JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Simultaneous Determination of Multiple (Fluoro)quinolone Antibiotics in Food Samples by a One-Step Fluorescence Polarization Immunoassay

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Supporting Information

ABSTRACT: This paper describes a rapid one-step fluorescence polarization immunoassay (FPIA) for the simultaneous determination of multiple (fluoro)quinolone antibiotics (FQs) in food samples. Several fluorescent tracers were synthesized and evaluated in the FPIA method based on a broad-specificity of monoclonal antibodies toward FQs. The heterogeneous tracer, SAR-5-FAM, was considered as the optimal choice to prepare the immunocomplex single reagent, which allows a rapid and sensitive displacement reaction by addition of analytes. Optimized single-reagent FPIA exhibited broad cross-reactivities in the range of 7.8−172.2% with 16 FQs tested and was capable of determining most FQs at the level of maximum residue limits. Recoveries for spiked milk and chicken muscle samples were from 77.8 to 116%, with relative standard deviation lower than 17.4%. Therefore, this method could be applicable in routine screening analysis of multiple FQ residues in food samples.

KEYWORDS: *fluorescence polarization immunoassay, (fluoro)quinolones, multiple residues analysis*

INTRODUCTION

In recent years, residues of veterinary drugs have been considered as an issue of worldwide concern due to their potential threat to human health by entering the food chain and contributing to bacterial resistance.[1,2] (Fluoro)quinolones (FQs) represent a large group of synthetic antibacterial agents with a broad spectrum of activities, which have been extensively applied in both human medicine and veterinary treatment. Now, several FQs are used exclusively for animals, such as enrofloxacin (ENR), danofloxacin (DAN), difloxacin (DIF), flumequine (FLU), marbofloxacin (MAR), orbifloxacin (ORB), oxolinic acid (OA), and sarafloxacin (SAR).[3] However, it is inevitable that some FQ-resistant bacteria of animal origin will express cross-resistance to other FQs used in human medicine, because these FQs show high similarity in chemical structures and almost the same antibacterial mechanism.[4] To minimize the risk of FQ exposure to humans via products from food-producing animals, maximum residue limits (MRLs) have been established for several FQs in different edible samples by a number of countries and organizations.[3] Therefore, it is necessary to monitor these chemical residues in food samples to obtain better food safety assurance.

Chromatography techniques are the most common methods for the determination of FQs in different food samples, which provide excellent analytical performance but require expensive equipment, complicated sample preparation, and trained personnel.[3,5,6] Therefore, they are not suitable for monitoring drug residues in a large number of food samples. Immunoassays have been confirmed as effective and economical screening methods due to their simplicity and high-throughput capabilities. The similarity in chemical structures of the FQ family makes it possible to produce broad selectivity antibodies against multiple FQs, and the current trend is to develop immunoassays capable of measuring multiple targets in a single test.[7] So far, several broad selectivity antibodies against FQs have been produced and used to develop immunoassays for the determination of multiple FQs, including enzyme-linked immunosorbent assay (ELISA),[7−9] lateral flow immunochromatographic assay (LFIA),[10] and biosensor.[11,12] Conventional ELISA is sensitive and reliable; however, its efficiency is constrained as a result of multiple washing steps and the long time required for immunoreaction, making it difficult to meet the fast, high-throughout, and automated demands on analytical screening methods in the future.

Homogeneous immunoassays (HIA) are promising alternative and complementary methods to overcome those shortcomings, because immunoreaction in solution phase can reach equilibrium in minutes or even seconds and no separation or washing steps are required. Fluorescence polarization (FP) is a typical homogeneous technique that allows rapid and quantitative analysis of binding of a small fluorescent ligand to a larger protein using plan-polarized light to detect the change in effective molecular volume.[13] This technique has been widely utilized in medical diagnosis, monitoring therapeutic drug levels in body fluids, high-throughput screening, and small molecule drug discovery.[14] Fluorescence polarization immunoassay (FPIA) is an antibody-based homogeneous analytical method and is usually performed in competitive format involving the competition between an unlabeled analyte and a labeled tracer for the antibody binding.

Received: May 31, 2013
Accepted: September 9, 2013
Published: September 9, 2013

FPIA can also be performed in a simplified format using the immunocomplex single reagent, which is a pre-equilibrated mixture of antibody and tracer. The analyte in the sample could displace the tracer from the complex, resulting in the change in the polarization value, which is related to the concentration of analyte.[15] Single-reagent FPIA (SR-FPIA) is a one-step immunoassay, so the advantage in detection speed makes it more suitable for screening determination in a large number of samples.[16] Throughout the past decades, FPIA has been used for the measurement of various small molecule analytes, such as toxins,[17−19] pesticides,[20,21] and veterinary drugs.[22−24]

In this work, we describe the development of a sensitive one-step FPIA based on a class-specific mAb for the simultaneous determination of multiple FQ antibiotics in food samples. One paper recently has described a noncompetitive FP assay for ENR using a molecularly imprinted polymer,[25] but the antibody-based FPIA for multiple-FQ determination has not been reported before.

MATERIALS AND METHODS

Reagents and Chemicals. *N*,*N*′-Dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), 5-carboxyfluorescein (5-FAM), 6-carboxyfluorescein (6-FAM), fluorescein isothiocyanate isomer I (FITC), 5-(4,6-dichlorotriazinyl)aminofluorescein (DTAF), and 5-aminofluorescein (AF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4′-(Aminomethyl)fluorescein (4′-AMF) was from Invitrogen (Carlsbad, CA, USA). The analytical standards of ciprofloxacin hydrochloride (CIP), enrofloxacin (ENR), flumequine (FLU), norfloxacin (NOR), pefloxacin methanesulfonate (PEF), sarafloxacin (SAR), and difloxacin (DIF) were purchased from the China Institute of Veterinary Drug Control (Beijing, People's Republic of China). Orbifloxacin (ORB), sparfloxacin (SPA), lomefloxacin (LOM), enoxacin (ENO), marbofloxacin (MAR), ofloxacin (OFL), danofloxacin mesylate (DAN), oxolinic acid (OA), and nalidixic acid (NAL) were obtained from Dr. Ehrenstorfer GmbH, (Ausburg, Germany). Structures of the (fluoro)quinolones used in this study are shown in Figure 1.

All other chemicals and solvents were of analytical grade or better from Beijing Chemical Reagent Co. (China). Deionized water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Buffers and Standard Solutions. PBS (0.02 M, pH 7.4) with 1 mM EDTA and 0.1% sodium azide was used as working buffer for all FPIA experiments. Stock solutions (1 mg mL^{-1}) of FQ antibiotics, and other analytes were prepared by dissolving 5 mg of drug in 5 mL of 0.03 M sodium hydroxide and were stored at −20 °C. Working standard solutions of analytes in the range from 0.1 to 1000 ng mL^{-1} were prepared by dilution of stock solution with assay buffer.

Apparatus. A SpectraMax M5 microplate reader from Molecular Devices (Downingtown, PA, USA) was used to measure fluorescence polarization (FP) and fluorescence intensity (FI) signal. Black microplates (96-well) with a nonbinding surface for FPIA were obtained from Corning Life Sciences (New York, NY, USA). Precoated silica gel $60GF_{254}$ glass plates (plate size = 10 × 10 cm; layer thickness = 0.15−0.2 mm, particle size = 2 μm) for thin-layer chromatography (TLC) were purchased from QingDao HaiYang Corp. (Shandong, China).

Synthesis of Fluorescent Conjugates (Tracer). *FQs-FITC and SAR-DTAF.* SAR (5 mg) was dissolved in 0.5 mL of methanol. Triethylamine (50 μL) and FITC or DTAF (4 mg) were added with mixing. After overnight reaction at room temperature, small portions (50 μL) of reaction mixture were separated by TLC using CH_2Cl_2/methanol (4:1, v/v) as the eluent. The new yellow band at R_f 0.1 was scraped from the plate, extracted with 0.5 mL of methanol, and stored in the dark at 4 °C. CIP, NOR, ENO, and LOM were also used to conjugate with FITC to obtain the corresponding tracers in the same way.

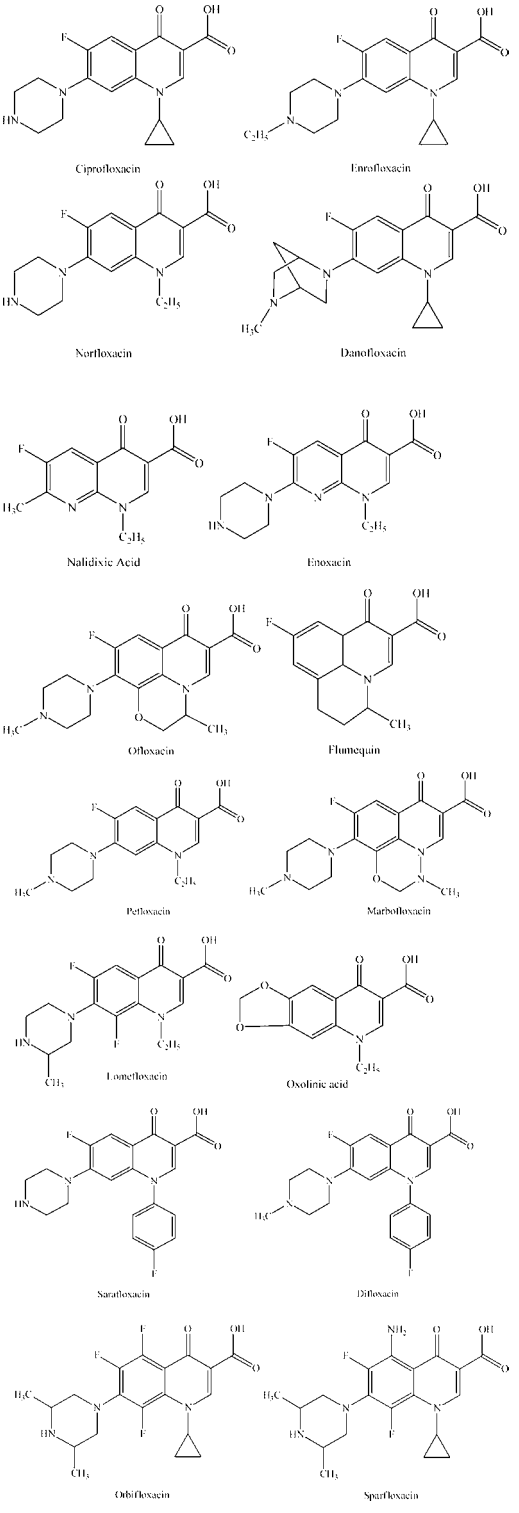
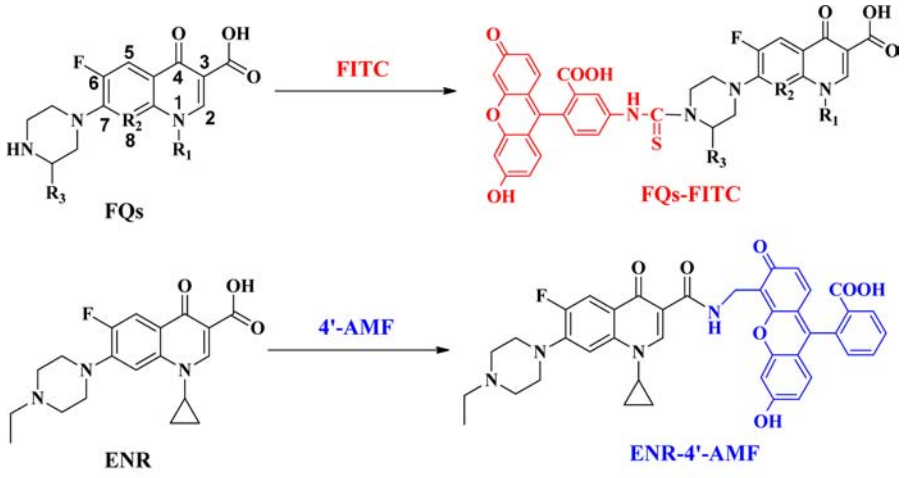


Figure 1. Structures of (fluoro)quinolones used in this study.

SAR-5(6)-FAM and SAR-GAF. Five milligrams of 5(6)-FAM was dissolved in 400 μL of absolute *N*,*N*-dimethylformamide (DMF) and then mixed with 2 mg of DCC and 1 mg of NHS for 4 h at room temperature. The reaction mixture was then centrifuged to remove the precipitate of dicyclohexylurea, and 3 mg of SAR was added. After stirring overnight at room temperature, a small portion of the reaction mixture was purified by TLC in methanol/AcOEt/NH_4OH (2:1:0.04, v/v/v). *N*-Glutaryl-5-aminofluorescein (GAF) was an AF derivative synthesized on the basis of a previous report,[26] and SAR-GAF was prepared using NHS ester method as described above. The main new

dx.doi.org/10.1021/jf403972r | J. Agric. Food Chem. 2013, 61, 9347−9355



FQs	CIP	NOR	ENO	LOM	SAR
R_1	△	C_2H_5	C_2H_5	C_2H_5	
R_2	CH	CH	N	C-F	CH
R_3	H	H	H	CH_3	H

Figure 2. Schematic routes for synthesis of (fluoro)quinolone tracers in two different modes.

bands obtained at R_f 0.4 for SAR-5-FAM, R_f 0.4 for SAR-6-FAM, and R_f 0.8 for SAR-GAF were scraped from the plate and extracted with 0.5 mL of methanol and then stored at 4 °C.

ENR-4′-AMF. The carboxylic group of ENR (5 mg) was activated with DCC and NHS in the same way as indicated above. The activated ENR was reacted with 5 mg of 4′-AMF. A small portion of the reaction mixture was purified by TLC in CH_2Cl_2/methanol (4:1, v/v). The main new band at R_f 0.5 was scraped from the plate and extracted with 0.5 mL of methanol and then stored at 4 °C.

Preparation of Antibodies. Three monoclonal antibodies (MAbs) against FQs, anti-CIP (named C4A9H1), anti-NOR (N2H3A8), and anti-ENR (E5B7E8), were previously prepared.[7] All antibodies were purified by conventional $(NH_4)_2SO_4$ precipitation, divided into aliquots, and stored at −20 °C until used.

Competitive FPIA and Single-Reagent FPIA Procedure. The FPIA approach was described as follows: 70 μL per well of tracer solution was mixed with 70 μL per well of diluted mAb. Subsequently, 70 μL per well of standard solution or sample extract was added, and the mixtures were shaken for 10 s in the microplate reader. After a short incubation period (5 min) at room temperature, the FP value was measured at λ_{ex} = 485 nm, λ_{em} = 530 nm (emission cutoff = 515 nm, *G* factor = 1.0).

The immunocomplex single reagent (SR) was prepared by mixing 50 mL of tracer SAR-5-FAM (diluted to 5 nM) with 50 mL of anti-CIP Ab (diluted to 1:1200) and stored at 4 °C. To generate the standard curve, 70 μL of FQ standard solution was mixed well with 140 μL of SR, and fluorescence polarization was measured under the same instrument settings without any incubation. The blank control containing all assay elements except SR was performed simultaneously for correcting polarization measurement by subtracting the background in assay buffer or sample matrix.

Curve Fitting and Cross-Reactivity Determination. The sigmoidal curve was used to fit FPIA data by OriginPro 8.0 (OriginLab Corp., Northampton, MA, USA). IC_{50} is the standard concentration at 50% of specific binding. The limit of detection (LOD) is defined as the standard concentration corresponding to the mean signal of 20 independent blank controls minus 3 times their standard deviation (SD). The limit of quantification (LOQ) is the standard concentration at IC_{80}, and the working range is defined as the standard concentration at the range of IC_{20}−IC_{80}. To normalize the FP value, the ratio mP/mP_0 (where mP_0 is the maximum FP value of the inhibition curve and mP is the current value) resulting in relative units was used. Cross-reactivity (CR) was calculated by both ELISA and FPIA methods according to the equation

$$\text{CR}\ (\%) = (\text{IC}_{50}\ \text{of CIP}/\text{IC}_{50}\ \text{of tested FQs}) \times 100$$

where IC_{50} obtained from calibration curves is the concentration at which 50% of the antibodies are bound to the analyte.

Sample Treatment. Two milliliters of negative whole milk samples was fortified with the appropriate FQ standard solution and then mixed with an equal volume of 1.5% trichloroacetic acid (TCA). The mixtures were agitated on a shaker for 2 min and then deproteinized through centrifugation for 10 min (8000*g* at 4 °C). The supernatants were diluted with assay buffer to fit the working range before measurement in FPIA.

Two grams of homogenized chicken muscle was placed in a 50 mL centrifuge tube and fortified with standard solution. Then 8 mL of PBS (0.02 M, pH 7.0) was added, and the mixture was vortex mixed for 10 min. After centrifugation at 8000*g* for 10 min, the supernatant was collected, and the extraction was repeated using another 4 mL of PBS. Half of the combined supernatants was mixed with 8 mL of dichloromethane for 5 min and then centrifuged at 3000*g* for 10 min. Four milliliters of the lower organic phase was dried under a gentle stream of nitrogen gas at 50 °C. The organic phase residue was redissolved in 1 mL of assay buffer and washed with 1 mL of hexane and then diluted to fit the working range before measurement in FPIA.

RESULTS AND DISCUSSION

Characterization of Antibodies and Tracers. Three monoclonal antibodies obtained in our previous study were chosen here for FPIA study. Anti-CIP (C4A9H1) and anti-NOR (N2H3A8) exhibited broad specificity for most FQ antibiotics (including ENR), which have been used in multiresidue ELISA for FQ determination,[7] and anti-ENR (E5B7E8) was a highly specific mAb against ENR. As schematic routes shown in Figure 2, three homologous tracers were first synthesized in two different modes. CIP-FITC and NOR-FITC were prepared by linking FITC with CIP and NOR through the piperazine moiety at position 7, whereas ENR-4′-AMF was obtained by linking 4′-AMF with the carboxylic group at position 3. Binding assays between each tracer and antibody were performed to screen the fluorescent conjugates available

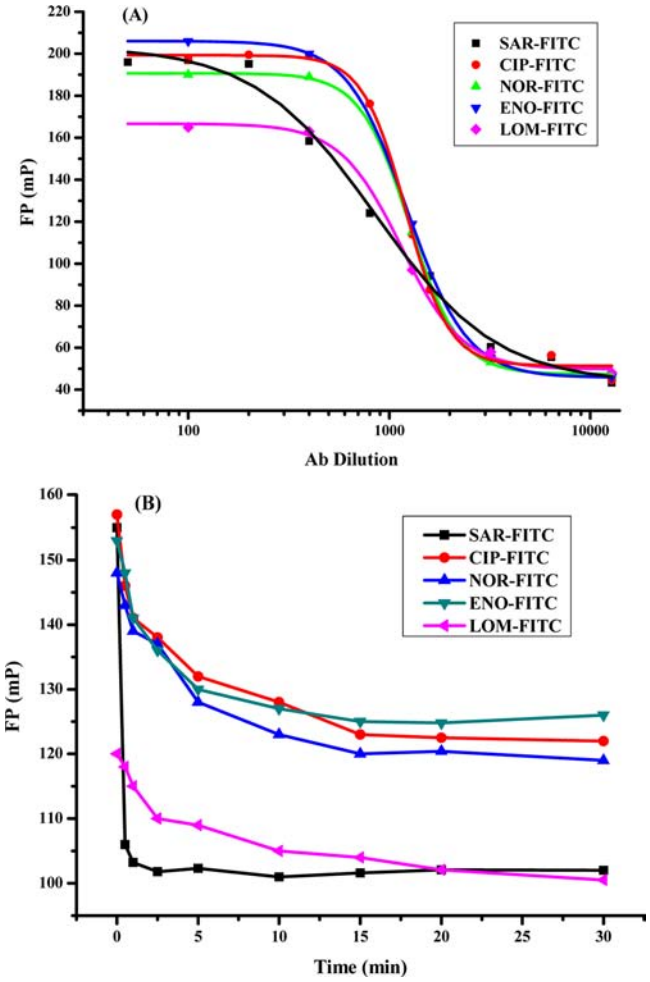


Figure 3. (A) Antibody binding curves for the anti-CIP with five FQ-FITC tracers. The antibody dilution curves were performed by mixing 2-fold serially diluted mAb (over the range from 1/200 to 1/102400) with each tracer at a fixed concentration (10 nM). (B) Comparison among five FQ-FITC tracers in sensitivity and dissociation rate in the SR-FPIA format. Each point represents the mean FP of three replicates measured at the CIP concentration of 10 ng mL^{-1}.

in FPIA and investigate the recognition sites for these MAbs. The appropriate tracers could be preliminarily characterized by observing the increase in FP signal after mixing with saturating amounts of specific antibody, and a higher FP difference will result in a higher signal-to-noise ratio.[27] As shown in Figure S1 (see the Supporting Information), both CIP-FITC and NOR-FITC exhibited sufficient binding with anti-CIP and anti-NOR, but almost no binding with anti-ENR. ENR-4′-AMF was recognized only by anti-ENR. These results showed that both broad specificity MAbs (anti-CIP and anti-NOR) recognized the quinolone main ring and β-keto acid in FQ molecules and must be raised through the corresponding immunogens prepared by conjugating the FQ molecule through the piperazine NH group at position 7 to the carrier protein, rather than the carboxyl group at position 3. Because both amino and carboxylic groups are present in some FQs (such as CIP, NOR, and SAR), it was inevitable that both functional groups in FQ molecules could react with the carrier protein when using a mixed anhydride or a carbodiimide reaction for conjugation in a one-step method. The present study was focused on the multiple residue analysis for FQs, so preliminary inhibition assays were performed to investigate the specificity and sensitivity of these MAbs. As displayed in Figure S2 (see the Supporting Information), a combination of the anti-CIP along with the homologous tracer CIP-FITC afforded higher sensitivity and broader specificity and was selected for further evaluation and optimization with the aim to improve detectability.

Tracer Optimization. To investigate the influence of the hapten structure of tracers on the assay characteristics, several heterologous tracers, NOR-FITC, LOM-FITC, ENO-FITC, and SAR-FITC, were synthesized subsequently and compared with the homologous tracer CIP-FITC. The binding of the various tracers with anti-CIP was further assessed by plotting antibody dilution curves with a fixed concentration (10 nM) of all tracers to obtain the respective maximum FP changes (δmP_{max}) and 50% antibody binding dilutions (antibody titers). Satisfactory binding was observed between anti-CIP and these five tracers (δmP_{max} = 116−157 mP), and similar antibody titers (∼1/1000) were observed for CIP-FITC, NOR-FITC, LOM-FITC, and ENO-FITC, whereas a lower antibody titer (∼1/800) was obtained for SAR-FITC (Figure 3A). It was not surprising that anti-CIP exhibits lower affinity to SAR-FITC than the other four tracers, because this mAb had a much lower cross-reactivity with SAR. Higher sensitivity and faster dissociation rate were observed when using the heterogeneous tracer SAR-FITC, presumably because SAR-FITC could be more easily replaced by competitors (see Figure 3B). Therefore, SAR was a better hapten for preparing fluorescent conjugates to perform a simplified FPIA in displacement format, which is generally called single-reagent FPIA (SR-FPIA).

Because the linker is also closely associated with tracer−antibody recognition, another four tracers, SAR-DTAF, SAR-5-FAM, SAR-6-FAM, and SAR-GAF, were prepared, which mainly differed in the linker structure or length as well as orientation of the attached fluorophore (see Figure 4). Using the same procedure as before, all of these tracers were assessed in antibody binding and competition assay. Three of the tracers were able to achieve higher FP change (δmP_{max} = ∼200mP for SAR-5-FAM and SAR-GAF, ∼180 mP for SAR-6-FAM) than those tested before (Figure 5A). Figure 5B and Table 1 show the standard curves and main analytical parameters obtained using anti-CIP with different SAR tracers at the same level (10nM). The highest sensitivity (lowest IC_{50}) was obtained using SAR-5-FAM with minimum linker length (one carbon), whereas the introduction of a triazine heterocycle linker (SAR-DTAF), long linker (SAR-GAF), or difference in attached orientation (SAR-6-FAM) caused a considerable decrease in sensitivity. Therefore, the combination of anti-CIP/SAR-5-FAM was finally selected for further studies.

Assay Optimization. To yield a more sensitive assay, concentration optimization was performed for the tracer SAR-5-FAM and anti-CIP. It is known that the tracer signal sets the sensitivity; a low tracer concentration will result in high sensitivity, but low precision of FP signal. Precision of <5 mP standard deviation (SD) was recommanded according to the technical specifications of the instrument used in the present study. Thus, we could drop the tracer concentration to 5 nM with satisfactory precision in the following experiments. The antibody concentration that produced maximal inhibition signal was used in the optimal assay.[28] As shown in Figure S3 (see the Supporting Information), two antibody dilution curves were performed in the absence and presence of competitors (3 ng

dx.doi.org/10.1021/jf403972r | J. Agric. Food Chem. 2013, 61, 9347−9355

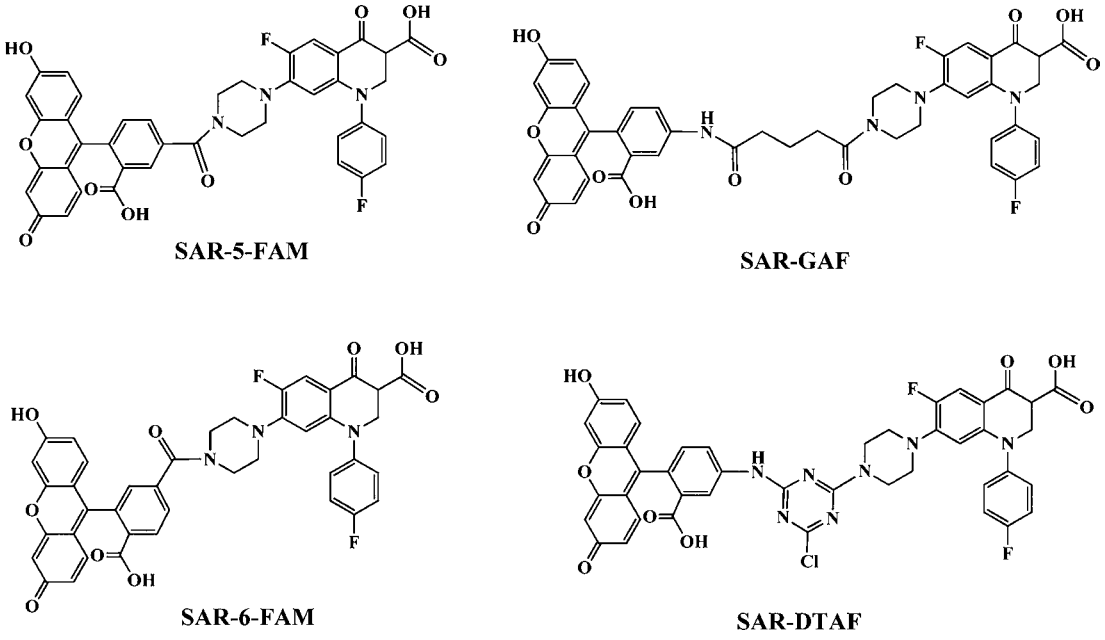


Figure 4. Structures of SAR tracers with different linkers.

mL^{-1} of CIP), and the Ab dilution of 1/1200 that yielded the maximal difference was selected as the optimal antibody dilution. Figure S4 (see the Supporting Information) shows the standard curves obtained using optimized one-step FPIA with different incubation times, demonstrating that the displacement of tracer from the immunocomplex was reached rapidly by addition of the analyte, which allows a real "mix and read" measurement without any incubation.

Effects of Physicochemical Conditions on Assay Performance. The effect of different physicochemical conditions on FPIA were assessed by comparing IC_{50} and δmP under various conditions. Fluorescein is a pH-sensitive dye, and Figure S5A (see the Supporting Information) shows the effect of solution pH value ranging from 6.0 to 11.0 on the fluorescent intensity of the tracer at the working concentrations of 5 nM. The fluorescence intensity increased as the pH value increased from 6.0 to 8.0 and then became nearly constant when the pH value was >8.0. Fluorescence intensity dropped very sharply at pH values below 7.5, leading to low precision (SD > 5 mP) in the FP signal. To study the influence of pH in the assay system, IC_{50} and δmP were obtained at different pH values from 6 to 11. The relationship of these parameters as a function of pH is shown in Figure S5B (see the Supporting Information). The results showed that the assay performed optimally at pH 7.5 with high sensitivity and maximal δmP. Both sensitivity and δmP were significantly reduced at the more basic pH value, which indicated that a negative effect occurred in the recognition between mAb and tracer. Although no significant deleterious effect on assay sensitivity was observed below 7.5, reliability was remarkably reduced due to fluorescence quenching.

It has been proved that divalent ions form complexes with FQs through the β-keto acid moiety[29] and strongly effect the performance of the immunoassay when this moiety has a great contribution on antibody recognition.[9] In the present study, the significant decrease in the δmP and the increase in IC_{50} were observed as a result of increasing divalent ion concentration, and the presence of magnesium had a much stronger effect than that of calcium (see Figure S6A in the Supporting Information). The divalent ions caused the decrease in assay sensitivity on the contrary to previous studies.[9] However, it was also demonstrated that this moiety probably makes a great contribution to antibody recognition. Therefore, EDTA, a divalent cation chelator, was introduced into the final assay buffer. In addition, the effects of methanol and DMF on the assay performance were investigated. As shown in Figure S6B (see the Supporting Information), the presence of <10% methanol could be tolerated in the current assay, whereas even 1% of DMF caused a considerable decrease of δmP and bad curve fitting (data not shown).

Assay Specificity. Antibody cross-reactivity was tested using both the SAR-5-FAM and CIP-FITC tracers in the FPIA toward structurally related (fluoro)quinolones and other antibiotics such as streptomycin (STR), cephalexin (CEP), and sulfamethoxazole (SMZ). As can be observed in Table 2, the anti-CIP antibody could recognize all 16 tested (fluoro)-quinolones to different degrees, and no cross-reactivity was observed with other antibiotics. In agreement with previous studies,[8,30] the heterologous tracer SAR-5-FAM not only significantly increased the assay sensitivity but also prompted the Ab to display a more even distribution level in cross-reactivity. This must be beneficial to develop a immunoassay for multiple-residue analysis. The tested (fluoro)quinolones can be approximately divided into three groups according to the cross-reactivity results. The first group contains the 11 drugs CIP, LOM, NOR, DAN, ENR, ENO, PEF, ORB, OFL, MAR, and SPA. They all present similar cross-reactivities close to that of CIP. The second group consists of two drugs, SAR and DIF, both of which had cross-reactivities below 10%, probably due to steric hindrance caused by the larger fluorophenyl ring at position 1. The third group contains three drugs, OA, NAL, and FLU, which showed cross-reactivities of much greater than 100%. It is surprising that the anti-CIP exhibited higher affinitiy for these three compounds, because they all lack the piperazinyl moiety that contained in almost all other FQs. More surprisingly, in the case of OA, the substitution of fluorine with an oxygen at position 6 exhibited the highest affinitiy with anti-CIP Ab. These results further supported the previous

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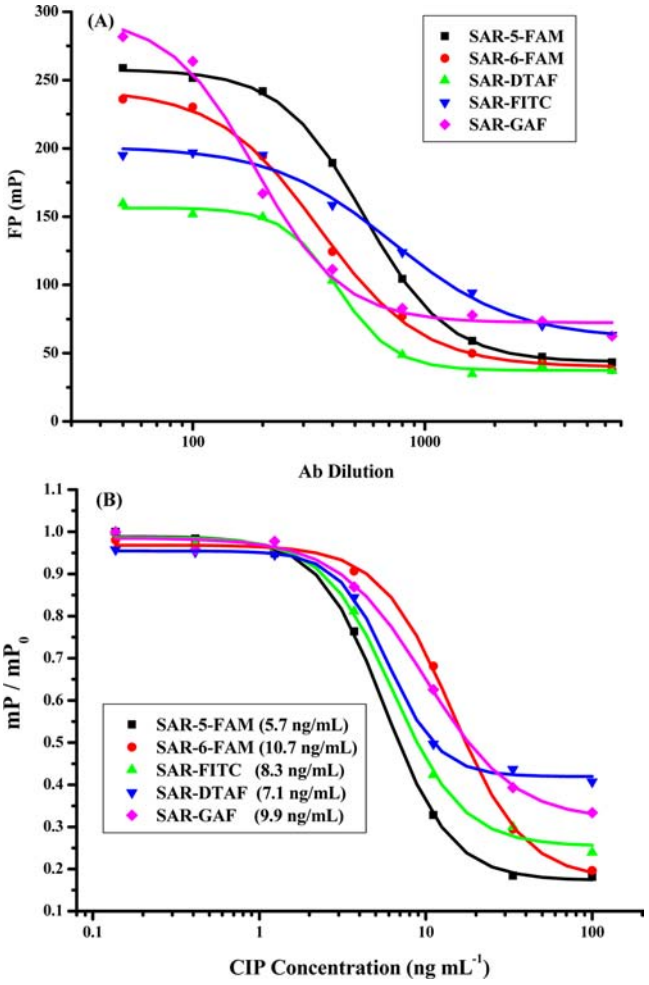

Figure 5. (A) Antibody binding curves for the anti-CIP Ab with five SAR tracers. The antibody dilution curves were performed by mixing 2-fold serially diluted mAb (over the range from 1/200 to 1/102400) with each tracer at a fixed concentration (10 nM). (B) Normalized FPIA standard curves obtained by using five SAR tracers (10 nM) and respective Ab dilutions providing 70% binding. Each point represents the mean FP of three replicates measured at the CIP concentration ranging from 0 to 100 ng mL^{-1}. Numbers in parentheses correspond to IC_{50} values.

hypothesis that the piperazinyl ring at position 7 in CIP is not as important as other sites in binding.[7] On the other hand, it was demonstrated that the immunogens must be prepared by conjugating the piperazine NH group to the carrier protein.

Application of the Assay to the Analysis of Milk and Chicken Muscle Samples. The optimized SR-FPIA method was applied for detecting FQs in two different food samples, milk and chicken muscle. Although immunoassays are

Table 2. Cross-Reactivity of Related Compounds in the SR-FPIA with Two Tracers, SAR-5-FAM and CIP-FITC, Respectively

	SAR-5-FAM		CIP-FITC	
compd	IC_{50} (ng mL^{-1})	CR (%)	IC_{50} (ng mL^{-1})	CR (%)
		Group 1		
CIP	2.17	100.0	25.07	100.0
ENO	1.87	116.0	22.28	112.5
ENR	1.92	113.0	19.95	125.7
LOM	1.96	110.7	13.3	188.5
PEF	1.96	110.7	26.36	95.1
ORB	2.13	101.9	29.95	83.7
DAN	2.15	100.9	19.61	127.8
NOR	2.20	98.6	13.73	182.6
SPA	2.25	96.4	41.62	60.2
MAR	2.34	92.7	39.43	63.6
OFL	2.72	79.8	36.96	67.8
		Group 2		
DIF	24.16	8.9	228.0	11.0
SAR	27.7	7.8	391.9	6.4
		Group 3		
OA	1.26	172.2	3.24	773.8
NAL	1.36	159.6	9.29	269.9
FLU	1.30	166.9	11.01	227.7
		Group 4		
SMZ	>10000	<0.02	>10000	<0.2
STR	>10000	<0.02	>10000	<0.2
CEP	>10000	<0.02	>10000	<0.2

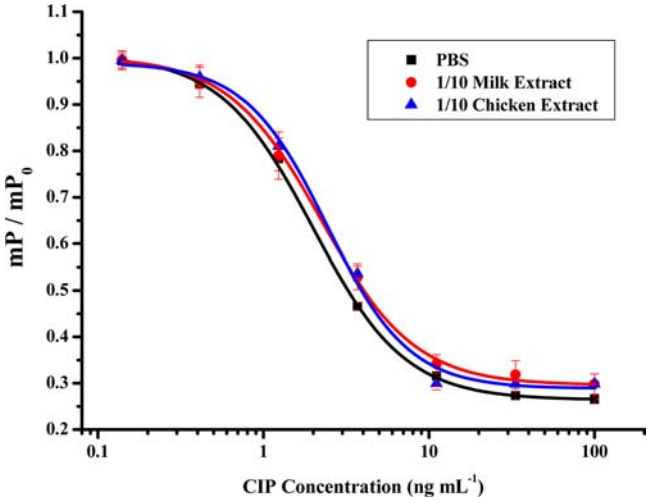

Figure 6. SR-FPIA standard curves for the determination of CIP in PBS (IC_{50} = 2.08 ng mL^{-1}), 1/10 milk extract (IC_{50} = 2.25 ng mL^{-1}), and 1/10 chicken muscle extract (IC_{50} = 2.42 ng mL^{-1}). Each point of the curve represents the mean FP ± SD (n = 3).

Table 1. Analytical Parameters Obtained Using Anti-CIP with Different Tracers (n = 3)[a]

tracer	Ab dilution	mP_{max}	mP_{min}	δmP	IC_{50} (ng mL^{-1})	slope	R^2
SAR-FITC	1:600	164.9	42.4	122.5	6.3 ± 0.6	2.07	0.994
SAR-5-FAM	1:400	201.7	35.3	166.4	5.7 ± 0.7	2.17	0.996
SAR-6-FAM	1:250	197.5	35.7	161.8	14.5 ± 1.2	2.01	0.998
SAR-DTAF	1:250	156.4	70.1	86.3	6.1 ± 0.7	2.08	0.993
SAR-GAF	1:200	183.2	58.7	125.5	9.9 ± 1.0	1.64	0.992

[a] mP_{max} and mP_{min} represent maximum (no inhibition) and minimum (complete inhibition) signal in standard curve; $mP_{max} - mP_{min}$, represents the assay window (δmP).

dx.doi.org/10.1021/jf403972r | J. Agric. Food Chem. 2013, 61, 9347−9355

Table 3. Analytical Features of the Optimized SR-FPIA for 7 FQs in Buffer and 10-Dilution of Blank Sample Matrix and the Corresponding MRLs

	buffer		milk			chicken muscle		
FQ	LOD (ng mL^{-1})	working range (ng mL^{-1})	LOD (ng mL^{-1})	CCβ (ng mL^{-1})	MRL (ng mL^{-1})	LOD (ng g^{-1})	CCβ (ng g^{-1})	MRL (ng g^{-1})
CIP	0.75	1.17−4.02	1.06	<15	100	1.14	<50	100
ENR	0.72	1.25−5.38	0.95	<15	100	1.20	<50	100
OA	0.46	0.76−2.67				0.85	<50	100
FLU	0.62	0.87−2.52	0.72	<15	50	1.08	<50	400
DAN	0.83	1.08−4.28	1.10	<15	30	1.31	<50	200
MAR	1.05	1.48−5.61	1.36	<15	75			
DIF	3.52	4.86−120.1				4.40	<50	300

Table 4. Recovery of FQs from Fortified Milk and Chicken Muscle Samples (n = 3)

	milk					chicken muscle			
compd	added (ng mL^{-1})	found (ng mL^{-1})	recovery (%)	CV (%)	compd	added (ng g^{-1})	found (ng g^{-1})	recovery (%)	CV (%)
CIP	50	45.8	91.6	10.0	CIP	50	42.1	82.2	10.6
	100	93.4	93.4	5.9		100	81.4	81.4	8.4
	200	174.6	87.3	4.8		200	179.6	89.8	7.6
ENR	50	42.6	85.2	8.0	ENR	50	40.4	80.8	11.4
	100	98.4	98.4	4.1		100	81.5	81.5	7.6
	200	182.6	91.3	2.4		200	170.4	85.2	9.0
FLU	25	22.1	88.4	14.5	FLU	200	175.6	87.8	10.5
	50	52.1	104	6.7		400	320.8	80.2	9.3
	100	87.6	87.6	3.8		800	628.8	78.6	9.0
DAN	15	16.1	107	17.4	DAN	100	88.6	88.6	9.0
	30	27.5	91.7	9.5		200	180.4	90.2	7.5
	60	48.4	80.7	8.0		400	366.8	91.7	7.7
MAR	37.5	39.0	104	9.5	OA	50	43.0	86.0	10.7
	75	87.1	116	6.5		100	82.6	82.6	9.9
	150	139.3	92.9	9.0		200	166.3	83.2	8.1
					DIF	150	163	108	13.8
						300	264.6	88.2	11.7
						600	466.8	77.8	9.4

comparatively simple and rapid analytical techniques, animal food is a very complex matrix consisting of different components (fats, proteins, various sugars, etc.), which can strongly interfere with the analytical determination.[24,31] FPIA is susceptible to some of these components in the food matrix. Therefore, sample extraction and a cleanup process are required for the recovery evaluation. Solid-phase extraction (SPE) was commonly chosen as a purification technique in the HPLC method, but it was relatively time-consuming, expensive, and not suitable for a screening method. The solution of TCA[22] or oxalic acid[32] had been successfully used for protein precipitation to determine sulfanilamides in milk by FPIA. In our work, 1.5% TCA was used for extraction and protein precipitation in milk sample, and then the extract was diluted 5 times (10 dilution in total) with assay buffer to neutralize acid and reduce matrix interference, but that was not an effective means for chicken muscle sample in the present assay. A re-extraction procedure with dichloromethane was carried out from chicken muscle extract under neutral conditions based on previous reports.[33,34] Figure 6 shows the optimized SR-FPIA standard curves performed in both assay buffer and sample matrixes, implying that the pretreatment procedures can obviously reduce the matrix effect from different food samples. Table 3 shows analytical features of the optimized SR-FPIA for these FQs in assay buffer, milk and chicken muscle, and the corresponding MRLs set by European Union legislation. Detection capability (CCβ) is the concentration at which only ≤5% false compliant results remain according to Commission Decision 2002/657/EC. Twenty blank samples were determined following fortification with target FQ at the level of ≤1/2 MRLs, respectively. The fortified levels were 15 ng mL^{-1} in milk and 50 ng mL^{-1} in chicken muscle. No false-negative results were obtained for those fortified samples, so the CCβ value was lower than the fortification level, which demonstrates that most of the FQs could be detectable at levels of MRLs. Known FQs were spiked in milk and chicken muscle samples at levels of 1/2, 1, and 2 × MRLs, extracted, and diluted to fit the working range of the optimized SR-FPIA before measurement. Recoveries of tested FQs ranged from 77.8 to 116%. Assay reproducibility was satisfactory, with CV ranging from 2.4 to 17.4% (see Table 4).

In conclusion, a new one-step fluorescence polarization immunoassay for the simultaneous measurement of multiple (fluoro)quinolones was developed on the basis of the

dx.doi.org/10.1021/jf403972r | J. Agric. Food Chem. 2013, 61, 9347−9355

displacement of tracer from the immunocomplex single reagent. The described method was very sensitive due to the use of the optimal tracer, SAR-5-FAM, which was derived from the heterologous hapten, sarafloxacin, indicating much lower cross-reactivity with the antibody than other FQs. No further incubation time was required before the measurement of fluorescence polarization due to rapid displacement of the tracer from the immunocomplex by addition of the analyte. Optimized SR-FPIA exhibited broad cross-reactivities in the range of 7.8−172.2% with 16 FQs tested and enabled determination of most of the FQs at the levels of MRLs. Several FQs spiked in milk and chicken muscle samples were determined with satisfactory recovery and precision. Therefore, the one-step FPIA could be applicable in routine screening analysis of multiple FQ residues in milk and chicken muscle samples. However, further work will be needed to validate this assay for real incurred samples and applications in other food samples.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental figures (Figures S1−S6) regarding preliminary evaluation on antibody and tracer interaction, assay optimization of Ab concentrations, and the effect of physicochemical conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work is supported by grants from Trans-Century Training Programme Foundation for the Talents by the Ministry of Education (NCET-12-0529), National Natural Science Foundation of China (No. 31372475), State Key Program of National Natural Science of China (No. 30830082), CAU Fundamental Research Funds (2012QJ111), and Sino-Russian International Scientific and Technological Cooperation (2011DFR30470).

Notes

The authors declare no competing financial interest.

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