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Determination of Marbofloxacin Residues in Beef and Pork with an Enzyme-Linked Immunosorbent Assay

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Marbofloxacin is a fluoroquinolone veterinary antibiotic. An indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed for the detection of marbofloxacin using polyclonal antibody. The half-maximum inhibition concentrations (IC_{50}) and limit of detection (LOD, calculated as IC_{20}) of the ELISA for marbofloxacin in phosphate buffer were 4.6 and 0.6 ng/mL, respectively. The assay showed little cross-reactivity with marbofloxacin structural analogues, except for ofloxacin (148%). Matrixes from the extracts of beef and pork muscle have shown a significant influence on the ELISA. Standard curves of ELISA for marbofloxacin in the extracts of the appropriate marbofloxacin-free control muscles were used in the analysis of marbofloxacin in the animal muscles without any cleanup. The average recoveries of intra- and interassay for marbofloxacin from fortified muscle samples, at five concentrations of 10, 50, 100, 500, and 1000 ng/g, were 87−93 and 84−95%, respectively. The LOD of this assay for marbofloxacin in real muscle extracts was 0.8 ng/mL. A survey of 55 animal muscle samples purchased from local markets by the ELISA was conducted, and marbofloxacin was detected in one of them at a concentration of 22 ng/g. This positive sample was validated by liquid chromatography−electrospray ionization−tandem mass spectrometry (LC−ESI−MS/MS) to be 28 ng/g of marbofloxacin.

KEYWORDS: Marbofloxacin; ELISA; beef; pork; food safety; food contamination; antibiotics

INTRODUCTION

Fluoroquinolones are important antimicrobial agents developed in recent years that are used to treat various human and animal infectious diseases. These compounds exhibit high activity against a broad spectrum of Gram-negative and Gram-positive bacteria through inhibition of their DNA-gyrase or topoisomerase II (*1*, *2*). Marbofloxacin is a fluoroquinolone antibiotic approved for veterinary clinics for dermatological, respiratory, and urinary tract infections. Pharmacokinetic studies in normal animals have shown that, following oral administration, marbofloxacin is totally and rapidly absorbed and distributed in peripheral tissues (*3*). The main pharmacokinetic characteristic of marbofloxacin is its long half-life (around 10 h), which allows for a once-a-day dosage administration (*4*).

Fluoroquinolones including marbofloxacin are widely used for treatment and prevention of diseases in food-producing animals and as feed additives to increase the animal mass over the last few decades (*5*, *6*). Their misuse can leave residues in edible animal meats, which may give rise to public health concerns over toxic effects, development of resistant strains of bacteria, allergic hypersensitivity reactions, etc. (*5*). The European Union (EU) has established maximum residue limits (MRLs) for certain veterinary medicines in foodstuffs of animal origin (Council Regulation EEC 2377/90, 1990).

Monitoring of fluoroquinolone residues in edible animal meats is crucial for proper assessment of human exposure to fluoroquinolones through foods. A number of analytical methods have been reported for the determination of marbofloxacin in biological samples by way of high-performance liquid chromatography (HPLC) (*7*, *8*), liquid chromatography−mass spectrometry (LC−MS) (*9*), capillary zone electrophoresis−mass spectrometry (CZE−MS) (*10*), etc. These methods are usually validated for the multiresidue analysis of fluoroquinolone antibiotics. In general, the expensive equipment, skilled personnel, and complex sample treatments are involved in instrumental methods, whereas immunoassay has been proven to be a rapid, sensitive, and cost-effective method, which was widely used for routine monitoring.

Recently, various immunoassay methods have been designed for determining individual (*11*−*14*) or generic fluoroquinolones (*15*−*19*) in several matrices. Broad-specificity enzyme-linked immunosorbent assays (ELISAs) developed for fluoroquinolones were also highly sensitive to marbofloxacin (*16*, *17*). Our interest was to develop a marbofloxacin-specific ELISA, which would permit the routine analysis of marbofloxacin in food-safety, pharmacokinetic, and clinical studies. In this study, an

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ELISA was developed for monitoring marbofloxacin residues in real-world animal muscles and some of the results were validated with LC−MS. To our knowledge, it is the first report on the survey of marbofloxacin residues in real-world animal tissues by immunoassay and complementary LC−MS.

MATERIALS AND METHODS

Reagents and Chemicals. All chemicals used were of analytical grade, unless otherwise specified. Marbofloxacin (99.0%) and other fluoroquinolones used in a cross-reactivity study were purchased from the China Institute of Veterinary Drug Control (Beijing, China). Oxolinic acid (OXO, 99.0%) and flumequine (FLU, 99.0%) were purchased from Dr. Ehrenstorfer, GmbH (Augsburg, Germany). Reagents obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO) were *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl), goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (IgG−HRP), bovine serum albumin (BSA), ovalbumin (OVA), dimethyl sulfoxide (DMSO), hydrogen peroxide, urea, and 3,3′,5,5′-tetramethyl benzidine (TMB).

Preparation of Marbofloxacin−Protein Conjugates. Marbofloxacin (0.1 mmol) was added to 1 mL of anhydrous dimethylformamide (DMF) containing 0.12 mmol of NHS and 0.12 mmol of EDC. The mixture was stirred at room temperature for 4 h and then centrifuged to remove precipitated urea. The resulting active ester (500 μL) was added slowly to 5 mL of protein solution (50 mg of BSA or 30 mg of OVA in 0.1 M borate buffer at pH 9.0). The reaction mixture was stirred at 4 °C overnight and then dialyzed against 0.01 M phosphate-buffered saline (PBS, pH 7.4) for 3 days.

Polyclonal Antibody Production. Two female New Zealand white rabbits were each injected subcutaneously 5 times with marbofloxacin−BSA conjugate (1 mg/mL) at 2 week intervals. The first injection consisted of 1 mL of the conjugate mixed with 1 mL of complete Freund's adjuvant. Incomplete adjuvant was used instead of complete in the subsequent boost injections. The rabbits were bled 10 days after the final injection, and antisera were purified with ammonium sulfate precipitation.

ELISA Performance. Microplate wells were coated with 100 μL per well of marbofloxacin−OVA diluted in 0.05 M carbonate−bicarbonate buffer (pH 9.6) (1:5000) by incubation overnight at 4 °C. The plate was washed 3 times with 0.01 M PBS containing 0.05% Tween-20 (PBST, pH 7.4) and blocked with 1% gelatin in PBS (150 μL/well) by incubation at 37 °C for 30 min. After washing, a solution of 50 μL per well of analytes or standards and 50 μL per well of antibody in PBST (1:5000) was added and incubated at 37 °C for 30 min. After the plate was washed again, an aliquot of 100 μL per well of goat anti-rabbit IgG−HRP in PBST (1:5000) was added and incubated for 30 min at 37 °C. The plate was washed again followed by the addition of 100 μL of TMB substrate solution into each well. The reaction was stopped with 2 M sulfuric acid solution after an incubation of 10−15 min at ambient temperature. The absorbance at 450 nm was read immediately, and the data were fitted with the four-parameter logistic equation using SigmaPlot 2000 (version 6.0).

LC−MS Analysis. LC−MS analysis was performed on an Agilent LC−MS system (Agilent Technologies, Palo Alto, CA) consisting of a 1100 model HPLC connected to a single octopole MSD operated in an electrospray ionization (ESI) positive mode. Chromatographic separation was achieved on a Symmetry C_{18} column (5 μm particle size, 150 × 4.6 mm inner diameter, Waters, Milford, MA). The mobile phase consisted of a mixture of acetonitrile and water with 0.1% formic acid (1:4, v/v), at a constant rate of 1 mL/min. The column temperature was maintained at 25 °C. An aliquot of 20 μL samples was injected. The MS conditions were as follows: gas (N_2) temperature, 350 °C; flow rate, 8.0 L/min; nebulizer pressure, 35 psi; multiplier voltage, 1577 V; dynode voltage, 7.0 KV; extracted ion, 363.2 [M + H]; and scan range, m/z 50−500.

Sample Preparation and Extraction. A total of 25 beef and 30 pork muscle samples were purchased from 11 supermarkets and 10 stall markets in Beijing, China. Some beef and pork samples with known backgrounds and certified as free of marbofloxacin were used as blank samples in this study. These samples were homogenized and then fortified with marbofloxacin to give the final concentrations at 10, 50, 100, 500, and 1000 ng/g. Spiked samples were prepared freshly prior to analysis.

For ELISA, a 2 g aliquot of sample in 10 mL of extraction buffer (13.6 g of KH_2PO_4 in 1 L of distilled water at pH 7.0) was homogenized for 1 min followed by centrifugation at 5000 rpm for 10 min. The supernatant was collected, and the pellet was extracted once again. The combined supernatants were centrifuged at 10 000 rpm for 10 min. The final supernatant (≈20 mL) was analyzed with the ELISA directly or at different dilutions.

For LC−MS, a 5.0 g aliquot of sample in 25 mL of acetonitrile containing 1% acetic acid (v/v) was homogenized for 1 min followed by centrifugation at 5000 rpm for 10 min. The supernatant was collected, and the pellet was extracted once again. The combined supernatant was concentrated to near dryness with a rotary evaporator under vacuum at 40 °C and further dried under a gentle stream of nitrogen at 40 °C. The residue was reconstituted with 2 mL of acetonitrile/water (1:4, v/v). This solution was defatted with *n*-hexane (5 mL × 2) and then passed through a filter (0.45 μm) prior to analysis.

RESULTS AND DISCUSSION

Antibody Characterization. The antisera collected after each boosting was subjected to titration with an indirect ELISA. Because the antisera finally bled from the two rabbits showed similar titers, they were pooled together. A checkerboard titer method was employed to screen the optimal concentrations of antibody and marbofloxacin−OVA in a competitive ELISA for marbofloxacin. **Figure 1** shows the standard inhibition curve of ELISA for marbofloxacin dissolved in PBST, using the optimized dilution ratio of 1:5000 for both antibody and marbofloxacin−OVA. The half-maximum inhibition concentration (IC_{50}, the value comes from the parameter determined by the least-squares fit of the four-parameter equation) of marbofloxacin and limit of detection (LOD, concentration calculated as IC_{20}) of this assay were 4.6 and 0.6 ng/mL, respectively, indicating that the antibody had high affinity to marbofloxacin.

The specificity of the antibody was evaluated by the determination of the cross-reactivity with marbofloxacin analogues, i.e., (fluoro)quinolones. All of the test compounds, except ofloxacin, showed low cross-reactivity (**Table 1**). As reported in other studies (*20*, *21*), when antibodies were developed from immunogen synthesized by linking a carboxylic acid group of fluoroquinolones to carrier proteins, high cross-reactivity was observed among those fluoroquinolones with similar subsitituents on R1 or R2 (I in **Table 1**). It is deduced that the substitution group (R1 or R2) on the basic structure of fluoroquinolones plays an important role in epitope determination. The high similarity of structures of substitutents (R1 and R2) between marbofloxacin and ofloxacin (II and III in **Table 1**) may explain their high cross-reactivity

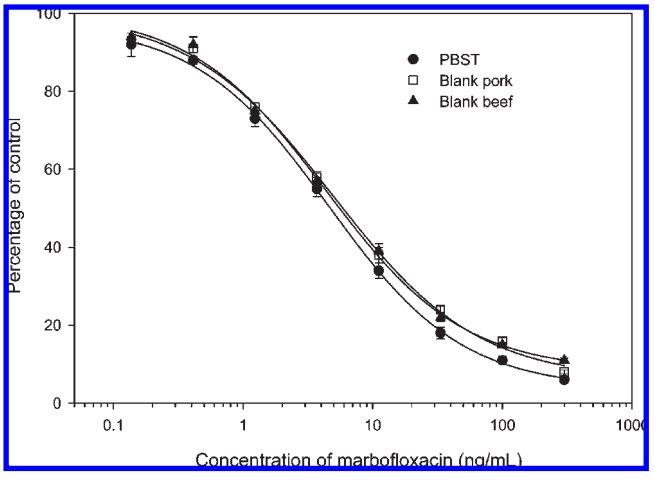
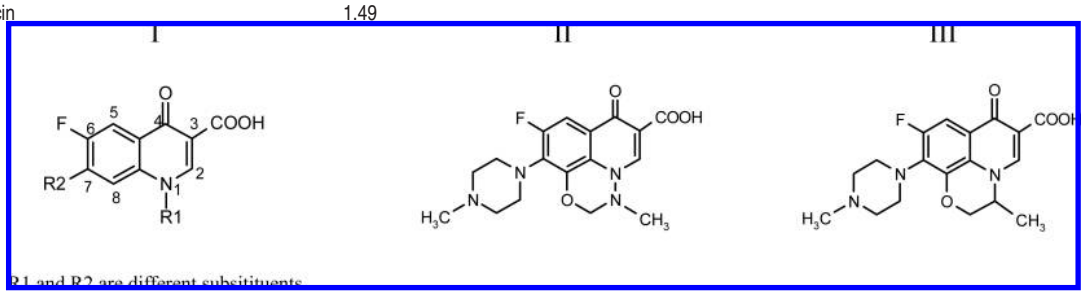


Figure 1. Standard inhibition curves of ELISA for marbofloxacin dissolved in PBST and in extracts of blank pork and blank beef without dilution. Data are mean of four replicates.

Table 1. Cross-reactivity of Anti-marbofloxacin Antibody with Various (Fluoro)quinolones[a]

compounds	cross-reactivity[b] (%)	compounds	cross-reactivity (%)
marbofloxacin	100	norfloxacin	0.02
ofloxacin	148	danofloxacin	0.2
enrofloxacin	2.16	oxolinic acid	<0.01
ciprofloxacin	0.02	flumequine	<0.01
difloxacin	1.49		



[a] Chemical structures listed are fluoroquinolones (I), marbofloxacin (II), and ofloxacin (III). [b] Cross-reactivity values were calculated as percentages of the IC_{50} of the standard marbofloxacin to the IC_{50} of the test compounds.

Table 2. Matrix Interference Effects on the ELISA Results as a Function of Dilution Factors

		detected concentration ± SD (ng/mL) (n = 3)					
			in PBS-diluted extracts				
matrix	fortified concentration (ng/mL)	in PBS	1×	5×	10×	20×	40×
beef	1.0	1.0 ± 0.1	11.2 ± 0.8	2.7 ± 0.1	2.4 ± 0.2	1.8 ± 0.2	**1.2 ± 0.1**
	5.0	5.2 ± 0.1	30.2 ± 1.8	9.2 ± 0.5	6.5 ± 0.7	**5.7 ± 0.9**	**4.5 ± 0.2**
	20.0	20.6 ± 0.8	69.3 ± 9.2	30.2 ± 4.3	**23.9 ± 5.9**	**23.0 ± 3.7**	**19.9 ± 2.6**
pork	1.0	1.1 ± 0.1	10.8 ± 2.4	2.8 ± 0.4	2.4 ± 0.3	1.8 ± 0.3	**1.2 ± 0.3**
	5.0	5.3 ± 0.2	25.5 ± 2.8	9.8 ± 0.8	7.8 ± 0.7	**6.1 ± 0.5**	**5.1 ± 0.4**
	20.0	21.2 ± 0.6	42.7 ± 9.8	26.4 ± 3.5	**21.8 ± 3.8**	**20.7 ± 2.4**	**17.1 ± 2.7**

(148%). This ELISA could potentially be applied to the determination of both marbofloxacin and ofloxacin.

Matrix Effects on ELISA. ELISAs are designed to be simple and rapid for sample screening; therefore, complex procedures involved in sample pretreatment are not recommended. In the present study, the beef and pork muscle samples were simply extracted with phosphate buffer and no cleanup step was employed prior to analysis. The influence of sample matrixes on the ELISA was evaluated by detecting marbofloxacin fortified in PBS-diluted extracts at concentrations of 1, 5, and 20 ng/mL (**Table 2**). It is clear that extracts with low dilution factors (≤5-fold) significantly affected the ELISA for marbofloxacin, because the detected concentrations were much higher than those fortified (**Table 2**). To reduce the matrix effects, the least dilutions for muscle extracts with a fortification level of 1, 5, and 20 ng/mL marbofloxacin were expected to be 40-, 20-, and 10-fold, respectively (data in bold in **Table 2**). It is noted that, the lower the concentrations of marbofloxacin fortified in the extracts, the higher the matrix effects on the ELISA. According to the extraction procedure, a 40-fold dilution for an unknown sample extract (≈20 mL) will allow for the LOD of this ELISA for marbofloxacin in beef and pork muscles to be around 240 ng/g, which is above the MRLs (150 ng/g) set by EU.

In general, dilution of sample extracts and preparation of the marbofloxacin standard in the blank muscle extracts are two effective ways to minimize the interference of matrixes on the ELISA. As discussed above, the dilution of sample extracts may make the marofloxacin residue level below the LOD when the concentration of marbofloxacin in samples is very low. The standard inhibition curve of the ELISA for marbofloxacin in the blank muscle extracts shifted moderately to the right from that in PBST (**Figure 1**), which was due to the nonspecific matrix interference. It is noteworthy that the optical density (OD) of ELISA for marbofloxacin in the blank muscle extract is lower than that in PBST (data not shown). However, the ELISA running in PBST is a little more sensitive than that running in the blank muscle extracts (**Figure 1**). The IC_{50} values obtained from the curves constructed in the blank extracts of beef and pork were 5.8 and 6.0 ng/mL, respectively. The IC_{20} values (LODs) from both curves were approximately 0.8 ng/mL, which is sensitive enough for the determination of marbofloxacin in beef and pork muscles. Thus, the ELISA running with the standard marbofloxacin dissolved in the blank muscle extracts was applied to the determination of marbofloxacin in samples in the present study.

Recoveries of Marbofloxacin in Beef and Pork. Marbofloxacin was fortified in beef and pork muscle samples each at five concentrations. The average recoveries of marbofloxacin by ELISA in intra- and interassays were in ranges of 87−93 and 84−95%, respectively (**Table 3**). The coefficients of variation (CVs) of intra- and interassays were 4−7 and 5−11%, respectively. In the analysis of LC−MS, the average recoveries and CVs were in a range of 92−116 and 2−18%, respectively. It is noticed that all of the recoveries of mabofloxacin by ELISA (< 100%) are less than those by LC−MS (**Table 3**). However, considering the ELISA as a screen method, the recoveries are good enough for the detection of marbofloxacin in animal muscle samples.

Survey and Validation of Marbofloxacin in Real-World Samples. This ELISA was applied to survey marbofloxacin residues in 25 beef and 30 pork samples collected from the local markets. Marbofloxacin was not detectable in all muscles but one pork sample, with an approximate concentration of 22 ng/g. This positive sample determined by ELISA along with six randomly selected negative beef and pork samples was then validated with LC−MS (**Figure 2**). Both methods showed consistent results for

Table 3. Recoveries and Precision of Marbofloxacin in Fortified Samples by ELISA and LC−MS

		ELISA					
		intra-assay[a] (n = 4)		interassay[b] (n = 4)		LC−MS (n = 3)	
sample	fortified concentration (ng/g)	average recovery (%)	CV[c] (%)	average recovery (%)	CV (%)	average recovery (%)	CV (%)
beef	10	87	6	85	7	114	11
	50	93	7	90	11	NA[d]	
	100	88	6	90	8	104	2
	500	89	4	84	5	115	8
	1000	91	6	88	7	92	6
pork	10	89	6	87	6	108	7
	50	89	6	92	8	NA[d]	
	100	92	4	92	7	111	4
	500	90	5	95	6	106	6
	1000	92	5	89	5	116	18

[a] Intra-assay variations were determined by four replicates on a single day. [b] Interassay variations were determined by a single test on 4 different days. [c] Coefficient of variation. [d] NA = no analysis was carried out by LC−MS.

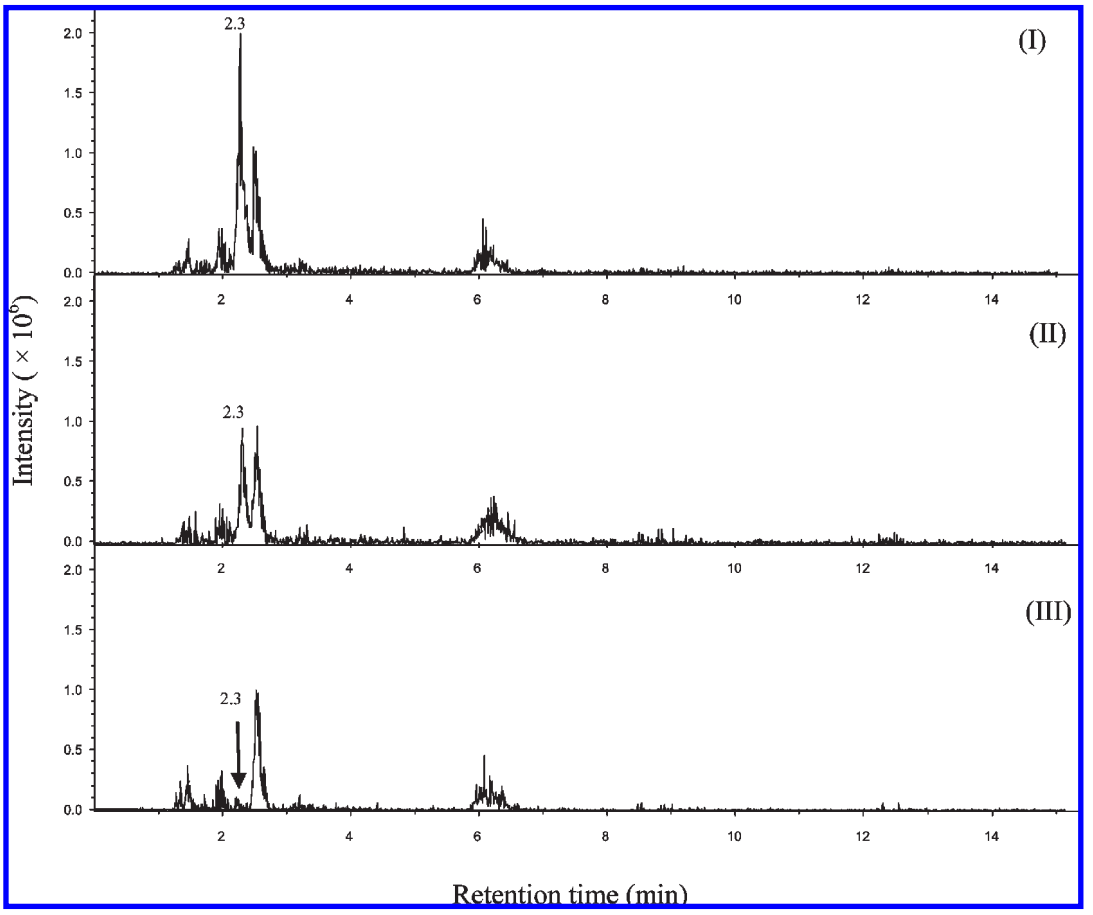


Figure 2. Extracted ion chromatograms of marbofloxacin in the fortified pork sample (100 ng/g) (I), positive pork sample (28 ng/g) (II), and negative pork sample (III). The retention time (RT) of marbofloxacin is 2.3 min.

these samples. The presence of the marbofloxacin residue in the positive sample was validated by LC−MS analysis (II in **Figure 2**) with a concentration of 28 ng/g, which is far below the MRLs of 150 ng/g in beef and pork.

Marbofloxacin is an approved veterinary medicine in China. The results of this very preliminary survey indicated that it was probably used in the local livestock industry for some food-producing animals. Although the MRL of marbofloxacin in food meats has not been set yet in China, routine monitoring of real-world samples would be necessary to ensure proper usage of marbofloxacin. The consistent results between ELISA and LC−MS suggest that the ELISA is a suitable method for screening marbofloxacin residues in real tissue samples. It may be used for the analysis of marbofloxacin not only in future food safety but in pharmacokinetic and clinical studies as well.

In summary, a sensitive polyclonal-based ELISA was developed for the analysis of marbofloxacin residues in beef and pork muscles. Because of the remarkable interference from the matrix, this ELISA was adjusted to construct the standard inhibition curve in blank animal muscle extracts and then applied to the analysis of marbofloxacin in samples. The recoveries of both intra-assays (87−93%) and interassays (84−95%) for marbofloxacin spiked in muscle samples were satisfactory. A total of 55 pork and beef meat samples

collected from the local markets were surveyed with this ELISA, and one of them was shown to be marbofloxacin-positive, which was confirmed with LC−MS analysis. This ELISA can be a useful method to detect marbofloxacin residues in animal muscle samples.

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