Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Development of an indirect competitive ELISA for simultaneous detection of enrofloxacin and ciprofloxacin*

Hai-tang ZHANG[1], Jin-qing JIANG[†‡1,2], Zi-liang WANG[2], Xin-yao CHANG[1], Xing-you LIU[2], San-hu WANG[2], Kun ZHAO[1], Jin-shan CHEN[1]

([1]*College of Animal Science, Henan Institute of Science and Technology, Xinxiang 453003, China*)

([2]*Henan Higher Education Engineering Technology Research Center for Animal Diseases Control and Residues Supervision, Xinxiang 453003, China*)

[†]E-mail: jjq5678@126.com

Received Mar. 12, 2011; Revision accepted May 26, 2011; Crosschecked Oct. 12, 2011

Abstract: Modified 1-ethyl-3-(3-dimethylaminopropy) carbodiimide (EDC) method was employed to synthesize the artificial antigen of enrofloxacin (ENR), and New Zealand rabbits were used to produce anti-ENR polyclonal antibody (pAb). Based on the checkerboard titration, an indirect competitive enzyme-linked immunosorbent assay (ELISA) standard curve was established. This assay was sensitive and had a linear range from 0.6 to 148.0 μg/kg (R^2=0.9567), with the half maximal inhibitory concentration (IC_{50}) and limit of detection (LOD) values of 9.4 μg/kg and 0.2 μg/kg, respectively. Of all the competitive analogues, the produced pAb exhibited a high cross-reactivity to ciprofloxacin (CIP) (87%), the main metabolite of ENR in tissues. After optimization, the matrix effects can be ignored using a 10-fold dilution in beef and 20-fold dilution in pork. The overall recoveries and coefficients of variation (CVs) were in the ranges of 86%–109% and 6.8%–13.1%, respectively. It can be concluded that the established ELISA method is suitable for simultaneous detection of ENR and CIP in animal tissues.

Key words: Enrofloxacin, Ciprofloxacin, Indirect competitive ELISA, Animal tissues
doi:10.1631/jzus.B1100076 **Document code:** A **CLC number:** S859

1 Introduction

Fluoroquinolones (FQs) are an important group of synthetic antimicrobials with a broad-spectrum antibacterial activity, good absorption after oral administration, and extensive tissue distribution (Yang *et al*., 2005). FQs act via inhibition of DNA-gyrase, abolishing its activity by interfering with the DNA rejoining reaction. Because gyrase is an essential enzyme in prokaryotes, but not found in eukaryotes, bacteria are an ideal target for these antibiotics (Yorke and Froc, 2000). Therefore, FQs have found a widespread application in the treatment and prevention of veterinary diseases in food-producing animals, and they have even been used as growth-promoting agents.

Enrofloxacin (ENR) is the first specified FQ developed for veterinary application, which belongs to the second generation of quinolone antibiotics fluorinated in position 6 and bearing a piperazinyl moiety in position 7 (Fig. 1). Similar to other FQs, ENR is used in the treatment of systemic infections including urinary tract, respiratory, gastrointestinal, and skin infections (Tong *et al*., 2010). Because of a very broad spectrum of activities against both Gram-negative and Gram-positive bacteria and lower side effects, ENR has also been widely used for the treatment of some infectious diseases in pets and livestock. However, ENR residues may persist in animal body and may result in the development of drug-resistant bacterial strains or allergies (Yan *et al*., 2011).

‡ Corresponding author

* Project supported by the Henan Innovation Project for University Prominent Research Talents (No. 2010HASTIT026), and the Key Scientific & Technological Project of Education Department in Henan Province of China (No. 2011A230003)

Ciprofloxacin (CIP) is a major metabolite of ENR in animals, and is also one of the most widely used FQs for the treatment of urinary tract infections, respiratory tract infections, and chronic bacterial prostatitis (Ahmad *et al*., 2006). On the other hand, the CIP residues in livestock products may cause serious public health problems. In the present years, awareness of residual antibiotics in animal-derived food is growing as their application increases in both human and veterinary medicine. Therefore, more and more countries have set the maximum residue levels (MRLs) and withdrawal periods for FQs (Huet *et al*., 2006; Lu *et al*., 2006). For example, an MRL of 100 μg/kg has been recommended for the sum of ENR and its major metabolite, CIP, in animal muscles (Huet *et al*., 2006). In China, the species of animal, usage, dosage, and withdrawal period of FQs have been determined by the Ministry of Agriculture of the People' Republic of China (No. 278, May 22, 2003).

Traditionally, FQ residue analysis has relied upon classical analytical techniques, such as high performance liquid chromatography (HPLC) (Hassouan *et al*., 2007; Zhao *et al*., 2007; Christodoulou *et al*., 2008), liquid chromatography-mass spectrometry (LC-MS) (Delepine *et al*., 1998; San Martín *et al*., 2007), LC-MS/MS (Volmer *et al*., 1997; Dufresne *et al*., 2007; Hermo *et al*., 2008), and other confirmatory methods. Chromatographic techniques generally require highly skilled personnel, laborious sample pretreatment, and high-cost complex equipment. Therefore, these techniques are not practical for screening large numbers of food samples. Immunological techniques are increasingly considered as alternative and/or complementary methods for residue analysis because of their rapidity, cost-effectiveness, portability, and high sample throughput.

In recent years, several papers have reported the development of a broad-specificity immunoassay for the determination of FQ residues (Bucknall *et al*., 2003; Huet *et al*., 2006; Wang *et al*., 2007; Zhu *et al*., 2008; Huang *et al*., 2010). However, there have not yet been any reports of immunoassays to directly identify the sum of ENR and CIP residues in animal muscles. The aim of the present study was to produce high-sensitivity polyclonal antibodies displaying a broad specificity towards ENR and CIP. The analytical performance of the developed indirect competitive enzyme-linked immunosorbent assay (icELISA) method is reported here and its applicability in different muscle matrices is discussed. This work potentially optimizes the procedures for FQ generic kits and test strip development.

2 Materials and methods

2.1 Chemicals and reagents

ENR, CIP, danofloxacin, norfloxacin, and ofloxacin were purchased from Sigma (St. Louis, MO, USA), while other FQs were provided by the China Institute of Veterinary Drug Control (Beijing, China). Goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (GaRIgG-HRP; whole molecule specific) was purchased from Sino-American Biotechnology Company (Shanghai, China). 1-Ethyl-3-(3-dimethylaminopropy) carbodiimide (EDC), Freund's complete adjuvant (FCA), and Freund's incomplete adjuvant (FIA) were obtained from Pierce (USA). *N*-hydroxysuccinimide (NHS) was obtained from Sigma-Aldrich (USA). Bovine serum albumin (BSA; 67000 Da) and ovoalbumin (OVA; 45000 Da) were supplied by Sigma while Dialysis bag (8000–14000 Da) was from Solarbio Company (Beijing, China). Transparent 96-well polystyrene microtitre plates (Boyang Experimental Equipment Factory, Jiangsu, China) were used for the colorimetric measurement. 3,3,5,5-Tetramethylbenzidine (TMB), phenacetin, and urea peroxide were obtained from Sigma Company (USA). All other solvents and reagents were of analytical grade or higher, unless otherwise stated.

Female New Zealand white rabbits weighing 2.0–2.5 kg were obtained from the Laboratory Animal Center, Beijing Medical University, China, and raised under strictly controlled conditions in our laboratory chamber.

2.2 Instruments

A spectrophotometric microtitre reader (Multiskan MK3, Thermo Company, USA), provided with a 450 nm filter, was used for absorbance measurements. Ultraviolet-visible (UV-vis) spectra were obtained by using a DU800 UV-vis spectrophotometer (Beckman-Coulter Company, USA). Infrared (IR) spectra were acquired with an IR spectrometer (TENSOR 27, Bruker Company, Germany). Deionized

water was prepared using an ultra class UV plus water purification system (SG Company, Germany). Immunoreactions were carried out in a DH-360A oven from Zhongxingweiye Instrument Co., Ltd. (Beijing, China).

2.3 Buffers

The buffers used were as follows: phosphate buffered saline (PBS) consisted of NaCl (137 mmol/L), $Na_2HPO_4{\cdot}12H_2O$ (10 mmol/L), KCl (2.68 mmol/L), and KH_2PO_4 (1.47 mmol/L), pH 7.4; carbonate buffer saline (CBS) contained Na_2CO_3 (15 mmol/L), $NaHCO_3$ (35 mmol/L), pH 9.6. Washing buffer consisted of PBS containing 0.05% Tween-20 (PBST); blocking buffer contained BSA (1%, w/v) in PBS; assay buffer consisted of adding Part A (500 ml) and Part B (500 ml) solutions together. Part A contained (per 1 L of water) 3.15 g of citric acid, 6.97 g of anhydrous sodium acetate, 0.08 g of phenacetin, and 0.05 g of urea peroxide adjusted to pH 5 with HCl. Part B contained 1.27 g of TMB dissolved in 500 ml of methanol and 500 ml of glycerol. The stopping solution was 2 mol/L H_2SO_4.

2.4 Synthesis of artificial antigen for ENR

A modified EDC method was employed to synthesize the artificial antigen of ENR-BSA, and the procedure is presented in Fig. 1. Briefly, 36 mg (0.1 mmol) of ENR was suspended in 3 ml dimethyl formamide (DMF) and then 12 mg (0.1 mmol) of NHS and 38 mg (0.2 mmol) of EDC were added. During the following 24 h incubation in dark chamber, the mixture was stirred with an HY-4 reciprocal shaker at 37 °C. To this solution, 67 mg (0.001 mmol) of BSA dissolved in 6 ml of PBS was added dropwise under stirring. The resulting mixture was stirred by rotor in a dark chamber at 37 °C for 1 h, and then incubated with a reciprocal shaker for 3 h. After centrifuged at 3000 r/min for 10 min, the obtained supernatant was dialyzed against distilled water and followed by PBS for 4 d. When the absorption peak of the dialyzed solution disappeared, checked by UV-vis spectra, the artificial immunogen of ENR-BSA was stored in ampoule and kept at −20 °C. The coating antigen of ENR-OVA conjugate was prepared by a similar method.

2.5 Artificial antigen identification

For UV-vis analysis, 0.1 mg/ml of ENR, 1 mg/ml of BSA and ENR-BSA were prepared with PBS, in which UV-vis spectra were recorded, and the conjugation rate of artificial antigen was calculated (Huang *et al*., 2010). The IR identification was performed as follows: heated under an IR lamp, 1 mg of ENR-BSA and 100 mg of KBr were even mixed and ground in an agate mortar. Taking part of the mixture into a mold, at the pressure of 10 MPa, and keeping it 3–5 min, a transparent KBr pellet with the thickness of 1 mm was made, for which IR spectra were acquired with an IR spectrometer.

2.6 Production of anti-ENR polyclonal antibody

New Zealand white rabbits were subcutaneously immunized with ENR-BSA conjugate to produce the polyclonal antibody (pAb). FCA was employed in the first immunization and FIA was used in the subsequent boost injections. Rabbits were immunized every three weeks with 500 μg of immunogen, and blood samples from the marginal vein of the ear were taken for ELISA identification. Ten days after the final boost, all rabbits were exsanguinated by heart puncture and the serum was separated from blood cells by storing at 4 °C overnight and centrifugation with 5000 r/min for 10 min. This crude serum was purified using saturated ammonium sulfate (SAS) precipitation method, and the characteristics of antibody were identified (data not shown). Sodium azide was added to the purified serum as a preservative at a final concentration of 0.02% (w/w), and the pAb was then stored at −70 °C.

2.7 Development of icELISA

These experiments were carried out according to the general assay conditions previously described

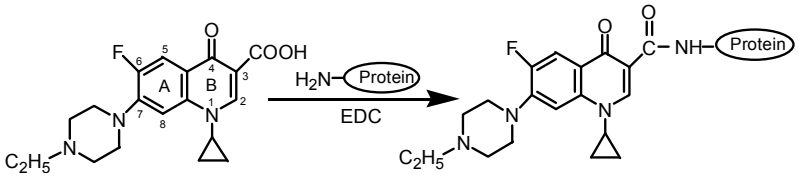

Fig. 1 Synthesis procedure of ENR artificial antigen through EDC method
EDC: 1-ethyl-3-(3-dimethylaminopropy) carbodiimide

(Liu *et al*., 2007; Wu *et al*., 2010). Briefly, ENR-OVA was appropriately diluted in CBS and 100 μl was added to each well of the 96-well microtitre plates. After 2 h incubation at 37 °C, the plates were washed with PBST three times and blocked with 250 μl/well of blocking buffer, followed by incubation for 1 h at 37 °C. After another washing procedure, varying concentrations of ENR or competitor (50 μl/well) were added, followed by equal volume of anti-ENR antiserum previously diluted in PBS. The plate was incubated for 25 min at 37 °C and followed by washing as described above. Then GaRIgG-HRP (1:1000, 50 μl/well) was added, and plates were washed again as above and 60 μl/well of TMB substrate solution was added, followed by incubation for 15 min at room temperature. The enzymatic reaction was stopped with sulfuric acid (2 mol/L, 100 μl/well) and the plate was spectrophotometrically read in a single wavelength mode at 450 nm. Preimmune withdrew serum and PBST were used as a negative control and blank control, respectively, in all assays.

2.8 Characteristics of icELISA standard curve

Based on the checkerboard titration protocol, an icELISA method was developed, and the calibration curve was fitted based on the average of three separate assays in triplicate. Sensitivity was evaluated according to the inhibition rate, and the data were calculated using the IC_{50} values, which represented the concentration of ENR that produced 50% inhibition of antibody binding to the hapten conjugate. The limit of detection (LOD) was defined as the lowest concentration that exhibits a signal of 15% inhibition (Wang *et al*., 2010). The dynamic range for the icELISA was calculated as the concentration of the analyte providing a 20%–80% inhibition rate (IC_{20}–IC_{80}) of the maximum signal. Specificity was defined as the ability of structurally related chemicals to bind to the specific antibody and cross-reactivity (CR) was calculated as: (IC_{50} of ENR)/(IC_{50} of FQs)×100%. The lower the CR, the higher the specificity of ENR pAb.

2.9 Sample preparation and determination

A total of 10 beef and 20 pork muscle samples purchased from the supermarkets and stall markets (Xinxiang, China) were used as blank samples in this study. For ELISA, a 5-g aliquot of ENR-free sample (determined by HPLC) was homogenized with 5 ml of extraction solvent consisting of a 1:1 (v/v) mixture of methanol and PBS adjusted to pH 7.4 with 6 mol/L HCl. The homogenate was mixed on a vortex mixer for 30 s, vigorously shaken for 5 min, and then centrifuged at 4 °C with a speed of 8000 r/min for 10 min. The supernatant layer was transferred into a calibrated flask, and diluted in assay buffer (total 1, 2, 5, 10, and 20-fold dilutions) before they were applied to the microtiter plate.

Based on the optimized results, these samples were homogenized and then fortified with ENR/CIP to give the final concentrations at 20, 100, and 200 μg/kg. Spiked samples were prepared freshly prior to recovery analysis.

3 Results and discussion

3.1 UV-vis spectrogram identification

UV-vis spectra for ENR-BSA, ENR, and BSA are shown in Fig. 2. The absorbance for ENR-BSA (277, 318, and 331 nm) gave a significant shifted peak at 277 nm compared with the 269 nm peak for ENR (269, 321, and 332 nm), while the maximum absorbance of BSA was at 278 nm, which indicated that ENR was successfully conjugated with BSA. The coating antigen of ENR-OVA gave a UV pattern similar to that of immunogen. Calculated from the formula (Huang *et al*., 2010), the molar ratio of 16:1 for ENR-BSA conjugate was obtained, and the conjugation ratio for ENR-OVA was 7.6:1.

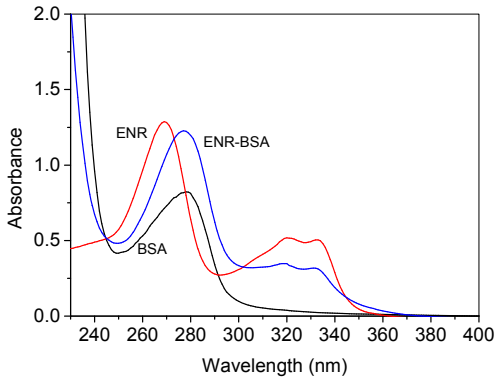

Fig. 2 Ultraviolet absorbance spectra for ENR, BSA, and ENR-BSA

3.2 IR spectra of artificial antigen

Fig. 3 shows the IR spectra of artificial antigen. The IR absorption bands were extremely similar in

3200–2500 cm^{-1} and 1680–1500 cm^{-1} between BSA and ENR-BSA, which were the characteristic absorption peaks of amino acids in BSA, showing the BSA character in the artificial antigen. As compared to ENR-BSA, strong absorption peaks at 1745–1596 cm^{-1} and 2825 cm^{-1} appeared in ENR, which proved the conjugation had been successful. The absorption peaks at 1687–1635 cm^{-1} and 1581–1500 cm^{-1}, which were ascribed to carbonyl and methyl groups, respectively, were enhanced obviously when ENR conjugated to BSA. This shows that the two characteristic functional groups of ENR remained in the artificial antigen. The results indicate that the artificial antigen was synthesized successfully.

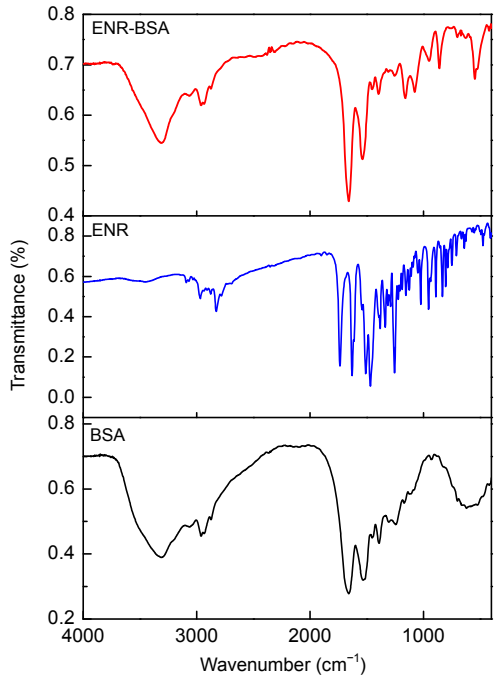

Fig. 3 IR spectra for ENR, BSA, and ENR-BSA

3.3 Establishment of icELISA standard curve

Antisera titer was monitored using an icELISA format with antigen-coated assays. Rabbits injected with ENR-BSA immunogens produced sera exhibiting high titer values after the third injection (second boost). Experimentally, the concentrations of antibody and immobilized antigen are very important in order to enhance the sensitivity for immunoassay. To determine the optimum concentrations of antibody and ENR-OVA providing the highest sensitivity, the absorbance value and IC_{50} were investigated simultaneously. The optimal reagent concentrations are determined as those that give the maximum absorbance (A_{max}) value ranging from 1.5 to 2.0 and have the lowest IC_{50}, using a checkerboard titration.

According to the data (not shown), an increase of the concentrations of the antibody and ENR-OVA enhanced the signal of the test, but with a decrease of the sensitivity of the method. A competitive binding reaction for the binding sites on a limited amount of antibody is carried out between ENR and the ENR-OVA immobilized on the plate, and lower concentrations of coating antigens can lead to a higher inhibition of the analyte in solution (Wang *et al.*, 2010). However, inadequate reagents give low signals and may cause less accurate results. Therefore, the optimal concentrations of the coated ENR-OVA and anti-ENR pAb were 1.0 and 0.5 μg/ml in 1:10000 dilution, respectively.

The blocking step is important to avoid nonspecific absorption in the ELISA. Otherwise, unoccupied sites of the plates may absorb the components such as antibody and GaRIgG-HRP during the subsequent steps, which may cause a high background. As a result, the blocking buffer containing BSA (1%, w/v) in PBS showed a better result because of the lower background value (0.08) than that containing 1% OVA (0.12) or 2% milk powder (0.13), so it was selected in the following experiments.

The organic solvents added to the assay solution to dissolve the ENR were also evaluated. Increasing the concentration of NaOH generally decreased and then increased the IC_{50} value, while increasing the concentration of methanol in the assay solution generally caused a continuous decrease in the A_{max} value but a continuous increase in the time for color development. The optimum combination of NaOH/methanol/PBS (10:20:70, v/v/v) was selected, where A_{max}/IC_{50} was the highest.

After optimization, a representative standard inhibition curve is shown in Fig. 4. As can be seen, this assay allowed the detection of ENR (20%–80% inhibition of color development) from 0.6 to 148.0 μg/kg, with an LOD value of 0.2 μg/kg. The sensitivity (IC_{50}) of the assay was determined to be 9.4 μg/kg.

3.4 Specificity

The specificity of the antibody was evaluated by determination of the cross-reactivity with ENR analogues, i.e., FQs. The cross-reactivity studies were

carried out by an icELISA using various free FQs to compete with binding of the antibody to the coating antigen, which values were measured by comparison of the IC_{50} of the competitor with that of ENR, and the results are shown in Table 1.

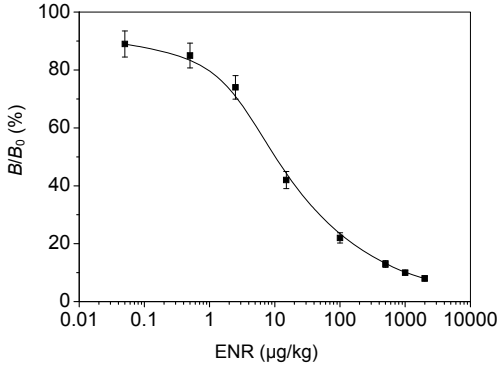

Fig. 4 Optimized standard icELISA inhibition curve for ENR

B and B_0 mean absorbances of each standard and positive control, respectively. Data (mean±SD) were obtained in triplicate

Table 1 Cross-reactivities (CRs) of related FQs in the ENR immunoassay

Analogue	IC_{50} (μg/kg)	CR (%)
Enrofloxacin	9.4	100.0
Ciprofloxacin	10.8	87.0
Norfloxacin	36.5	25.8
Ofloxacin	82.1	11.4
Marbofloxacin	125.6	7.5
Sarafloxacin	158.5	5.9
Lomefloxacin	>94000	<0.01
Danofloxacin	>94000	<0.01
Flumequine	>94000	<0.01

In our research, the immunogen was synthesized by the linkage of the carboxylic acid group of ENR with the amino group of the carrier protein (Fig. 1). In this method, the furthest group of ENR from the linking point is the piperazinyl moiety. The high similarity of structures of substitutents (Positions 1 and 7) between ENR and CIP may explain their high reactivity (87%). Therefore, this ELISA could potentially be applied to the determination of both ENR and CIP.

Although norfloxacin has the same piperazinyl moiety at Position 7, the extra ethyl group located in Position 1 may be responsible for the different electronic structures. Thus, medium cross-reactivity (25.8%) is not surprising. The second group consists of marbofloxacin, ofloxacin, and sarafloxacin, which lack the cyclopropyl group at Position 1 of ENR, but have some heteroatom structures. A comparison of the IC_{50} values demonstrates that the loss of this group and substitution with other groups has a diverse degree of effects on binding. The combined effect of changing the group at Position 1 and at the piperazinyl ring can be evaluated by comparing the IC_{50} values for lomefloxacin, danofloxacin, and flumequine. These results reveal the importance of spatial volume of the group at Positions 1 and 7 for antibody recognition, and the aromatic ring as well as the electronegative fluorine would be expected to involve a number of binding interactions. It is believed that the hapten- or antigen-antibody interaction is dependent on molecular shape and low-energy interactions such as hydrogen bonding and hydrophobic interactions (Wang *et al*., 2007).

3.5 Matrix effect determination

One of the most common challenges of immunosorbent assays for food analysis is matrix interference. Immunosorbent assays often have a high potential for nonspecific binding to nontarget analysis. Chemical substances present in samples or sample extracts, such as solvents, fat, salt, and other compounds, might affect the binding between the antibody and analyte, reduce the sensitivity, and lower the extent of color development. So removing the matrix effects is important in the ELISA assays. In the present study, the beef and pork muscle samples were simply extracted and no cleanup step was employed prior to analysis. Fig. 5 shows the comparison of the standard curves in dilutions of extracts and in PBS buffer alone. If the two curves are superposable, the matrix effect is not significant and the samples can then be analyzed according to the calibration curve.

As the dilution of beef extracts increased from 1:2 to 1:20, the absorbance gradually increased to approach the PBS buffer values. The average B_0 (antibody binding with no competitor present) values for dilutions at 1:2, 1:10, and 1:20 had absorbencies of 1.515, 1.864, and 1.902, respectively, as compared to 1.898 in PBS. It can be seen that matrix effects actually can be ignored at a dilution of 1:10 in beef. When compared the inhibition curves in pork, similar analytical sensitivity was observed between 1:20 dilution and PBS, indicating that matrix interference was sufficiently low and 1:20 dilution allowed a significant gain in the detectability of the analyte.

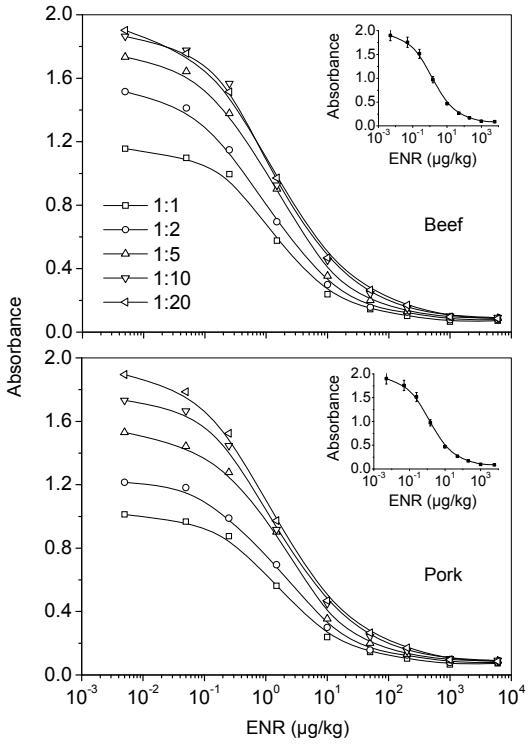

Fig. 5 ENR inhibition curves in the diluted muscle samples
Each point represents the average absorbance at 450 nm of three separate assays in triplicate. Insets indicate the icELISA standard curves in PBS

Although dilution of the sample is an effective means of reducing the matrix effect, it leads to a reduction of assay sensitivity. Considering the dilution schedule, the LODs for ENR in blank samples of beef and pork were 2.1 and 4.6 μg/kg, respectively, which were much lower than MRLs for ENR in these matrices. Based on the LODs of ENR in diluted muscle extracts, the same values for CIP (CR, 87%) in blank samples of beef and pork should be 2.4 and 5.3 μg/kg, respectively. Thus, the loss in assay sensitivity was acceptable.

3.6 Analysis of spiked samples

The analytical performance of ELISA is commonly assessed by spiking matrix samples with the target analyte. Each spiked sample was evaluated three times in duplicate to verify the repeatability of the developed icELISA, and the results are summarized in Table 2. In the analysis of beef, the average recoveries for ENR and CIP ranged from 89% to 104% with coefficient variations (CVs) between 6.8% and 10.2%, and from 93% to 109% with CVs between 9.6% and 13.1%, respectively. Meanwhile, the pork samples spiked with different amount of analytes were calculated. The recoveries were in the ranges from 86% to 101% for ENR, and 87% to 103% for CIP, respectively, and the CVs listed in the acceptable ranges.

Table 2 Recovery studies from animal samples fortified with ENR or CIP by icELISA

Analyte	Sample	Spiked level (μg/kg)	Recovery (%)	CV (%)
ENR	Beef (1:10)	2.1	104	10.2
		50.0	95	6.8
		100.0	89	9.2
	Pork (1:20)	4.6	101	11.4
		50.0	90	8.3
		100.0	86	7.5
CIP	Beef (1:10)	2.4	109	9.6
		50.0	100	12.2
		100.0	93	13.1
	Pork (1:20)	5.3	103	9.3
		50.0	94	7.8
		100.0	87	12.5

Since the presence of water in pork samples makes the amount of the whole extract bigger than that of PBS that had been added prior to extraction, it is reasonable to conclude that the pork sample shows a lower recovery than the beef sample. These results demonstrate that the values obtained are acceptable, and that the sample extraction methods and the icELISA developed for the analysis of FQ residues in animal tissues are satisfactory.

4 Conclusions

In summary, a polyclonal-based ELISA for the simultaneous detection of ENR and CIP residues has been developed. The acceptable recoveries from spiked samples in beef and pork suggested that this ELISA is a reasonable quantitative/screening method in animal tissues. Under the 10-fold dilution in beef and 20-fold in pork, the LODs (2.1–5.3 μg/kg) were low enough under the levels of MRLs (Huet *et al*., 2006). It can be concluded that the ELISA method is reliable and relatively economic, and moreover, the analytical approach proposed is easy to use and can be performed in any laboratory with basic equipment. As

the generic ELISA cannot distinguish the components of a mixture, a noncompliant result would require a confirmatory LC-MS/MS assay.

References

Ahmad, B., Parveen, S., Khan, R.H., 2006. Effect of albumin conformation on the binding of ciprofloxacin to human serum albumin: a novel approach directly assigning binding site. *Biomacromolecules*, **7**(4):1350-1356. [doi:10.1021/bm050996b]

Bucknall, S., Silverlight, J., Coldham, N., Thorne, L., Jackman, R., 2003. Antibodies to the quinolones and fluoroquinolones for the development of generic and specific immunoassays for detection of these residues in animal products. *Food Addit. Contam.*, **20**(3):221-228. [doi:10.1080/0265203021000055388]

Christodoulou, E.A., Samanidou, V.F., Papadoyannis, I.N., 2008. Development of an HPLC multi-residue method for the determination of ten quinolones in bovine liver and porcine kidney according to the European Union Decision 2002/657/EC. *J. Sep. Sci.*, **31**(1):119-127. [doi:10.1002/jssc.200700297]

Delepine, B., Hurtaud-Pessel, D., Sanders, P., 1998. Simultaneous determination of six quinolones in pig muscle by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *Analyst*, **123**(12):2743-2747. [doi:10.1039/a804911d]

Dufresne, G., Fouquet, A., Forsyth, D., Tittlemier, S.A., 2007. Multiresidue determination of quinolone and fluoroquinolone antibiotics in fish and shrimp by liquid chromatography/tandem mass spectrometry. *J. AOAC Int.*, **90**(2):604-612.

Hassouan, M.K., Ballesteros, O., Zafra, A., Vílchez, J.L., Navalón, A., 2007. Multiresidue method for simultaneous determination of quinolone antibacterials in pig kidney samples by liquid chromatography with fluorescence detection. *J. Chromatogr. B*, **859**(2):282-288. [doi:10.1016/j.jchromb.2007.10.002]

Hermo, M.P., Nemutlu, E., Kir, S., Barrón, D., Barbosa, J., 2008. Improved determination of quinolones in milk at their MRL levels using LC-UV, LC-FD, LC-MS and LC-MS/MS and validation in line with regulation 2002/657/EC. *Anal. Chim. Acta*, **613**(1):98-107. [doi:10.1016/j.aca.2008.02.045]

Huang, B., Yin, Y., Lu, L., Ding, H., Wang, L., Yu, T., Zhu, J.J., Zheng, X.D., Zhang, Y.Z., 2010. Preparation of high-affinity rabbit monoclonal antibodies for ciprofloxacin and development of an indirect competitive ELISA for residues in milk. *J. Zhejiang Univ.-Sci. B (Biomed. & Biotechnol.)*, **11**(10):812-818. [doi:10.1631/jzus.B1000055]

Huet, A.C., Charlier, C., Tittlemier, S.A., Singh, G., Benrejeb, S., Delahaut, P., 2006. Simultaneous determination of (fluoro)quinolone antibiotics in kidney, marine products, eggs, and muscle by enzyme-linked immunosorbent assay (ELISA). *J. Agric. Food Chem.*, **54**(8):2822-2827. [doi:10.1021/jf052445i]

Liu, Y., Zhang, C.Z., Yu, X.Y., Zhang, Z.Y., Zhang, X., Liu, R.R., Liu, X.J., Gong, Z.M., 2007. Development and evaluation of immunoassay for zeranol in bovine urine. *J. Zhejiang Univ.-Sci. B*, **8**(12):900-905. [doi:10.1631/jzus.2007.B0900]

Lu, S., Zhang, Y., Liu, J., Zhao, C., Liu, W., Xi, R., 2006. Preparation of anti-pefloxacin antibody and development of an indirect competitive enzyme-linked immunosorbent assay for detection of pefloxacin residue in chicken liver. *J. Agric. Food Chem.*, **54**(19):6995-7000. [doi:10.1021/jf061309q]

San Martín, B., Cornejo, J., Iragüen, D., Hidalgo, H., Anadón, A., 2007. Depletion study of enrofloxacin and its metabolite ciprofloxacin in edible tissues and feathers of white leghorn hens by liquid chromatography coupled with tandem mass spectrometry. *J. Food Prot.*, **70**(8):1952-1957.

Tong, C., Zhuo, X., Liu, W., Wu, J., 2010. Synchronous fluorescence measurement of enrofloxacin in the pharmaceutical formulation and its residue in milks based on the yttrium (III)-perturbed luminescence. *Talanta*, **82**(5):1858-1863. [doi:10.1016/j.talanta.2010.07.082]

Volmer, D.A., Mansoori, B., Locke, S.J., 1997. Study of 4-quinolone antibiotics in biological samples by short-column liquid chromatography coupled with electrospray ionization tandem mass spectrometry. *Anal. Chem.*, **69**(20):4143-4155. [doi:10.1021/ac970425c]

Wang, L., Zhang, Y., Gao, X., Duan, Z., Wang, S., 2010. Determination of chloramphenicol residues in milk by enzyme-linked immunosorbent assay: improvement by biotin-streptavidin-amplified system. *J. Agric. Food Chem.*, **58**(6):3265-3270. [doi:10.1021/jf903940h]

Wang, Z., Zhu, Y., Ding, S., He, F., Beier, R.C., Li, J., Jiang, H., Feng, C., Wan, Y., Zhang, S., *et al*., 2007. Development of a monoclonal antibody-based broad-specificity ELISA for fluoroquinolone antibiotics in foods and molecular modeling studies of cross-reactive compounds. *Anal. Chem.*, **79**(12):4471-4483. [doi:10.1021/ac070064t]

Wu, J.X., Zhang, S.E., Zhou, X.P., 2010. Monoclonal antibody-based ELISA and colloidal gold-based immunochromatographic assay for streptomycin residue detection in milk and swine urine. *J. Zhejiang Univ.-Sci. B (Biomed. & Biotechnol.)*, **11**(1):52-60. [doi:10.1631/jzus.B0900215]

Yan, H., Wang, H., Qin, X., Liu, B., Du, J., 2011. Ultrasound-assisted dispersive liquid-liquid microextraction for determination of fluoroquinolones in pharmaceutical wastewater. *J. Pharm. Biomed. Anal.*, **54**(1):53-57. [doi:10.1016/j.jpba.2010.08.007]

Yang, G., Lin, B., Zeng, Z., Chen, Z., Huang, X., 2005. Multiresidue determination of eleven quinolones in milk by liquid chromatography with fluorescence detection. *J. AOAC Int.*, **88**(6):1688-1694.

Yorke, J.C., Froc, P., 2000. Quantification of nine quinolones in chicken tissues by HPLC with fluorescence detection. *J. Chromatogr. A*, **882**(1-2):63-77. [doi:10.1016/S0021-9673(00)00165-5]

Zhao, S., Jiang, H., Li, X., Mi, T., Li, C., Shen, J., 2007. Simultaneous determination of trace levels of 10 quinolones in swine, chicken, and shrimp muscle tissues using HPLC with programmable fluorescence detection. *J. Agric. Food Chem.*, **55**(10):3829-3834. [doi:10.1021/jf0635309]

Zhu, Y., Li, L., Wang, Z., Chen, Y., Zhao, Z., Zhu, L., Wu, X., Wan, Y., He, F., Shen, J., 2008. Development of an immunochromatography strip for the rapid detection of 12 fluoroquinolones in chicken muscle and liver. *J. Agric. Food Chem.*, **56**(14):5469-5474. [doi:10.1021/jf800274f]