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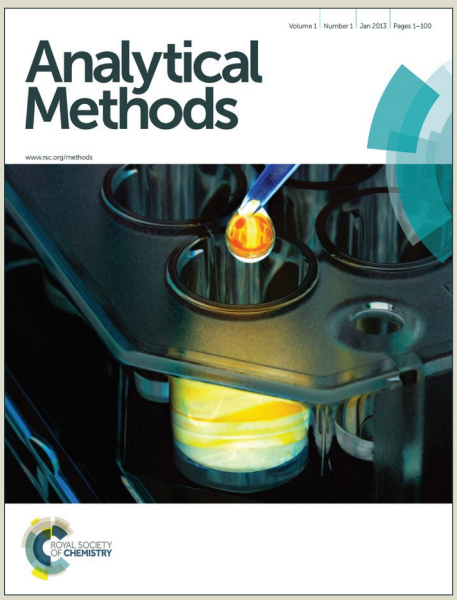

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Determination of sarafloxacin and its analogues in milk using an enzyme-linked immunosorbent assay based on monoclonal antibody

BN Tochi[1, 2], Juan Peng[1], Shanshan Song[1], Liqiang Liu[1], Hua Kuang[1], Chuanlai Xu[1, *]

1. State Key Lab of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu, 214122, China
2. Food Science and Technology, Pwani University, Kilifi Kenya.

Abstract

A heterologous immunoassay has been developed for the determination of sarafloxacin (SRFX) and its analogues residues in milk. A novel hapten with molar mass 499 g was synthesised by introducing a six carbon molecule [6-Bromohexanoic acid (BR)] as a spacer arm. This greatly improved the effect of SRFX inducing immune response in mice and enhanced the chance of producing a monoclonal antibody capable of recognising analogues to SRFX. The synthesised hapten [*7-(4-(5-carboxypentyl)piperazin-1-yl)-6-fluoro-1-(4-fluorophenyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid* (SRFX-BR)], to induce an immune response, was conjugated to bovine serum albumin (BSA) (SRFX-BR-BSA) via carbodiimide active ester method while mixed anhydride reaction was used to prepare the coating antigen [SRFX conjugated to ovalbumin (OVA) (SRFX-OVA)] to pursue the heterologous sensitivity. Based on the checkerboard titration, an indirect competitive enzyme-linked immunosorbent assay (icELISA) was developed for the quantitative detection of SRFX and three of its analogues in cattle milk. After optimisation, the immunoassay was found to tolerate up to 15% methanol at a physiological pH (7.4) and a salt (NaCl) concentration of 1.2%. The results of this assay showed a good cross-reactivity to Tosufloxacin (64.94%), Nadifloxacin (58.14%), Pazufloxacin (42.02%), Fleroxacin (40.04%), Pipemidic Acid (34.25%), and Ofloxacin (20.08%). These findings demonstrated that the immunoassay is able to detect FQs in milk samples.

Keywords: Monoclonal antibodies; Fluoroquinolones; ELISA; Antibiotic residues; Sarafloxacin.

E-mail: xcl@jiangnan.edu.cn; Tel: 0510-85329076

Introduction

Infectious diseases are a serious problem in animal husbandry prompting the use of various kinds of antibiotics and synthetic antibacterials in prevention and treatment. The fluoroquinolones (FQs) are the most important group of synthetic antibacterial, which are widely used in clinical practice because of their excellent antibacterial activity, wide spectrum of activity, and high degree of bioavailability [1, 2]. The chemical structures of twenty FQs are shown in Figure 1. Among these FQs, sarafloxacin (SRFX) which works by inhibition of bacterial DNA-topoisomerase II is commonly used in the treatment of cattle, pigs, poultry, sheep and fish. It is used in the drinking water of poultry to treat bacterial disease, and in fish feed to treat diseases such as furunculosis, vibriosis and enteric redmouth where a dosage at a rate of 10 mg/kg body weight is administered over a five days period [3]

This wide use has however brought with it other potential negative effects in terms of environmental degradation where SRFX for example has been reported to be very persistent in both soil and water [4]. The misuse of these antibacterials has consequently led to the presence of these compound residues in foodstuffs of animal origin [5]. This presence in foodstuffs and therefore exposure to these compounds has seen an increase of resistant human pathogens constituting a public health hazard, primarily through the increased risk to allergies [6] and treatment failures [7, 8].

Overwhelming evidence point to the transfer of these drug-resistant strains of pathogens from animals to humans [9-11] and this trend is expected to increase [12]. On the other hand, these antimicrobial residues have adverse effect on the indigenous human gastrointestinal tract micro-flora which are essential component of human physiology. These flora acts as a barrier against colonisation of the gastrointestinal tract by pathogenic bacteria [13] which plays an important role in the digestion of food. Several authors [14-16] have reported that food processing methods such as cooking and pasteurisation do not degrade FQs in food and their residues remain relatively stable during processing.

Due to these concerns, countries and organisations including the European Union (EU), United States (US), Japan, People's Republic of China (PRC), and Food and Agriculture Organization of the United Nations (FAO) have set maximum residue limits (MRLs) for several FQs [17]. These MRLs take into account the animal species, usage, dosage and withdrawal periods for each of the FQ in question. Taking SRFX as

an example, among the EU members, MRLs of 30 μg kg-1 is recommended for fish muscle, 100 μg kg^{-1} for chicken liver and 10 μg kg^{-1} for skin and fat [3]. In PRC, standards have been established by the Ministry of Agriculture through circular (No. 278, 22 May 2003).

In sum of these circumstances, monitoring foodstuffs of animal origin for the presence of veterinary drug residues is not optional to governments and food safety experts so as to safeguard public health and prevent their illicit use through regulation and surveillance [18, 19].

The most commonly used instrumental techniques for determination of FQs include HPLC with programmable fluorescence detection [20-22] and confirmatory analysis by liquid chromatography-mass spectrometry (LC-MS) [23, 24] and liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been reported [13, 20, 21, 25]. While these methods provide sensitive and accurate results, they rely on highly trained personnel to operate expensive and sophisticated instruments and interpret complicated chromatograms or spectrums. They are also labour intensive and employ the use of harmful organic solvents that are injurious to personnel and contribute to environmental degradation; therefore, they fail to fulfil the need for on-site, rapid screening tests for monitoring residues in foodstuffs. Compared to instrumental methods, enzyme linked immunosorbent assay (ELISA) methods are of low cost, sensitive and capable of screening large numbers of samples in a single test. So far, several authors have reported detection of FQs residues by the ELISA method [26-33].

The primary reagent in ELISA methods is the antibody whose activity and other analogues recognition ability is highly dependent on the hapten design steps. In these reports,[26-29, 31, 33] the parent FQ drugs were directly coupled to the carrier protein via the carboxyl group in their molecules. These conjugates on presentation to the animal immune system, give rise to antibodies which in most cases only show narrow cross-reactivity,[26, 28, 34-36] except those raised when the parent FQ is either ciprofloxacin or norfloxacin antibodies of which have been shown to simultaneously recognise more than 9 fluoroquinolone drugs.[30, 33, 37] Li and associates [38] introduced an amino group on the piperazine ring of the norfloxacin hapten through which they coupled to the carrier protein with the resultant antibody having cross reactivity to 13 analogues at a range of 16-112%. The same norfloxacin was reported [39] to have given rise to a polyclonal antibody that recognised 10 FQs with cross-reactivity range between 1-143% when it was coupled to carrier protein directly via the secondary amine on the

piperazinyl moiety. In all these cases, the common structure of the FQ drugs were presented to the animal immune system hence the antibodies obtained showed broad cross-reactivity. In the present work, a novel haptens of SRFX containing a six carbon molecule as a spacer arm on the piperazine ring was synthesised and coupled to carrier protein via the introduced carboxyl group to prepare an immunogen presented to the animal's immune system. The aim of this study was to produce a generic monoclonal antibody for multi-determination of FQs residues in milk.

Materials and methods

Materials and equipments

SRFX and other FQ standards were purchased from Shanghai HOPE Industry Co., Ltd, (Shanghai-PRC). Horseradish peroxidase-conjugated goat anti-mouse IgG, gelatine, 3,3′,5,5′-tetramethylbenzidine (TMB), and Freund's complete and incomplete adjuvants were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were obtained from Pierce (US). Bovine serum albumin (BSA) and ovalbumin (OVA) were acquired from Sunshine Biotechnology Co., Ltd. (Nanjing, PRC). All other reagents and chemicals were obtained from the National Pharmaceutical Group Chemical Reagent Co., Ltd. (Beijing, PRC). A vacuum rotary evaporator was bought from Shanghai shenshun technology Co., Ltd. (Shanghai, PRC). Female BABL/c mice 8 weeks old were obtained from the Shanghai Laboratory Animal Centre (Shanghai, China). A spectrophotometric microtitre plate reader (EON-Biotek instruments inc. highland park USA) was used for absorbance measurements.

Solutions and buffers

Solutions and buffers used during the course of this study were as follows: (1) 0.05 M carbonate buffer (CB), pH 9.6, as coating buffer; (2) 0.05 M CB containing 0.2% w/v gelatine as blocking buffer; (3) 0.01 M phosphate-buffered saline (PBS) containing 0.1% w/v gelatine and 0.05% v/v Tween 20 as antibody dilution buffer; (4) PBS with 0.05% v/v Tween 20 as washing buffer (PBST); (5) 0.03 M NaOH, pH 9.6, as standard stock solution buffer; (6) 0.01 M PBS, pH 7.4, as standard dilution buffer; (7) a freshly prepared was solution of 0.06% (w/v) TMB in glycol and 0.1 M citrate phosphate buffer (pH 5.0) at a ratio of 1:5 (v/v) containing 180 µl L-1 of 30% hydrogen peroxide as substrate reagent; (8) 2 M H_2SO_4 as stop solution; and (9) ethyl

ethanoate : HCl (1:1, v/v) and dichloromethane (DCM) : ethyl acetate (1:1, v/v) as extraction solutions.

Hapten and Immunogen synthesis

The SRFX hapten was synthesised and bound to carrier protein (BSA) to prepare immunogenic agent. The hapten synthesis route is summarised in Fig. 2 shown below.

Briefly, SRFX (0.6 g), 6-Bromohexanoic acid (0.6 g), Potassium carbonate (0.8 g) and Sodium iodide (14.2 mg) were refluxed in 100 mL acetonitrile for 12 days. Thereafter, the contents were allowed to sit for 8 days in the dark at room temperature prior to purification. Preceding purification, acetonitrile was evaporated to dryness in a vacuum rotary evaporator leaving behind a white precipitate. This precipitate was washed with 0.03 M NaOH solution and extracted with a mixture of HCl and ethyl ethanoate (1:1). The resultant precipitate was dissolved again in NaOH solution like before and re-extracted with a mixture of dichloromethane (DCM) and ethyl ethanoate (1:1). The hapten [*7-(4-(5-carboxypentyl)piperazin-1-yl)-6-fluoro-1-(4-fluorophenyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid* (SRFX-BR)] which was recrystallised as a white precipitate on addition of HCl, was successively washed twice with water, acetone and water. The LC-MS and the HPLC were used to decipher the molecular weight and purity of the hapten respectively (Fig. 3A and 3B)

The immunogen of SRFX-BR-BSA was prepared by a modified carbodiimide active ester method as previously described by Huang and associates [17]. A total of 15.26 mg (0.03 mmol) SRFX-BR, 9.46 mg (0.06 mmol) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 3.58 mg (0.03 mmol) of N-hydroxysuccinimide (NHS) were added to 1.0 ml Dimethylformamide (DMF) in order. The mixture solution was incubated for 24 h at room temperature in dark while continually being stirred on a magnetic stirrer. This solution was added drop-wise to 3 ml of PBS (0.01 mol/L, pH 7.4) containing 2% (w/w) BSA and re-incubated for a further 24 h like before. The reaction mixture was finally dialysed [molecular weight cut-off (MWCO): 14000 Da] under stirring against PBS (0.01 mol/L, pH 7.4) for 6 d with a 6 h repeated changes of the dialysis solution to remove the unconjugated free hapten. Two coating antigens were prepared, the first (SRFX-BR-OVA) following the above described route and

the second SRFX-OVA conjugate prepared based on the mixed anhydride reaction described by Jinqing and associates [31]. Briefly, 10 mg of OVA was dissolved in 5 ml of deionised water before 50 mg of EDC in 2 ml of deionised water was added. This mixture was stirred at 25°C for 45 minutes. Thereafter, 2 mg of SRFX in 1.0 ml of DMF was added drop wise to the activated mixture and further incubated overnight. Fig. 4 depicts a diagrammatic pathway to conjugating the coating antigen through the mixed-anhydride method. The reaction mixture was dialysed as previously described. The immunogen and coating antigens were both characterized by spectrophotometry Fig 5. The products were stored at −20 °C until use.

Immunization and cell fusion

SRFX-BR-BSA conjugate was emulsified in Freund's complete adjuvant (FCA) and administered subcutaneously as injections (100 µg/mouse) at multiple sites in the back of female BALB/c mice (8-10 weeks old). Six booster immunisations (50 µg/mouse) in Freund's incomplete adjuvant (FIA) were given on 28 days basis. A week after the third injection and in subsequent boost injections, the mice were tail bled to collect the antiserum. The mouse exhibiting the highest affinity and inhibition ratio by icELISA was administered one more injection (25 µg) intraperitoneally. The spleen of this mouse was fused with SP 2/0 murine myeloma cells [40] and cultured using HAT and HT solutions in 96-well plates. Eight days post-culture, supernatants were screened by icELISA for the secretion of mAb against SRFX. Selected hybridoma cells were cloned and subcloned thrice by limiting dilution.

Production and purification of mAb

A mature female BALB/c mouse was injected intraperitoneally (i.p) with 0.5 mL of paraffin 10 days before receiving an i.p injection of the positive hybridoma cells. Ascites fluid was collected 10 days after the injection and then stored at -20°C until use. Purification of mAb was performed according to the modified caprylic acid-ammonium sulphate precipitation method as previously described [41-43]. The protein content of the antibody was determined according to the following formula: protein concentration (mg/mL) = $1.45OD_{280nm}$ $-1.74OD_{260nm}$, where OD value is the optical density. The mAb were labelled and stored at -20°C until use.

icELISA establishment and optimisation

The half maximal inhibitory concentration (IC_{50}) was used to estimate the sensitivity of monoclonal antibodies which was the SRFX concentration that produced a 50% inhibition of antibody binding to the coating antigen. Additionally, the SRFX concentration that gave an optical density (OD) inhibitory of 20% was defined as the limit of detection (LOD) [30] in this study. Conversely, the SRFX concentration that gave an inhibitory effect between 20-80% of the maximum optical density was considered to be the detection range of icELISA [44].

Chemical parameters such as pH values, ionic strengths and organic solvent concentration are commonly acknowledged to affect immunoassay performance in this kind of studies [45]. To optimise, these parameters were estimated by running standard curves under various conditions. The A_{max}, (maximum absorbance value at zero concentration of SRFX) and IC_{50} (half-maximum inhibition concentration) values were calculated, and the condition that gave the maximal A_{max} /IC_{50} ratio was chosen as most ideal for this work.

ELISA procedure

The icELISA was carried out as described elsewhere [30]. Briefly, microtiter plates were incubated with 100 µl per well of the coating antigen (SRFX-OVA or SRFX-BR-OVA) diluted in coating buffer for 2 h at 37°C, washed thrice in PBST and blocked with blocking solution (200 µl per well) for 2 h at 37°C. Following post-block washing (twice), 50 µl of standard solution and 50 µl of diluted mAb were added to each well and incubated for 30 min at 37°C. After a third wash, 100 µl of goat-anti-mouse IgG-HRP (1:3000 in antibody dilution buffer) was added into each well and the plates incubated for 30 min at 37°C. Subsequently, the plates were washed four times and TMB (100 µl) substrate solution was added. The enzymatic reaction was terminated with 50 µl of stop solution following 15 min incubation at 37°C. Optical density (OD) was measured at 450 nm in a microplate reader.

Specificity of monoclonal antibodies

The specificity of the monoclonal antibodies was defined as the ability of structurally related analogues to combine with the monoclonal antibodies. The cross-reactivity (CR) values were calculated according to equation 1.

$$CR\% = \frac{\text{IC}_{50}\text{ value of Hapten}}{\text{IC}_{50}\text{ value of Competitor}} \text{ x } 100 \qquad (1)$$

Sample preparation and detection

Milk sample were purchased from a local supermarket and used for recovery tests. The residue-free status of the samples was confirmed by HPLC-MS/MS [46] prior to use.

Matrix effects in cattle milk samples

A total of 5 mL of milk samples were centrifuged at 4ºC with a speed of 10000 rpm for 20 min, and the fat discarded. In order to assess residual matrix interference and reduce the background noise, extracted milk samples were diluted [31, 35, 47, 48] in PBS (25, 50, 100, 150 and 200 fold) before they were dispensed unto the microtiter plate. The resultant experimental absorbance values were plotted to typical response curves, compared with that generated from the PBS buffer to determine the optimal milk dilution level.

Recovery studies

Under the optimal dilution regime, recovery studies were carried out by spiking a negative pool of milk samples with different SRFX concentrations (50, 100, and 200 μg L^{-1}). The concentration measured and concentration fortified was compared to validate the effectiveness of the developed immunoassay. Intra-assay variability in this study is defined as evaluation of nine repeat samples analysed on a single day, while inter-assay variability was determined from three triplicate spiked samples analysed on three different days. Accuracy means the recovery data of the spiked concentrations and precision was expressed as the coefficient of variation (CV).

Curve fitting and statistical analysis

A four-parameter logistic equation was used to fit the immunoassay data. Calculations were performed using OrginPro 8.5 software (OriginLab Corporation, Northampton, MA-USA).

Results and discussion

Hapten, Immunogen and coating antigen Synthesis

The key step during production of antibodies showing high affinity is attributed to the design of a suitable hapten for immunisation; therefore, the structure of the target

molecule should be maintained [49]. Various authors previously employed the use of norfloxacin, enrofloxacin, ciprofloxacin and sarafloxacin as haptens [5, 27, 30, 48]. Among these works, Huet and associates [5] reported detecting 15 FQs including 13 shown in Figure 1 in a heterologous direct competitive ELISA based on SRFX antibody. In this study, SRFX-BR hapten was successfully synthesised and used to immunise the animal. The LC-MS and HPLC were used to decipher the structure and purity of the hapten respectively, giving the molecular mass of the hapten as 499 g compared to the parent SRFX at 385.36 g and was about 63% pure (Fig. 3A and 3B). Despite the change in mass, the UV spectra showed the new hapten was very similar to the parent SRFX since it depicted the presence of characteristic peaks of SRFX. The introduction of BR as the spacer arm was meant to fully expose the parent heterocyclic organic molecule quinoline, which is a common structure in fluoroquinolone antibiotics with the aim of achieving broad cross-reaction to other analogues in the same family. Franek et al. [50] showed an immunogen with a long spacer arm between hapten and carrier could likely generate the antibody with high degree of binding for the hapten and its structurally similar analogues. By linking the parent hapten to a six carbon spacer arm, this ensued the animal's immune system was fully exposed to the hapten and therefore antibody produced had a wide cross-reactivity to similar analogues.

ELISA optimisation

The IC_{50}, maximal absorbance (A_{max}), and standard curve linearity are used to evaluate the ELISA performance. The A_{max}/IC_{50} ratio is an estimate of ELISA sensitivity; high ratios are indicative of high ELISA sensitivity [49]. The parameters pH, organic solvents and NaCl ions concentration were evaluated on the performance and sensitivity of this ELISA kit. Using the optimised conditions, a standard calibration curve (Fig. 6) with SRFX concentrations between 0.01-2.7 ng mL^{-1} was established. The limit of detection (LOD) or the least detectable dose was evaluated as the concentration of SRFX giving a 10% inhibition [51].

pH tolerance

To study the influence of pH on the assay characteristics, competitive curves were prepared using standards with pH values of 5.0, 6.0, 7.4, 8.0 and 9.0 in PBS. Fig. 7

presents the pH effects on the icELISA performance. Using the methods described above, these parameters (A_{max} and IC_{50}) were considered and the ratio of A_{max}/IC_{50} was used to estimate the optimum pH value. Although IC_{50} was lowest at pH 5, the A_{max} value was equally low and therefore the ratio of A_{max} /IC_{50} was low. This phenomenon was seen with pH 9 although the IC_{50} value at this pH was elevated. There was no significant fluctuation in A_{max} values between pH 6 and 8, but the IC_{50} at pH 6 was elevated. Overall, the highest A_{max} value and lowest IC_{50} was achieved at pH value 7.4, an indication that neutral assay buffer provides the best conditions for the binding of antibody and hapten.

Organic solvents tolerance

The effects of Methanol on the icELISA system was estimated by preparing standard curves containing varying amounts (0, 5, 10, 15, 20, and 25%) of organic solvent in PBS. The normalised dose-response curves at various solvent concentrations are shown (Fig. 8). The results showed progressive decrease and increase in the A_{max} and IC_{50} values respectively with increasing amount of organic solvents. The fluctuations were not significant up to 15% solvent presence, beyond which a significant drop in A_{max}/IC_{50} ratio was evident. From these results, it conclusively indicates that SRFX is better determined in assay buffers with minimal methanol.

NaCl ions tolerance

The effect of NaCl ions concentration on the icELISA system was evaluated. The results presented in Table 1 showed the optimal condition to be 1.2% NaCl in 0.01 M PBS standard dilution buffer (pH 7.4).

These optimised conditions (pH, organic solvent and NaCl concentration) were applied for the development of icELISA for subsequent steps in this kit.

ELISA sensitivity and specificity

Specificity was evaluated by determination of the cross-reactivity values from analogues within this family of antibiotics. Analogues that do not react with the antibody would produce absorbance near 100%; conversely, those that do react

with the antibody would decrease in percentage of absorbance. The IC_{50} value and cross-reactivity rate for each compound are presented (Table 2). According to this assay using a heterologous coating antigen (SRFX-OVA), 6 analogues (Tosufloxacin (65.2%), Nadifloxacin (58.14%), Pazufloxacin (42.02%), Fleroxacin (40.04%), Pipemidic acid (34.21%) and Ofloxacin (20.08%)) exhibited a middle cross-reactivity defined as a range between 40-80% [52]. Twelve (12) of the tested analogue, (Lomefloxacin, Enoxacin, Norfloxacin, Pefloxacin, Ciprofloxacin, Oxolinic Acid, Danofloxacin, Flumequine, Nalidixic Acid, Enrofloxacin, Gatifloxacin and Cinoxacin) did show low cross-reactivity ranging from 17.20% (Lomefloxacin) to 1.04% (Cinoxacin). One (1) analogue-Q-ACID (7-Chloro-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxoquinoline-3-carboxylic acid) had a cross-reactivity of below 1%. It proves that this immunoassay can simultaneously detect effectively six kinds of veterinary FQs residues. This result was achieved with SRFX-OVA a heterologous coating antigen to the immunogen used. Previously, we attempted to use a homologous coating antigen (SRFX-BR-OVA) but the result was unsatisfactory with IC_{50} value of 1.73 and poor cross-reactivity to other analogues. Liang and colleagues [53] did note that heterology is a proper strategy for the improvement of assay sensitivity in immunoassays which has also been demonstrated in this test kit's data (Table 2).

In this study, the immunogen was synthesised by the linkage of carboxylic acid group of the spacer arm BR with the amino group of BSA. In this linkage, the furthest group of SRFX from the linking point is the main heterocyclic part of the parent organic molecule which is a common structure in fluoroquinolone antibiotics. It is generally accepted in immunology that elicited to haptenic conjugates show a preferential recognition to the part of molecule that is furthest from the attachment site of the hapten [39]. Sarafloxacin molecule has the most complex heterocyclic body of all the fluoroquinolone antibiotics used in this study. Tosufloxacin which had the highest cross-reactivity is structurally quite very similar to sarafloxacin except it has extra fluorine attached to the phenol group off position 1 off the heterocyclic group. Generally, it is observed that the closer the heterocyclic part of the FQ is to SRFX hapten, the higher the cross-reactivity exhibited towards it by this antibody. However, there were exceptions (danofloxacin-2.5%, enrofloxacin-1.78% and gatifloxacin-1.72%) and it was difficult to speculate why these could not elicit high cross-reactivity yet they are structurally close to sarafloxacin around their heterocyclic ring.

Despite this, with the MRLs set by the EU ranging from 10 μg kg^{-1} for skin and fat to 100 μg kg^{-1} for chicken liver [3], and the fact that it shouldn't be used in animals producing milk for human consumption [54], this antibody is able to detect and discriminate against food matrices containing within this range for all the 19 FQs tested for cross-reactivity in this study.

Matrix effects

Matrix interference is common in recovery studies for ELISA [27, 41] as it affects antigen-antibody interaction. Dietary components, higher ionic strengths and the pH parameters all do strongly suppress the IC_{50} and the maximum absorbance values. In this study, a comparison between calibration plots for SRFX prepared in PBS and those prepared in different dilutions of milk clearly show the effect of matrix interference (Fig. 9). It was observed that the more dilute the milk was, the greater the reduction in matrix interference. In this case, a dilution 1:200 in cattle milk gave the inhibition curve almost the same as that of PBS buffer.

Recovery studies for ELISA

Sarafloxacin (SRFX) and the three analogues; Tosufloxacin (TSFX), Nadifloxacin (NDFX), and Pazufloxacin (PZFX) that exhibited a fairly good cross-reactivity (Table 2), were used for spiked cattle milk samples analysed by the developed icELISA. As shown in Table 3, the recovery values were 86.1 to 103.7% and 90.2 to 104.7% for SRFX, 96.4 to 109.8% and 92.1 to 103.2% for TSFX, 89.6 to 98.4% and 82.3 to 110.2% for NDFX and 97.9 to 105.8% and 93.7 to 106.1% for PZFX intra and inter assay respectively. In this study, matrix interferances were substantially narrowed by simple dilution [35, 47, 55] with water at a ratio of 1:200. These findings are within the range of those reported by others [5, 56-58]. Therefore, the developed method is reliable for real sample analyses.

Conclusions

In this study, a novel hapten of SRFX containing a six carbon molecule as a spacer arm on the piperazine ring was synthesised and coupled to carrier protein via the introduced carboxyl group to prepare an immunogen which was successfully applied to induce immune response in mice. Subsequently, the mouse that showed the highest titre on sera screening was sacrificed and its splenocytes fused with SP 2/0 murine myeloma cells, hybridoma cell lines cloned before being used to produce anti-SRFX monoclonal antibody (mAb). Two coating antigens (homologous and heterologous) were investigated and results revealed that the latter coating gave improved sensitivity as compared to the former. Using this coating, a highly sensitive icELISA was developed able to detect four of the twenty tested FQs analogues to SRFX in a food matrix system. Among the twenty FQs tested for cross-reactivity, TSFX showed the highest (64.94%) while 7-Chloro-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (Q-ACID) had the least (0.76%). The recovery values from spiked milk ranged from 86.1-109.8% and 82.3-110.2% intra and inter-assay respectively across the tested drugs. Following screening, the obtained mAb was found to be very sensitivity (IC_{50} value of 0.5ng mL^{-1} and an LOD value of 0.055ng mL^{-1}) and suitable for detecting SRFX and its cross-reactive analogues' residues in food samples.

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Author contributions

Chuanlai Xu conceived the original idea for the study and with Hua Kuang they were responsible for recruitment and follow-up. BN Tochi and Juan Peng were responsible for data collection and cleaning. Purchase of materials and supplies was done by Shanshan Song. Liqiang Liu together with BN Tochi carried out data analyses. BN Tochi drafted the manuscript, which was revised by Chuanlai Xu. All authors read and approved the final manuscript.

Compliance with Ethical Standards

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Conflict of interest: Authors declare no conflict of interest.

Ethical approval: This article does not contain any studies involving human subjects. All animal studies were carried out under the guidance of animal welfare committee of Jiangnan University.

Informed Consent: Not applicable

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Captions

Figure 1. Chemical structures of the 20 fluoroquinolone drugs used in this study

Figure 2. Diagrammatic depiction showing the route of hapten synthesis

Figure 3A. The LC-MS chromatogram for the synthesised hapten product

Figure 3B. The HPLC chromatogram showing the % purity for the synthesised hapten product

Figure 4. SRFX-OVA synthesis procedure through the mixed-anhydride method

Figure 5. Conjugates as characterised by spectrophotometry

Figure 6. The standard curve for SRFX under optimized conditions n=4

Figure 7. Effects of pH on the immunoassay with each point representing a mean of three replicates. Insets indicate the fluctuation of A_{max}/IC_{50} values

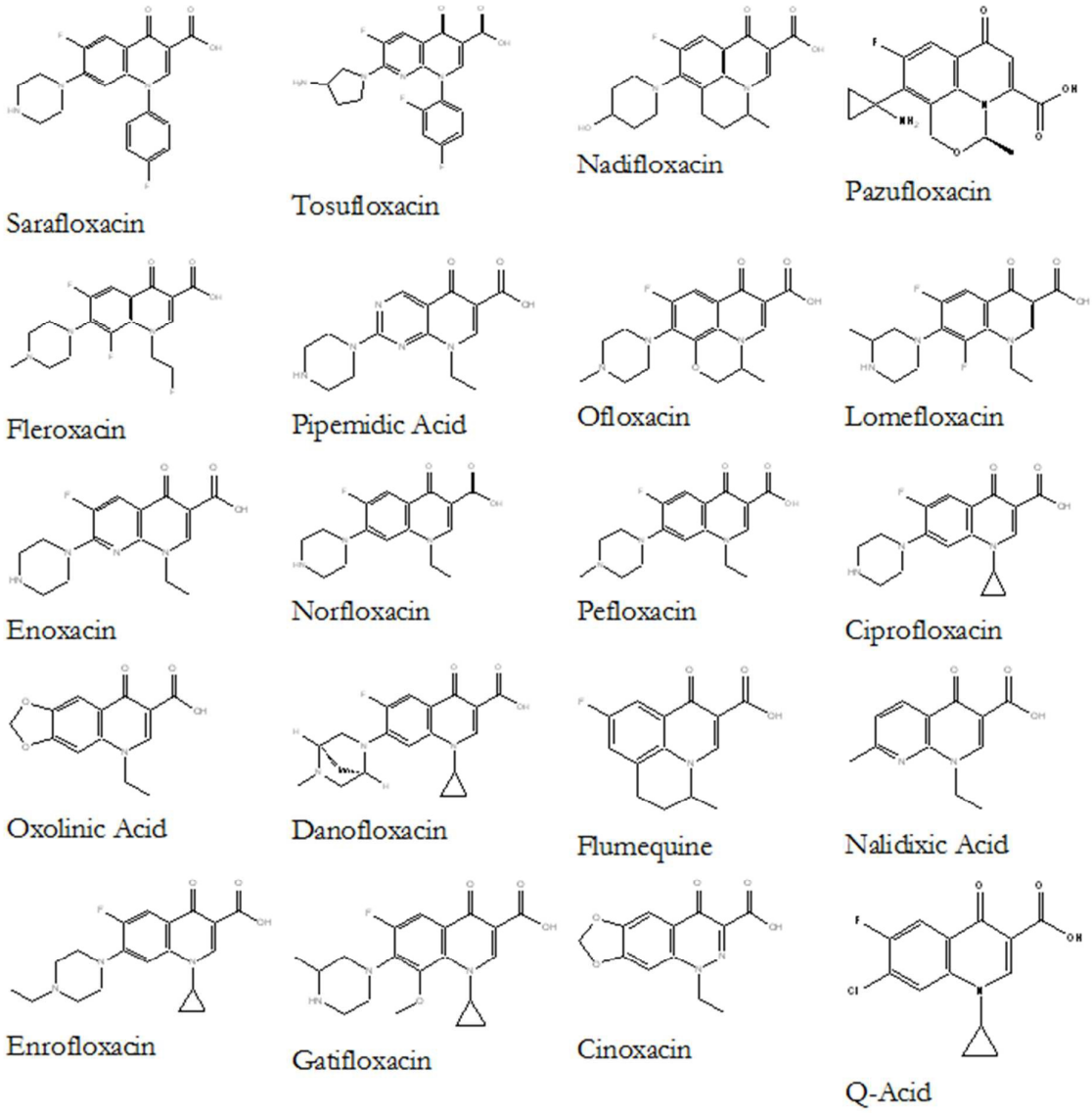
Figure 8. Effects of Methanol on the immunoassay with each point representing a mean of three replicates. Insets indicate the fluctuation of A_{max}/IC_{50} values

Figure 9. SRFX standard curves in the diluted milk samples. In the PBS buffer, 10-fold, 50-fold, 100-fold, 150-fold and 200 fold dilutions. Each point represents an average of three separate assays in triplicate.

Table 1. Effect of NaCl ions on the performance of icELISA

Table 2. Cross-reactivity values of 19 analogues to SRFX

Table 3: Recovery values from analysis of spiked cow's milk samples



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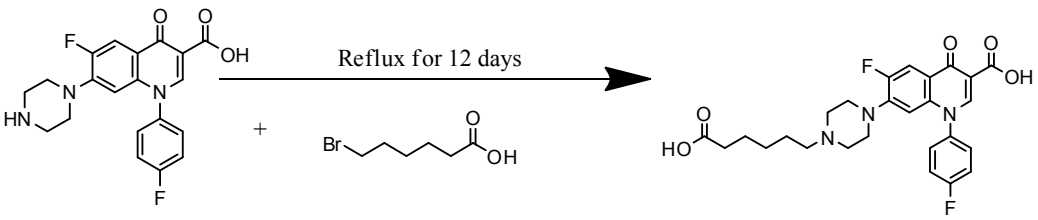

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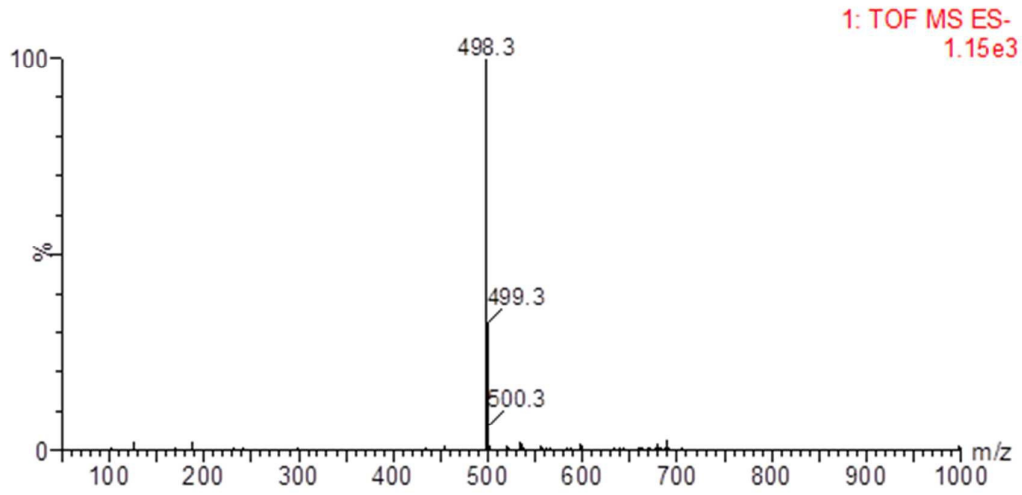
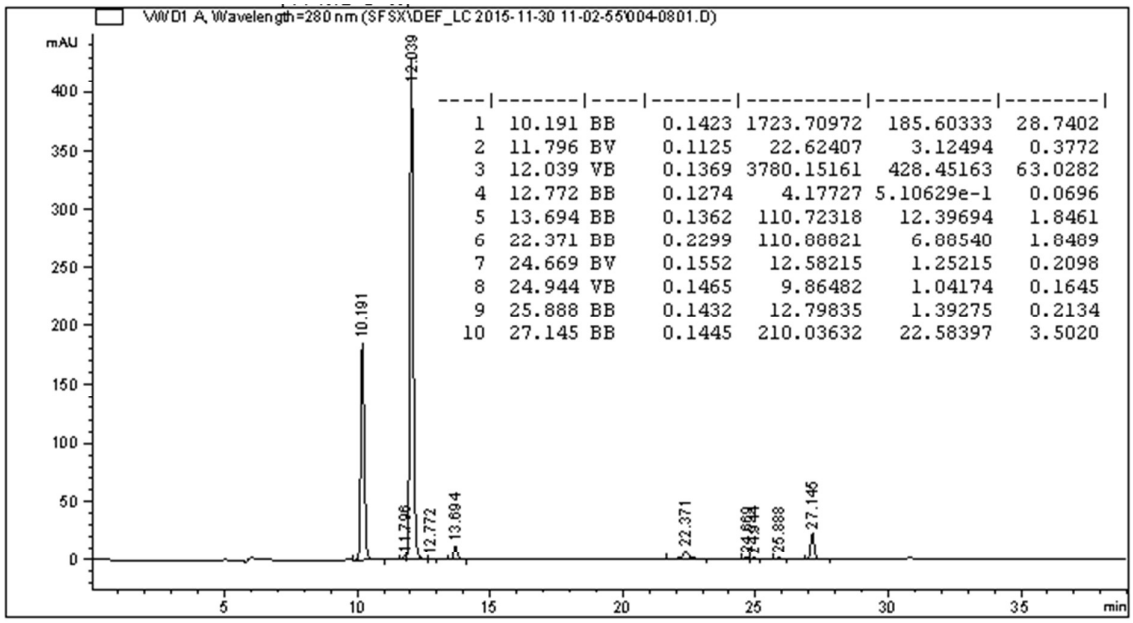


Fig. 3A: the LC-MS chromatogram for the synthesised hapten product



1	10.191	BB	0.1423	1723.70972	185.60333	28.7402
2	11.796	BV	0.1125	22.62407	3.12494	0.3772
3	12.039	VB	0.1369	3780.15161	428.45163	63.0282
4	12.772	BB	0.1274	4.17727	5.10629e-1	0.0696
5	13.694	BB	0.1362	110.72318	12.39694	1.8461
6	22.371	BB	0.2299	110.88821	6.88540	1.8489
7	24.669	BV	0.1552	12.58215	1.25215	0.2098
8	24.944	VB	0.1465	9.86482	1.04174	0.1645
9	25.888	BB	0.1432	12.79835	1.39275	0.2134
10	27.145	BB	0.1445	210.03632	22.58397	3.5020

Fig. 3B: The HPLC chromatogram showing the % purity for the synthesised hapten product

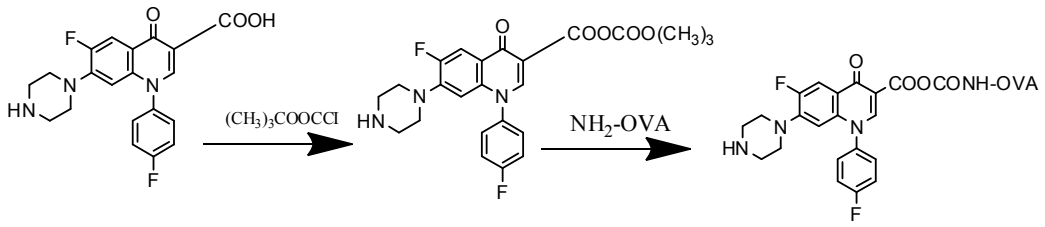
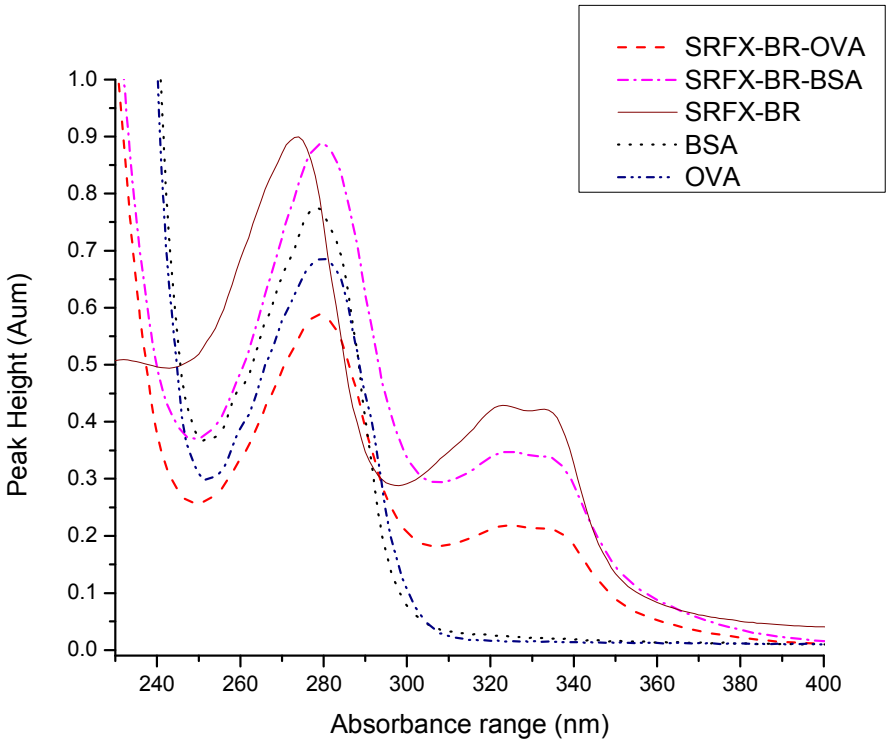


Fig. 4: SRFX-OVA synthesis procedure through the mixed-anhydride method



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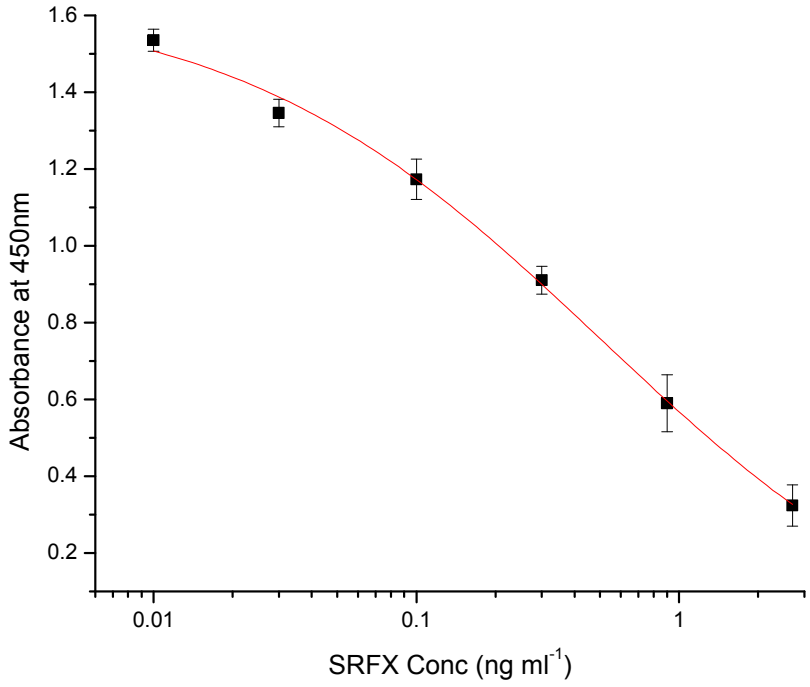

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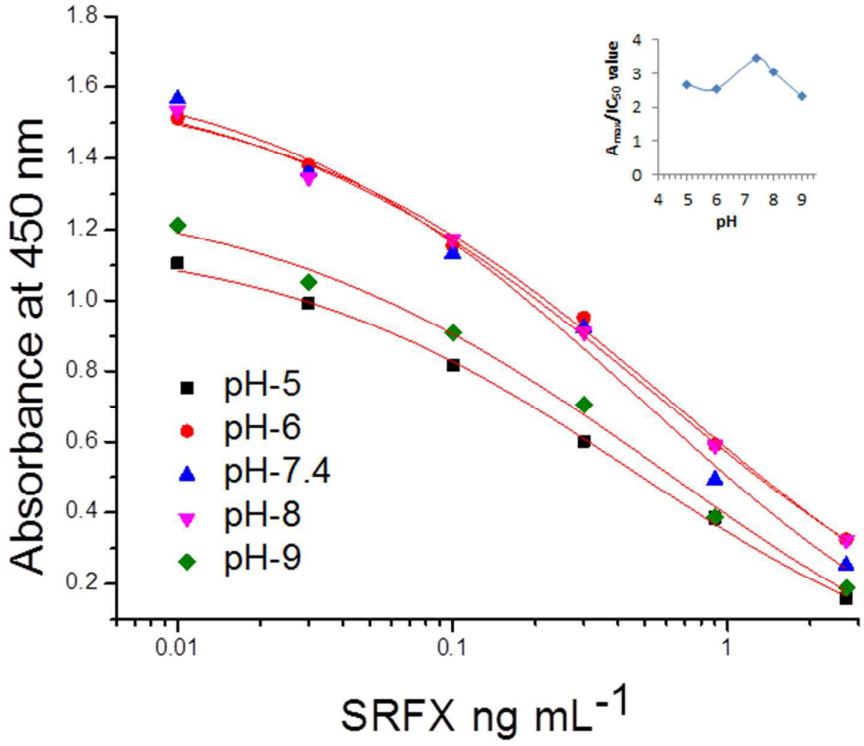

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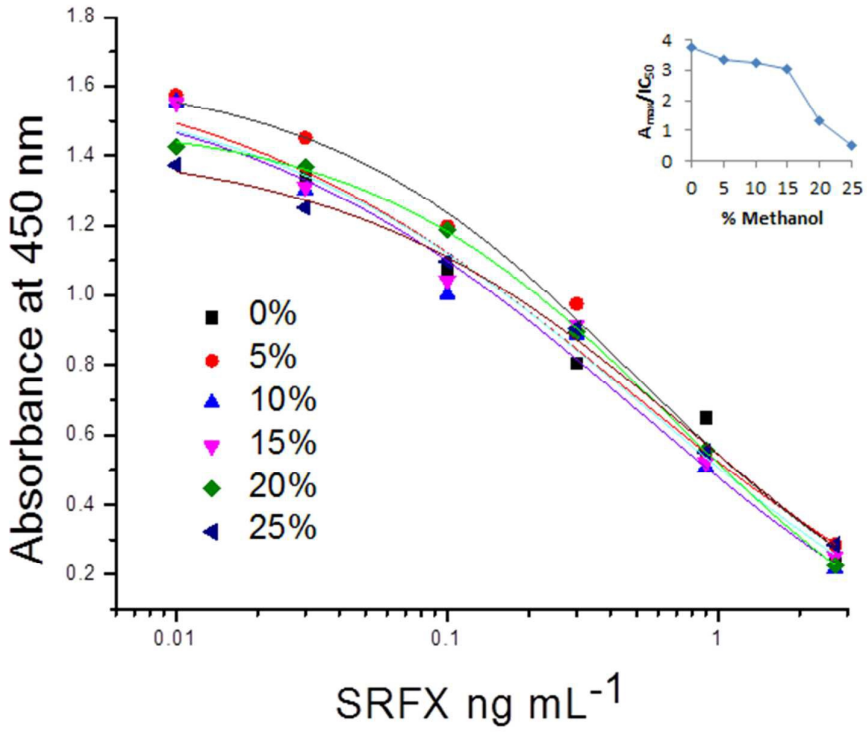

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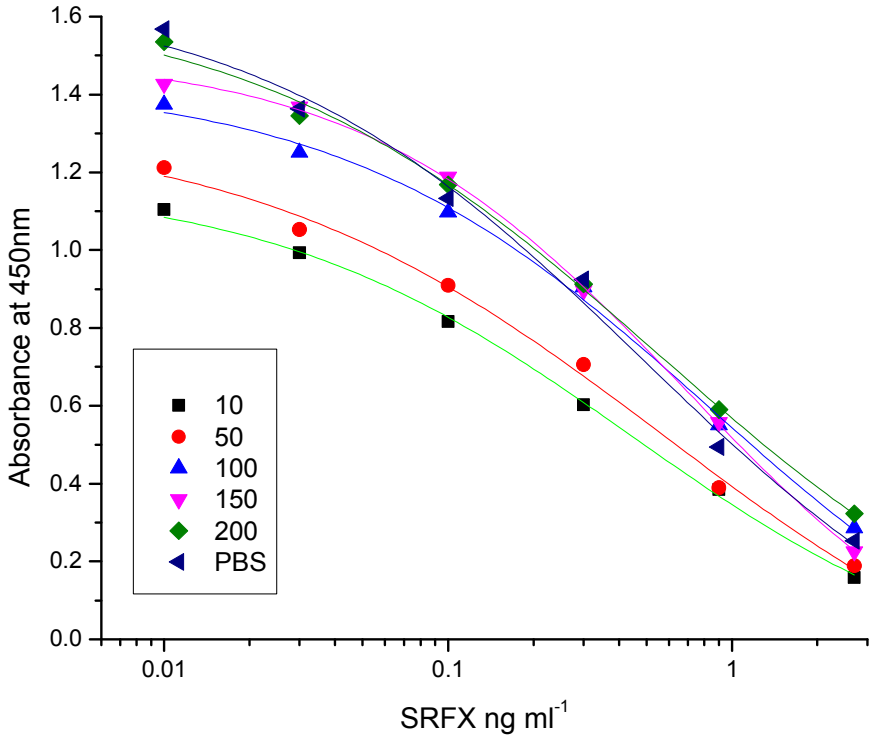

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Table 1: Effect of NaCl ions on the performance of icELISA

%NaCl	A_{max}	IC_{50}	A_{max}/IC_{50}
0.30%	1.508	0.68	2.20
0.60%	1.64	0.54	3.05
1.20%	1.662	0.48	3.44
2.40%	1.623	0.64	2.53
4.80%	1.334	0.57	2.34
9.60%	1.167	0.84	1.39

Table 2: Cross-reactivity values of 19 analogues to SRFX

	SRFX-OVA		SRFX-BR-OVA	
COMPOUND	**IC_{50} (ng ml^{-1})**	**CR%**	**IC_{50} (ng ml^{-1})**	**CR%**
Sarafloxacin	0.50	100.00	1.73	100.00
Tosufloxacin	0.77	64.94	23.26	7.44
Nadifloxacin	0.86	58.14	49.57	3.50
Pazufloxacin	1.19	42.02	169.15	1.02
Fleroxacin	1.25	40.04	131.48	1.32
Pipemidic Acid	1.46	34.25	125.92	1.37
Ofloxacin	2.50	20.08	241.26	<1
Lomefloxacin	2.91	17.18	394.74	<1
Enoxacin	3.25	15.38	575.06	<1
Norfloxacin	4.14	12.08	314.28	<1
Pefloxacin	13.96	3.58	284.21	<1
Ciprofloxacin	14.91	3.35	100.79	1.72
Oxolinic Acid	15.42	3.24	407.79	<1
Danofloxacin	20.05	2.50	594.15	<1
Flumequine	19.35	2.58	123.99	1.40
Nalidixic Acid	25.70	1.95	174.63	<1
Enrofloxacin	28.15	1.78	303.83	<1
Gatifloxacin	29.14	1.72	522.47	<1
Cinoxacin	48.19	1.04	1344.34	<1
Q-Acid	65.94	<1	982.08	<1

Table 3: Recovery values from analysis of spiked cow's milk samples

Sample	Spike level ($\mu g\ Kg^{-1}$)	Intra-assay (n=9)		Inter-assay (n=9)	
		Recovery (%)	CV (%)	Recovery (%)	CV (%)
SRFX	50	86.1	9.36	95.5	8.62
	100	94.2	5.18	90.2	10.16
	200	103.7	7.42	104.7	6.45
TSFX	50	96.4	6.6	92.1	2.6
	100	105.6	4.89	103.2	4.7
	200	109.8	6.75	97.9	8.9
NDFX	50	89.4	10.1	82.3	10.1
	100	92.1	5.8	96.5	5.32
	200	98.4	8.72	110.2	7.44
PZFX	50	97.9	3.6	93.7	9.8
	100	105.8	3.98	102.9	5.03
	200	101.2	7.31	106.1	4.99