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*Full Length Research Paper*

# Development and Optimization of a Heterologous Indirect Competitive ELISA for the Detection of Enrofloxacin Residues in Poultry Muscles

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**A heterologous indirect competitive enzyme linked immuno sorbent assay (icELISA) has been developed for the determination of enrofloxacin (ENR) residues in poultry. For this purpose, carbodiimide active ester method was employed to synthesize the artificial antigen of ENR- bovine serum albumin (BSA) while mixed-anhydride technique was used to synthesize the coating antigen of ENR- ovalbumin (OVA), to pursue the heterologous sensitivity. By square matrix titration, an icELISA method was developed, and the linear range was from 0.02 to 86.3 ng/mL, with limit of detection (LOD) and $IC_{50}$ value of 0.8 and 0.01 ng/mL, respectively. After optimization, 5% of NaOH was used in the assay buffer and this ELISA system can tolerate methanol not higher than 30%. The correlation coefficients ($R^2$) between concentration spiked and concentration determined were 0.9975 in chicken muscle and 0.9959 in duck muscle, respectively. Therefore, this assay has the potential to be incorporated into a quantitative monitoring program for the rapid screening of ENR residue in poultry muscles.**

**Key words:** Enrofloxacin, artificial antigen, polyclonal antibody, indirect competitive enzyme linked immuno sorbent assay (ELISA), heterologous, poultry.

## INTRODUCTION

Enrofloxacin (ENR) is the first specific fluoroquinolone 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. Similar to other fluoroquinolones, enrofloxacin is used in the treatment of systemic infections including urinary tract, respiratory, gastro-intestinal and skin infections (Tong et al., 2010). Due to the very broad spectrum of activity 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. In the present years, awareness of residual antibiotics in animal-derived food is growing as their application increases in both human and veterinary medicine (Yan et al., 2011).

In order to monitor enrofloxacin residue levels in livestock and poultry products, simple and rapid analytical methods are required. Various analytical methods, such as high performance liquid chromatography (HPLC) (Christodoulou et al., 2008), liquid chromatography-mass spectrometry (LC-MS) (Delepine et al., 1998; San Martin et al., 2007), and LC-MS/MS (Dufresne et al., 2007;

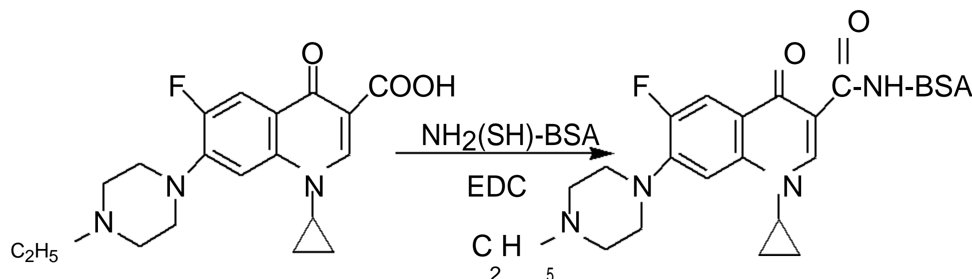


**Figure 1.** The synthesis procedure for ENR immunogen through EDC method.

Hermo et al., 2008) have been reported. However, many of the instrumental methods used to monitor the residues are time-consuming, solvent intensive, and costly; therefore, chromatographic and mass spectrometry methods are not practical for screening large numbers of food samples for ENR. The enzyme-linked immunosorbent assay (ELISA) was widely employed because of its sensitivity of detection and ease of use. Several papers have reported the development of an immunoassay for the determination of ENR residue (Watanabe et al., 2002; Wang et al., 2007; Zhang et al., 2011). However, there have not yet any reports of preparation a rabbit polyclonal antibody (pAb) for ENR and development of a heterologous immunoassay. In this study, we aimed to prepare the artificial antigen of ENR and produce anti-ENR pAb. We have also developed an indirect competitive heterologous immunoassay for monitoring enrofloxacin residues in poultry muscles.

## MATERIALS AND METHODS

### Materials and equipment

Enrofloxacin, ciprofloxacin and other fluoroquinolones (FQs) were provided by Sigma (St. Louis, MO). 1-ethyl-3-(3-dimethylaminopropyl) (EDC), freund's complete adjuvant (FCA) and freund's incomplete adjuvant (FIA) were obtained from Pierce while N-hydroxysuccinimide (NHS) was from Japan; MSDS available. O-(Carboxymethyl) hydroxylamine hemihydrochloride, succinic anhydride, bovine serum albumin (BSA) and ovalbumin (OVA) were supplied by Sigma while Dialysis bag (8000-14000 Da) was from Solarbio company. The peroxidase-conjugated rabbit anti-mouse IgG (GaRIgG-HRP) was purchased from Sino-American Biotechnology Company (Shanghai, China). 3,3,5,5-tetramethylbenzidine (TMB), phenacetin, urea peroxide were obtained from Sigma Company. All other solvents and reagents were of analytical grade or higher, unless otherwise stated. A spectrophotometric microtitre reader, Multiskan MK3 (Thermo company, USA), provided with a 450 nm filter, was used for absorbance measurements. Fresh meat samples of poultry (chicken tissue and duck) were purchased in retail outlets in Xinxiang, China. Female New Zealand white rabbits weighing 2–2.5 kg were obtained from the Laboratory Animal Center, Beijing Medical University, China, and raised under strictly controlled conditions in our laboratory chamber.

### Synthesis of immunogen and coating antigen

The immunogen of ENR-BSA was prepared by carbodiimide active ester method. Briefly, a total of 4.18 mg of ENR, 20.64 mg of EDC, and 5.76 mg of NHS were added to 2 mL dimethylformamide (DMF) respectively. The mixture solution was incubated for 24 h at room temperature in dark. Then 2.5 mL of phosphate buffer saline (PBS) (0.01 mol/L, pH 7.4) with 26.4 mg BSA was added slowly to the mixture solution with stirring, followed by 4 h incubation at room temperature. The synthesis procedure is shown in Figure 1. Finally, the reaction mixture was dialyzed under stirring against PBS for 6 days with repeated changes of the PBS solution to remove the unconjugated hapten. The solution was stored at -20°C. An ENR-OVA conjugate was prepared as a modified previous paper (Huang et al., 2010) and the mixed-anhydride technique route was as shown in Figure 2.

### Polyclonal antibody generation

Two female New Zealand white rabbits were subcutaneously immunized at multiple sites in the back with ENR-BSA conjugate, and under control of the local Ethical Committee for Research. The initial immunization was injected with 500 µg of conjugate in 0.5 mL of PBS and 0.5 mL of FCA. Subsequent 4 booster injections (0.5 mg of conjugate in 0.5 mL of PBS plus 0.5 mL of FIA) were performed three weeks later and then at 15 days intervals. Ten days after the last boost, all rabbits were exsanguinated by heart puncture under general anesthetic. The antiserum was prepared by allowing the blood to clot overnight at 4°C, followed by centrifugation at 10000 r/min for 20 min to remove particulate materials. The crude serum was purified by saturated ammonium sulfate (SAS) precipitation method (purified three times using SAS). The purified serum was then aliquotted and stored at -70°C.

### Development of a heterologous icELISA

The checkerboard procedure was used to optimize the coating antigen and the primary antibody concentrations, resulting in the following optimized protocol. To each well of a 96 well plate, 100 µL of selected coating antigen was added and incubated for 2 h at 37°C. The plate was washed three times with PBST (PBS containing 0.05% Tween-20) 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 competitive fluoroquinolones (50 µL/well) was added, followed by equal volume of ENR antiserum previously diluted 10, 000 folds in PBS according to the optimal data. The following steps were similar to the indirect ELISA (Jiang et al., 2011). Absorbances were corrected by blank

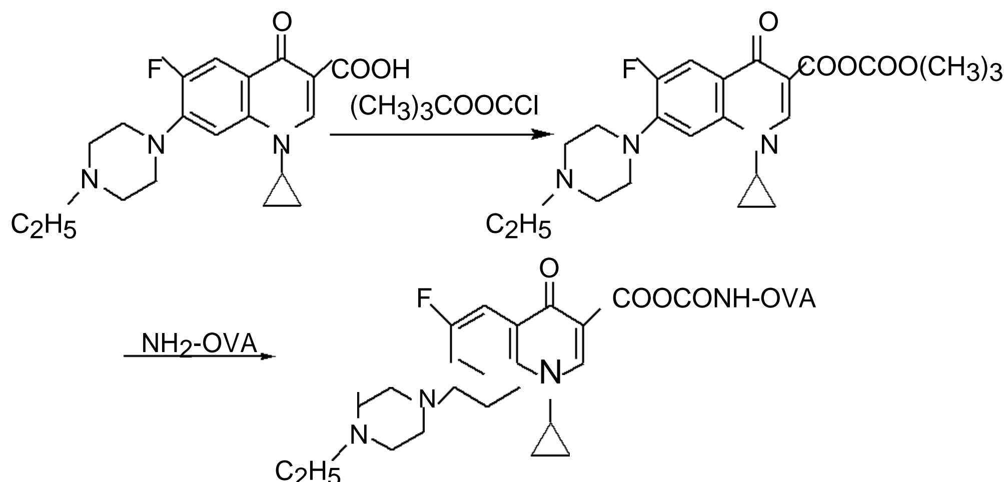


**Figure 2.** The synthesis procedure for ENR-OVA coating antigen through mixed-anhydride technique.

reading and the results were expressed in percent inhibition rate. 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 antiserum binding to the hapten conjugate. The limit of detection (LOD) was defined as the lowest concentration that exhibits a signal of 15% inhibition (Jiang and Wang, 2011). The dynamic range for the icELISA was calculated as the concentration of the analyte providing a 20–80% inhibition rate ($IC_{20}$–$IC_{80}$ values) of the maximum signal. Specificity was defined as the ability of structurally related chemicals to bind to the specific antibody. The cross-reactivity was calculated as: ($IC_{50}$ of ENR)/ ($IC_{50}$ of competitors) ×100. The lower the CR, the higher the specificity of ENR pAb (Lei et al., 2010).

#### Chemical effects on assay performance

It is commonly acknowledged that immunoassay performance is often affected by chemical parameters such as ionic strength, pH values, organic solvent concentration, and other substances in the sample matrix. The effects of these parameters were estimated by the maximum absorbance ($A_{max}$, the absorbance value at zero concentration of ENR) and half-maximum inhibition concentration ($IC_{50}$, the value represents the concentration of ENR that produce 50% inhibition of antibody binding to the hapten), and the maximal $A_{max}/IC_{50}$ ratio was chosen (Hao et al., 2009). In our study, the concentrations of NaOH and methanol contents in the assay buffer have determinant effects on the standard inhibition curve, which was checked for the optimal data.

#### Spiking tests in poultry samples

Prior to running the assay, poultry samples (chicken and duck muscles) were homogenized, then 1 g of each meat sample was weighed into a 10 mL polypropylene centrifuge tube. Next 2 mL of 0.1 mol/L sodium hydroxide-acetonitrile (1:10) was added, and the mixture was agitated on a shaker for 10 min. The samples were centrifuged at 5000 r/min for 10 min, and 1 mL of supernatants was diluted with 9 mL of PBS (0.01 mol/L ). 50 μL of this buffer solution was used in the ELISA. For spiking tests, a standard stock of ENR was prepared by diluting the initial solution prepared in HCl (0.03 mol/L) to give a final stock solution at 1 mg/mL. The stock solution was serially diluted with PBS to give the working standard solutions. The recoveries were calculated by interpolation of the mean absorbance values on a standard curve constructed by icELISA in PBS.

## RESULTS AND DISCUSSION

### Hapten conjugation

UV-vis spectrum for ENR-BSA, ENR, and BSA are presented in Figure 3. The absorbance for ENR-BSA (279 and 321 nm) gave a significant shifted peak at 279 nm compared with the 269 nm peak for ENR (269, 321, and 333 nm), which indicated the 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, molar ratio of 18:1 for ENR-BSA conjugates was obtained.

### Heterologous icELISA Standard curve

Checkerboard titrations were performed, taking into account the optimal dilutions. The optimal reagent concentrations were determined when the maximum absorbances ($A_{max}$) were between 1.5 and 2.0, and the dose-response curve of inhibition ratio versus the ENR concentration pursued the lowest $IC_{50}$ values. From the checkerboard assays, a representative standard inhibition curve was obtained (Figure 4). As can be seen, the optimum concentration of coating antigen was 1.0 μg/mL and pAb was 1:10,000 dilutions. This assay allowed the detection of ENR (20-80% inhibition of color development) from 0.02 to 86.3 ng/mL, with an $IC_{50}$ value

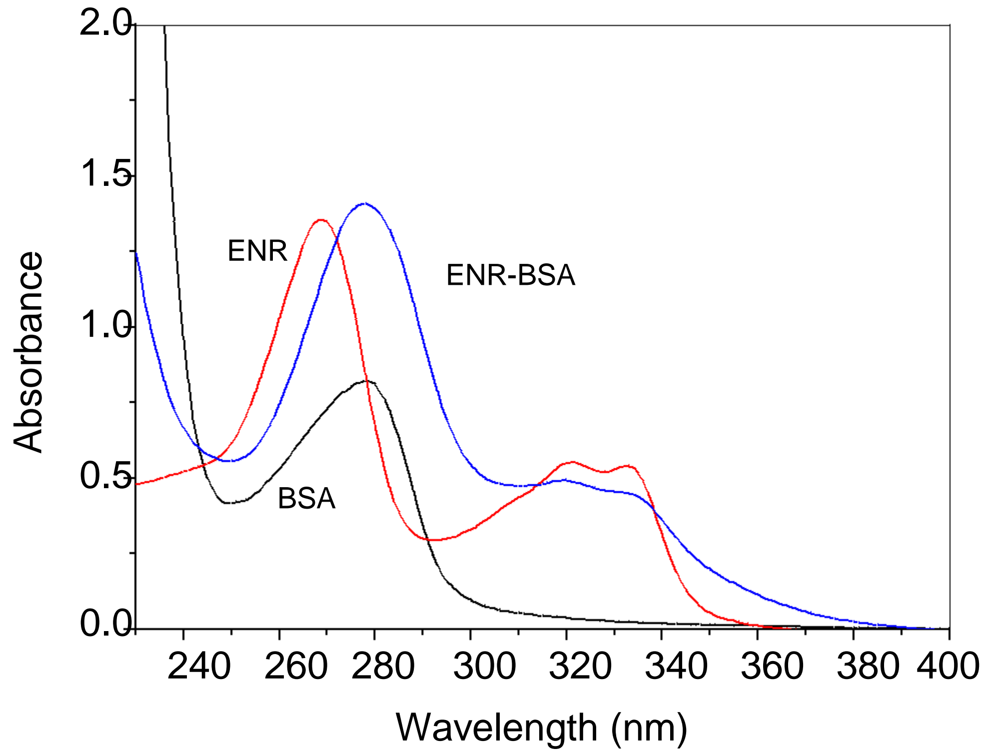


**Figure 3.** UV-vis spectrum for artificial antigen of ENR-BSA, BSA and ENR.

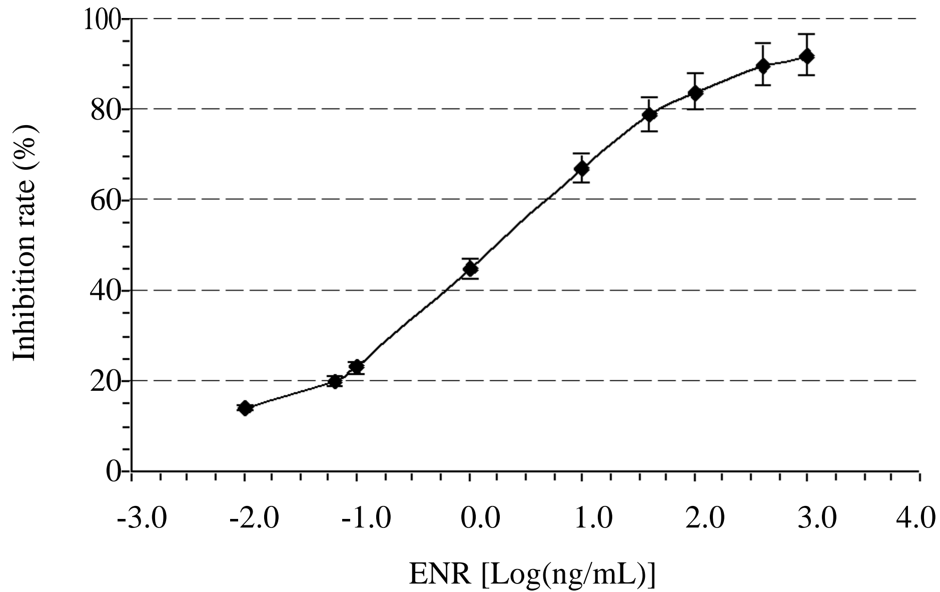


**Figure 4.** Optimized standard heterologous icELISA inhibition curve for ENR. Data were obtained by averaging three independent curves, each run in triplicate. ENR-OVA (1 μg/mL) as coating antigen was prepared in CBS (pH 9.6), purified anti-serum produced by ENR-BSA as immunogen was diluted 1:10 000 in PBS (pH 7.4), ENR was prepared in PBS, containing 10% methanol; GaRIgG-HRP was diluted 1:1000 in incubation buffer.

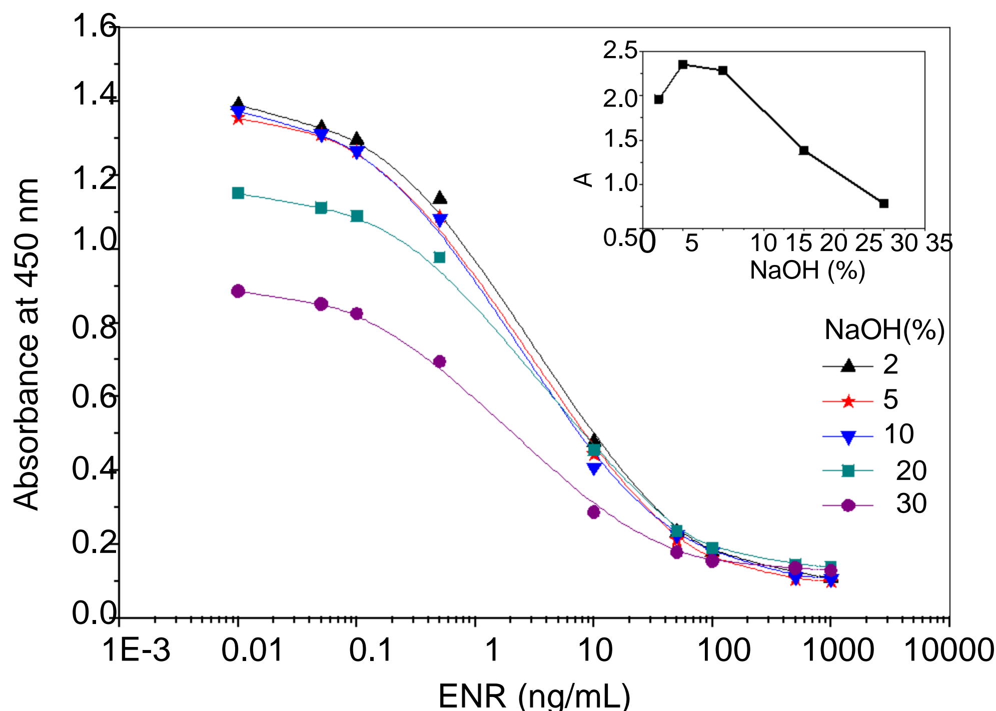


**Figure 5.** Effects of NaOH on the icELISA inhibition curve. Insets indicate the fluctuations of $A_{max}/IC_{50}$ (Y-axis) as a function of NaOH concentration (X-axis). Each value represents the mean of three replicates.

of 0.8 ng/mL. The LOD of the assay was 0.01 ng/mL.

## Chemical effects

To study the influence of NaOH on the assay characteristics, competitive curves were prepared using standards in PBS which contained 2, 5, 10, 20, and 30% of NaOH. Figure 5 presents the effects of NaOH in assay solution on the ELISA. This result indicate that higher NaOH concentrations may change the ionization state of the antibody binding site, disrupt the ionic interactions, and resist the entrance of the standards. Accordingly, 5% NaOH in assay buffer provides the best conditions for the binding of antibody and coating antigen that was selected for the immunoassays.

Methanol that may interfere with antigen-antibody binding and increase the solubility of analytes, was tested for its effects on the ELISA. Figure 6 shows the normalized dose-response curves at various solvent concentrations. The results show that the $IC_{50}$ values of the immunoassay were varying obviously when increasing the amounts of methanol. It indicated that a reproducible inhibition curve could be observed, only when the concentration of the solvent was not higher than 30%. Therefore, to accurately determine the concentration of ENR, methanol contents in the assay buffer should be minimized.

## Specificity

Specificity is a phenomenon inherent to all immunoassays, which was evaluated by determination of the cross-reactivity based on the $IC_{50}$ values of individual chemicals. In this work, the study was undertaken by adding various competitors of functional related analogues. The cross-reactivity rate for each compound is presented in Table 1. Of all the cross-reacting analogues, this assay exhibited a high cross-reactivity to ciprofloxacin (56%), but negligible cross-reactivity to other chemicals.

## Correlations between concentrations spiked and determined in poultry samples

The accuracy of the analysis was studied by comparative detection of fortified ENR in polutry muscle samples, and the measurement correlations between the fortified and analyzed concentrations are shown in Figure 7. We can find that the data spots were nearly distributed on both

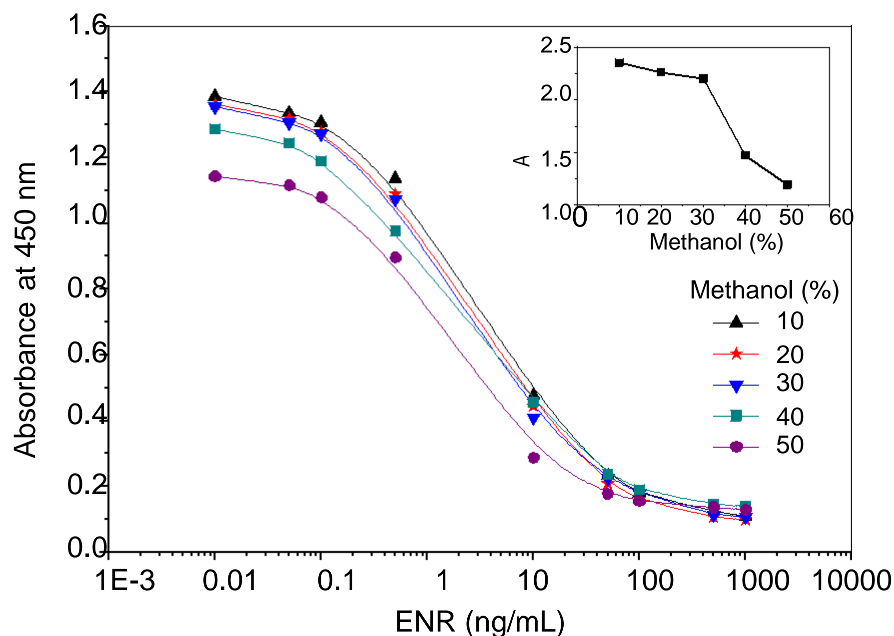


**Figure 6**. Effects of methanol on the icELISA inhibition curve.

**Table 1**. Cross-reactivities of FQs analogues in the ENR immunoassay.

| Analogue | $IC_{50}$ ( ng/mL ) | CR (%) |
|---|---|---|
| Enrofloxacin | 0.8 | 100 |
| Ciprofloxacin | 1.43 | 56 |
| Pefloxacin | >1600 | <0.05 |
| Danofloxacin | >1600 | <0.05 |
| Sarafloxacin | >1600 | <0.05 |
| Norfloxacin | >1600 | <0.05 |
| Lomefloxacin | >8000 | <0.01 |
| Ofloxacin | >8000 | <0.01 |
| Enoxacin | >8000 | <0.01 |
| Flumequin | >8000 | <0.01 |

sides of the trendline. This indicates that an excellent correlation between concentration spiked and concentration determined was found, and the results also suggest the veracity of the icELISA method for detecting ENR residue in poultry tissues.

## Conclusions

We have prepared a high-quality polyclonal antibody with high specificity for ENR. The feasibility to apply this antibody in a competitive ELISA has been explored, and the results show that this heterologous icELISA has been shown to be capable of detecting ENR residue in muscle, and it can also be potentially applied in other matrices.


## ACKNOWLEDGMENT

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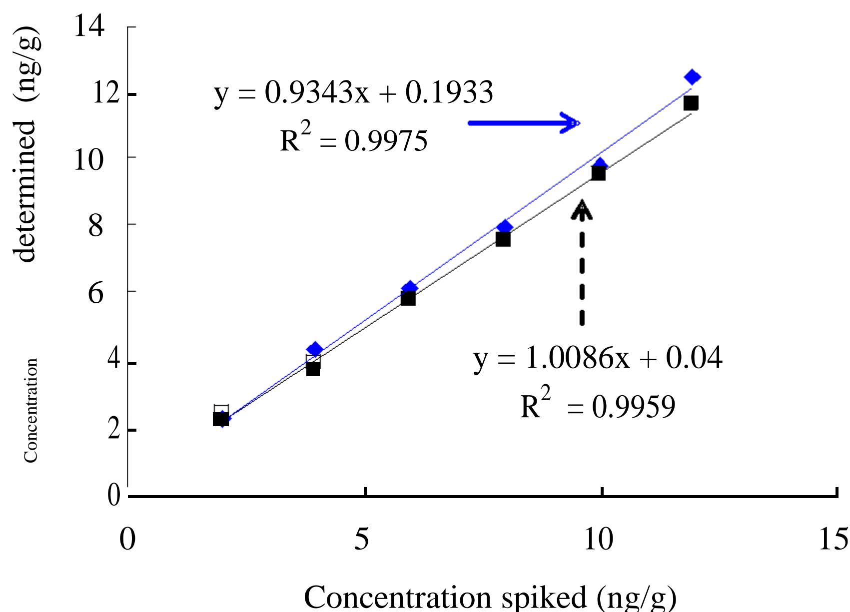


**Figure 7.** Correlations between concentration spiked and concentration determined in poultry muscle samples fortified with ENR. ◆, Chicken muscle; ■, duck muscle.

**Abbreviations: ENR,** Enrofloxacin; **icELISA,** indirect competitive enzyme linked immuno sorbent assay; **pAb,** polyclonal antibody; **BSA,** bovine serum albumin; **OVA**, ovalbumin; **FCA,** Freund's complete adjuvant; **FIA,** Freund's incomplete adjuvant; **GaRIgG-HRP,** the peroxidase-conjugated rabbit anti-mouse IgG; **$A_{max}$,** maximum absorbance; **$IC_{50}$,** half-maximum inhibition concentration; **LOD**, limit of detection; **CR**, cross-reactivity; **EDC,** 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; **NHS**, N-hydroxysuccinimide; **FQs**, fluoroquinolones; **PBS,** phosphate buffer saline; **TMB,** 3,3,5,5-tetramethylbenzidine; **HPLC,** high performance liquid chromatography; **LC-MS,** liquid chromatography-mass spectrometry; **pAb,** polyclonal antibody.

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