, 20 field pork samples were analyzed by the developed CL-ciELISA, traditional ELISA established in this study and LC-MS/MS with the LODs of 0.2 μg kg^{-1} for FF and 1.0 μg kg^{-1} for TAP (Table 3).[32] In Table 3, the results of field pork samples (for example P1, 3.7±0.2 μg kg^{-1} VS 3.5±0.2 μg kg^{-1}) measured by CL-ciELISA and traditional ELISA, were consistent. However, the CL-ciELISA may underestimate the FF+TAP residue concentrations when compared to those produced by the LC-MS/MS method (for example P11, 9.8±0.8 μg kg^{-1} VS 6.8 μg kg^{-1} for FF and 8.9 μg $kg^{-1}$for TAP). This underestimation occurred because the sum of FF and TAP was represented as μg FF/kg with the CR of 48.4% for TAP. The apparently limitation of the CL-ciELISA does not affect its usefulness as a screening tool because it will still indicate the presence of FF+TAP above their detection limits. The results demonstrated that the developed CL-ciELISA could screen FF and TAP in the incurred samples as the LC-MS/MS and traditional ELISA did. Thereafter, the developed CL-ciELISA was

reliable for screening of trace FF and TAP residues in pork.

Conclusion

Chemiluminescent detection has been proved to be an effective analytical technique for use in veterinary drugs monitoring owing to its high sensitivity, low cost and ease of handling. We have firstly developed a sensitive CL-ciELISA for quantitation of FF and TAP in pork with a good accuracy and reliability, which makes it a useful tool for screening purposes.

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Legends of Figures and Tables

Figure 1 The scheme of CL-ciELISA for determination of FF and TAP in pork

Figure 2 Normalized standard curve of CL-ciELISA for FF under optimized conditions compared to the standard curve obtained by traditional ELISA for FF

The standard inhibition curve concentrations of FF for CL-ciELISA were 0, 0.01, 0.05, 0.2, 0.6, 1.8, 5.4, 16.2 μg L^{-1} and for traditional ELISA were 0, 0.1, 0.5, 2.0, 6.0, 18.0, 54.0, 162.0 μg L^{-1}. The IC_{50} values for CL-ciELISA and traditional ELISA were 0.15 and 2.13 μg L^{-1}, respectively. The linear working ranges for FF determined as the concentrations causing 20%—80% inhibition for both assays were 0.028—4.77 μg L^{-1} and 0.33—45.59 μg L^{-1}, respectively.

Figure 3 Inhibition curves of FF in 0.02 M PB and extraction of pork matrix

Table 1 Percentage of cross reactivities of some structurally related and unrelated compounds in CL-ciELISA

Table 2 Intra- and inter-assay variations of pork spiked with FF and TAP

Table 3 Determination of field pork samples collected from retail outlets in Chongqing by the CL-ciELISA, traditional ELISA and LC-MS/MS

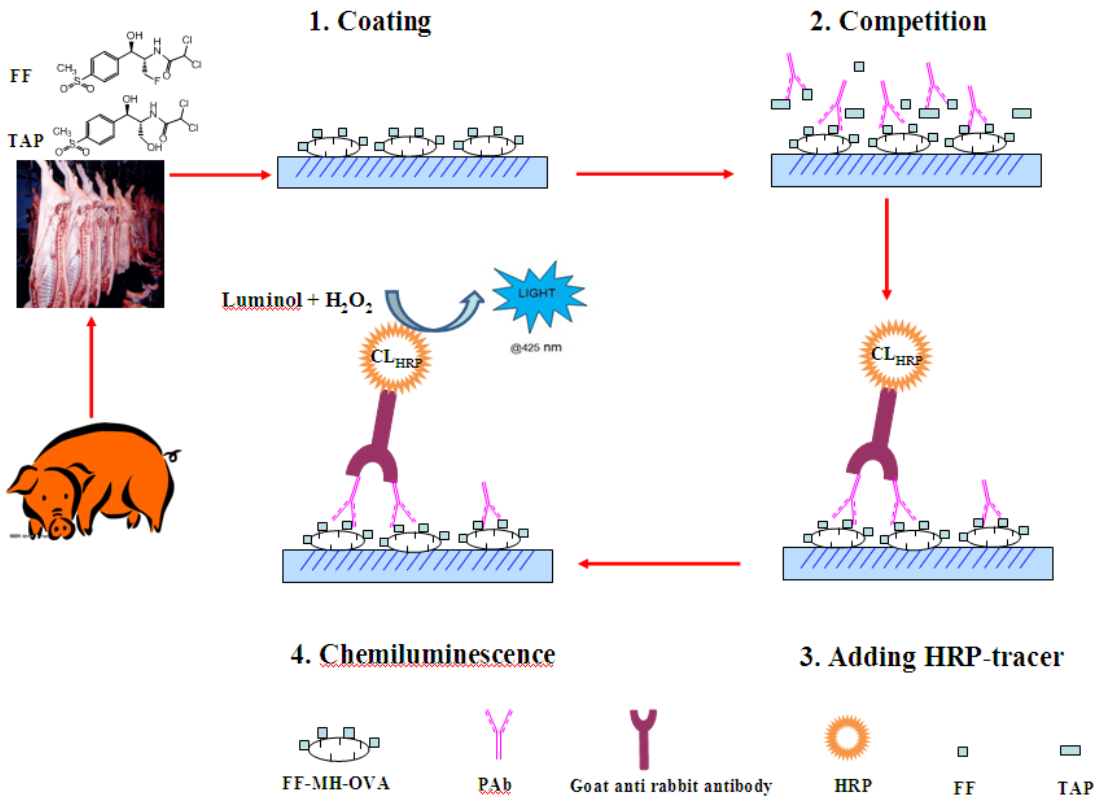


Figure 1 The scheme of CL-ciELISA for simultaneous determination of FF and TAP in pork

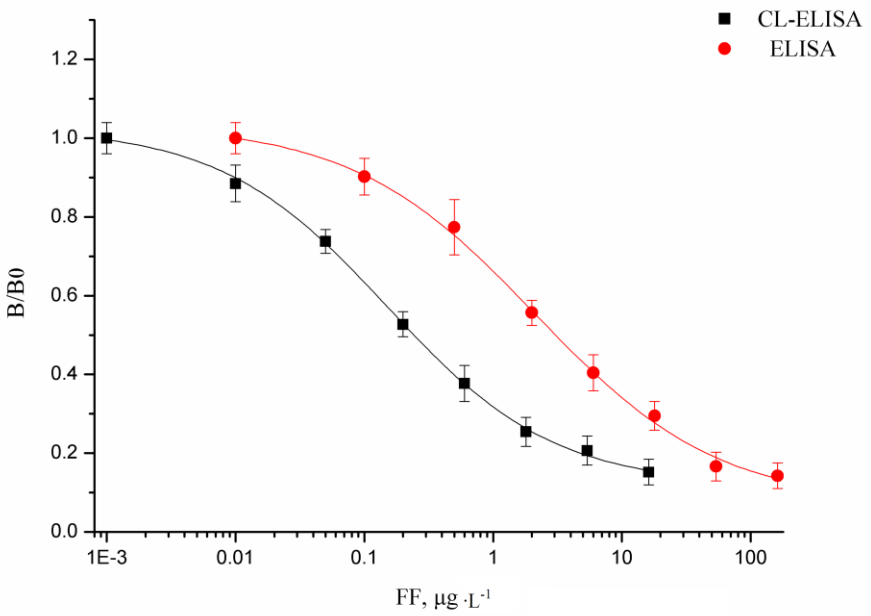
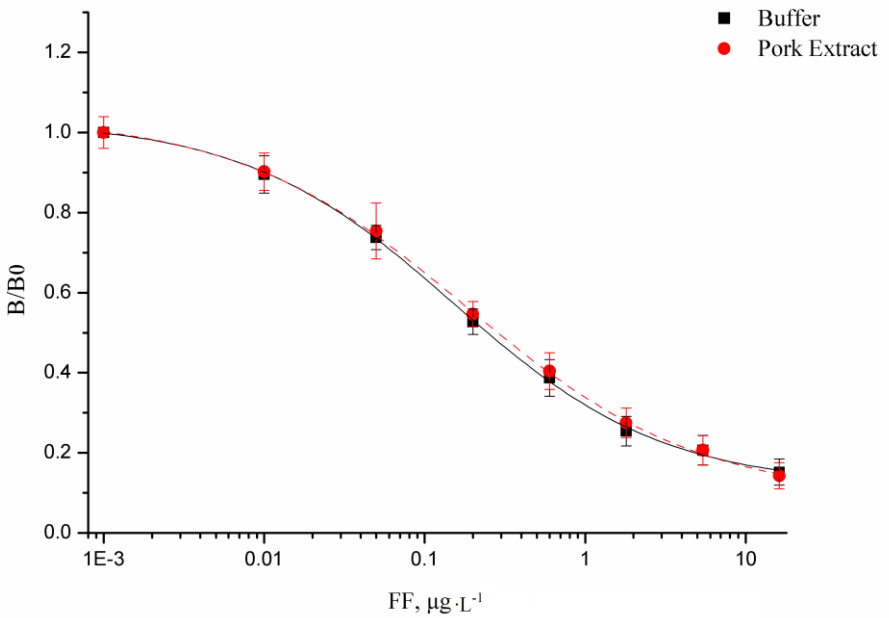


Figure 2 Normalized standard curve of CL-ciELISA for FF under optimized conditions compared to the standard curve obtained by traditional ELISA for FF



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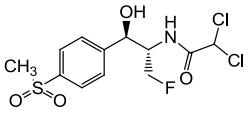
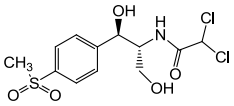
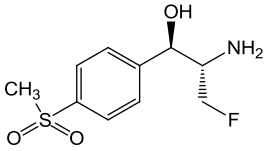
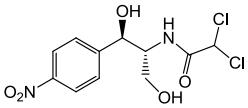
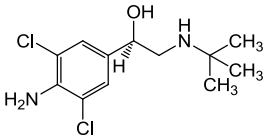
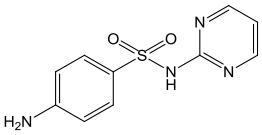
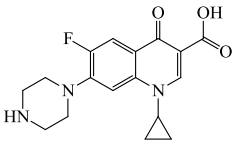
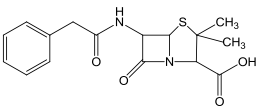
423 Figure 3 Inhibition curves of FF in 0.02 M PB and extraction of pork matrix

Table 1 Percentage of cross reactivity of some structurally related and unrelated compounds

Compound	Structure	IC_{50} (μg Ł$^{-1}$)	CR (%)
FF		0.15	100.0
TAP		0.31	48.4
FFA		50	0.3
CAP		>180	<0.1
CLE		>1000	ND*
SUL		>1000	ND
CIP		>1000	ND
PEN		>1000	ND

* not detectable

Table 2 Intra- and inter-assay variations of pork spiked with FF and TAP

Drug	Added ($\mu g \cdot kg^{-1}$)	Intra-assay[a]			Inter-assay[b]		
		Measured ($\mu g \cdot kg^{-1}$)	Recovery (%)	CV (%)	Measured ($\mu g \cdot kg^{-1}$)	Recovery (%)	CV (%)
FF	0.0075	0.012±0.006	160.0	50.0	0.013±0.007	173.3	53.8
	0.015	0.013±0.0009	86.7	9.0	0.014±0.001	93.3	7.1
	0.030	0.027±0.002	90.0	7.4	0.025±0.003	83.3	12.0
	0.15	0.12±0.009	80.0	7.5	0.13±0.009	86.7	9.0
	1.50	1.23±0.10	82.0	8.1	1.29±0.14	86.0	10.8
TAP	0.015	0.022±0.008	146.7	36.4	0.009±0.009	60.0	100.0
	0.030	0.025±0.003	83.3	12.0	0.026±0.003	86.7	11.5
	0.060	0.051±0.005	85.0	9.8	0.053±0.006	88.3	11.3
	0.30	0.25±0.02	83.3	8.0	0.24±0.03	80.0	12.5
	3.0	2.4±0.2	80.0	8.3	2.6±0.3	86.7	11.5

[a]Intra-assay variation was determined by 10 replicates on a single day

[b]Inter-assay variation was determined by 10 replicates on 3 consecutive days

435 Table 3 Determination of field pork samples collected from retail outlets in
436 Chongqing by the CL-ciELISA, traditional ELISA and LC-MS/MS
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Samples	LC-MS/MS		CL-ELISA	ELISA
	FF (μg ŀkg^{-1})	TAP (μg ŀkg^{-1})	FF +TAP (μg ŀkg^{-1})	FF +TAP (μg ŀkg^{-1})
P1	2.4	2.8	3.7 ±0.2 [a]	3.5 ±0.3
P5	9.8	5.0	12.2 ±0.8	15.3 ±1.1 [a]
P9	27.5	15.2	40.6 ±3.2	38.6 ±3.8
P11	6.8	8.9	9.8 ±0.8	8.7 ±0.5
P15	21.0	13.7	30.2 ±2.0	27.5 ±3.4
P20	0.9	< LOD	0.9 ±0.04	< LOD
P2-P4，P6-P8，P12-14，P16-19	< LOD			

438 [a]Each value is the mean of five replicates