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Monoclonal Antibody-Based Fluorescence Polarization Immunoassay for Sulfamethoxypyridazine and Sulfachloropyridazine

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In this paper, a new monoclonal antibody (Mab) against sulfamethoxypyridazine (SMP) was produced, and a fluorescence polarization immunoassay (FPIA) based on the produced Mab was developed and optimized for the qualitative screening analysis of SMP. The Mab was raised from mice immunized with SMP linked to bovine serum albumin (BSA) by carbodiimide activated ester formation, using a succinic anhydride spacer molecule between SMP and BSA. Fluorescein labeled sulfachloropyridazine (SCP) and SMP (tracer) were synthesized and purified by thin layer chromatography (TLC). The developed screening FPIA method can tolerate up to 20% methanol, and satisfactory assay sensitivity can be obtained between pH 4 and pH 8 and at lower salt concentration. The anti-SMP Mab exhibited a high cross-reactivity with SCP. The effect of the tracer structure on the analytical characteristic of the determination and on antigen−antibody binding constants was studied. The limits of detection (LOD) were 0.7 ng/mL for SMP and 0.25 ng/mL for SCP in buffer, respectively, whereas negligible cross-reactivities were exhibited by related sulfonamides. Analysis of SMP and SCP-fortified milk samples by the FPIA showed average recoveries from 60 to 145%.

KEYWORDS: Fluorescence polarization immunoassay; monoclonal antibody; sulfamethoxypyridazine; sulfachloropyridazine; milk

INTRODUCTION

Sulfamethoxypyridazine (SMP) and sulfachloropyridazine (SCP) are examples of sulfonamide veterinary drugs and have similar chemical structures (**Figure 1** and **Table 1**). They are effective antimicrobial drugs for the prevention of infections in cattle, poultry, and swine (prophylaxis), to treat veterinary diseases, and to promote growth (*1*). As a result of the extensive use of sulfonamides in the animal industry, residues of these drugs in food samples are a major concern because of their contribution to the development of antibiotic resistant pathogenic bacteria (*2*, *3*). To minimize this risk, maximum residue limits (MRLs) have been established for total or individual sulfonamides including SMP and SCP at 0.1 mg/kg in milk and meat by China Ministry of Agriculture (No. 278, 2003.5.22), which are similar to MRLs in the United States and Europe (*4*, *5*).

Although not as widely used as other available sulfonamides such as sulfamethazine and sulfaquinoxaline, SMP and SCP can be used as an alternative, and their application is increasing (*6*). Consequently, it is proposed to include them in screening projects to satisfy increasing public concerns about food safety.

The relatively low historical usage of the pyridazine sulfonamides in the food-production animal industry has resulted in few analytical methods being reported for the detection of residues of SMP or SCP individually in food samples. Recently, a number of papers have reported some sulfonamide multiresidue procedures in differing samples for SMP or SCP. These are mostly conventional instrumental analytical methods such as liquid chromatography (*7*−*11*), liquid chromatography−mass spectrometry (*12*−*14*), capillary electrophoresis−mass spectrometry (*15*), and chemiluminescence detection (*16*). However, instrumental analytical methods require high cost, highly skilled workers, and time-consuming sample preparation steps and are not suitable for routine analysis of a large number of samples. Therefore, there is a growing demand for more rapid and more economical methods.

The immunoassay such as enzyme-linked immunosorbent assay (ELISA) could be a solution to this problem (*17*, *18*). In previously reported immunoassays for sulfonamides, most have shown little or no cross-reactivity for SMP or SCP, because these antibodies were raised to specific haptens. Haasnoot et al. reported one Mab, named Mab 21C7, raised against

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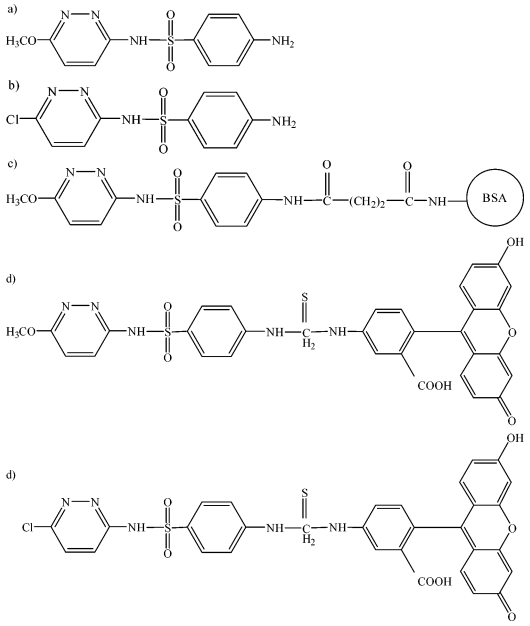
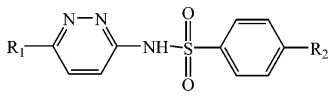


Figure 1. Chemical structures of sulfonamides (**a**) SMP and (**b**) SCP, (**c**) immunogen, and tracers (**d**) SMP–FITC and (**e**) SCP–FITC.

Table 1. Chemical Structures of Sulfanilamide Drugs, Hapten–Protein Conjugate (Immunogen), and Fluorescence Labeled Analyte (Tracer)



compound	R_1	R_2
SMP	OCH_3	NH_2
SCP	Cl	NH_2
immunogen	OCH_3	$NH–CO(CH_2)_2CO–NH–BSA$
SMP–FITC	OCH_3	NH–FITC
SCP–FITC	Cl	NH–FITC

sulfamethazine which was identified as an antibody recognizing SMP and SCP. This MAb was used in a biosensor immunoassay for the detection of eight sulfonamides including SCP in chicken serum (*19*). A lanthanide fluoroimmunoassay for the simultaneous screening of 18 sulfonamides using a mutant engineering antibody (M.3.4) was also described (*20*). Cliquet et al. described one ELISA method for the determination of SCP in porcine tissues, and the polyclonal antibody used in the study was obtained after immunization with a sulfathiazole derivative (*21*). To the authors' knowledge, three papers have reported the production polyclonal antibodies specifically to SMP and SCP and developed ELISA methods to detect SMP or SCP residues in food samples, respectively (*6*, *22*, *23*). The cross-reactivities of all sulfonamides with ovine polyclonal antisera against hemisuccinate derivative of SCP or SMP were less than 1% except for SMP antisera, which showed a cross-reactivity of 55.4% with SCP (*23*). The polyclonal antibodies against SMP could not recognize SCP (*22*), while the SCP antibodies showed 3.1% cross-reactivity with SMP (*6*).

Simplifying the assay and minimizing the analysis time are the primary goals in developing screening methods for large numbers of samples. An alternative method to ELISA is fluorescence polarization immunoassay (FPIA). FPIA does not require separations or multiple steps, giving the advantages of simplicity and high precision. The principles of FPIA have been reviewed (*24*). In brief, it is a competitive immunoassay method based on differences in the polarization of the fluorescence of the fluorescent-labeled analyte in the antibody-bound and nonbound fractions. A number of FPIAs for pesticides and toxins have been developed in the past few years (*25*−*27*). Several articles have been published for the determination of sulfonamides using FPIA (*28*−*30*). Polyclonal antiserum was used in most of these investigations, and to date, immunoassays specific for SMP or SCP using monoclonal antibodies instead of polyclonal antibodies have not yet been developed. In our present paper, we produced for the first time one Mab against SMP showing good cross-reactivity with SCP and developed an FPIA for the detection of SMP and SCP in milk.

MATERIALS AND METHODS

Materials and Apparatus. Bovine serum albumin (BSA), fluorescein isothiocyanate (FITC) isomer I, SMP, SCP, and other sulfonamides used in cross-reactivity studies, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDC), and Freund's complete and incomplete adjuvants were obtained from Sigma (St. Louis, MO). Polyethylene glycol (PEG 2000) was purchased from Merck-Schuchardt (Darmstadt, Germany). Common solvents and salts were supplied by Beijing Reagent Corporation (Beijing, P.R. China). The myeloma cell line SP2/O was a gift from Professor Jixun Zhao (China Agricultural University, Beijing, P.R. China). Cell culture media (DMEM) was obtained from Huamei (Beijing, P.R. China). Fetal calf serum and supplements were obtained from GIBCO BRL (Carlsbad, CA). Pre-coated silica gel 60G F_{254} coated glass plates for thin layer chromatography (TLC) were purchased from Qingdao Haiyang (Qingdao, P.R. China). All other chemicals and solvents were analytical reagent grade.

Borate buffer (BB; 50 mM, pH 8.0) with 0.1% sodium azide was used as working buffer for all FPIA experiments. Stock solutions (1 mg/mL) of SMP, SCP, and other analytes were prepared by dissolving 10 mg of drug in 10 mL of methanol and were stored at −20 °C. Aqueous standard solutions of analytes in the range 0.001−100 000 ng/mL were prepared by dilution of stock solution with BB buffer. Standard solutions were stored at 4 °C.

A Cary Eclipse fluorescence spectrophotometer (Varian Corporation, Palo Alto, CA) with a pair of polarizers was used to measure the value of the fluorescence polarization (FP). The excitation and emission monochromators were set at 492 and 514 nm, respectively, and the excitation and emission slits were set at 10 nm. An IBM PC microcomputer was used for on-line data acquisition and for data analysis.

Synthesis of Immunogen. The SMP hapten was synthesized as described by Eremin et al. with some modifications (*30*). SMP (6 g) and succinic anhydride (3 g) were refluxed in 60 mL of anhydrous ethanol for 90 min. The preparation was cooled to room temperature, and the resulting crystals were collected. This crude fraction was redissolved in a mixture of 20 mL of ethanol and 30 mL of water, refluxed for 30 min, and cooled to room temperature, and the crystals were collected by filtration. The prepared succinyl hemisuccinate of SMP (SMP-HS) was dried in a vacuum oven and stored, desiccated, at room temperature.

Two hundred milligrams of SMP-HS was dissolved in 2 mL of dimethylformamide, and an amount of 150 mg of EDC was dissolved in 500 μL of water and then added to the hapten solution. The mixture was stirred for 2 h at room temperature. The above reaction mixture was added dropwise to 2 g of BSA in 8 mL of sodium carbonate (0.01 M, pH 7.2) and placed at 4 °C overnight. The obtained immunogen (**Figure 1**) was dialyzed against phosphate-buffered saline (0.01 M, pH 7.4) for 7 days (one change every day) and stored in freezer in aliquots (*31*).

Synthesis of Fluorescent Conjugates (Tracers). SMP (5 mg) was dissolved in 0.5 mL of methanol. Triethylamine (50 μL) and FITC (4 mg) were added with mixing. After overnight reaction at room temperature, small portions (50 μL) of reaction mixture were separated by TLC using chloroform/methanol (4:1, v/v) as the eluent. The main yellow band at R_f 0.1 was scraped from the plate and extracted with 1

mL of methanol. SCP tracers were prepared in the same way (**Figure 1** and **Table 1**), and the tracers were stored in the dark at −20 °C (*30*).

Production of Monoclonal Antibody. The procedures for generating the immune response in mice and producing monoclonal antibodies were similar to those described by Zhang et al. (*32*). One hundred micrograms of the SMP-HS−BSA conjugate was emulsified with an equal volume of Freund's complete adjuvant and given as an intraperitoneal injection to each of five female BALB/c mice, 8 weeks of age. Booster injections were given 2, 4, and 6 weeks later with the same immunogen in an equal volume of Freund's incomplete adjuvant. One week after the third injection, serum was collected from the caudal vein of each mouse, and antibody titers were determined by the indirect ELISA. Three days before cell fusion, the mice that produced antibody with high titers were given another intraperitoneal booster injection of the immunogen without adjuvant.

Immunized mouse spleen cells (1×10^6) were fused with myeloma cells SP2/0 (6×10^6) using 1 mL of 50% PEG 2000. After fusion, the cells were selected with HAT medium (DMEM medium with 15% fetal calf serum, 100 units/mL penicillin, 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine). Aliquots of the cell suspension (50 μL) were added to the wells of the 96-well plates and incubated at 37 °C in 5% CO_2 atmosphere. After 12 h, an additional 50 μL of the selection medium was added to the wells. After 2 days, the medium was replaced with fresh selection medium. After day 12, aminopterin was omitted from the medium. Between days 12 and 14, supernatants from the 96-well plates were screened for antibody activity by the indirect ELISA in the absence and presence of 100 ng/mL SMP. ELISA-positive hybridoma cells were cloned by the limiting dilution method in aminopterin-free selection medium (HT medium), and one stable clone was obtained.

Curve Fitting and Statistical Analysis. FP was calculated according to the formula $P = (I_v - I_h)/(I_v + I_h)$, where I_v and I_h are the vertical and horizontal components of the emitted fluorescence intensities and are expressed in "millipolarization" units (mP).

The four-parameter logistic equation as defined below was used to fit the immunoassay data

$$Y = \frac{A - D}{[1 + (X/C)^B + D]}$$

where A = response at high asymptote, B = slope factor, C = concentration corresponding to 50% specific binding (IC_{50}), D = response at low asymptote, and X = calibration concentration.

The IC_{50} value, the assay dynamic range, and the limit of detection (LOD) serve as criteria to evaluate the FPIA. These characteristics represent the analyte concentrations that provide a tracer binding inhibition in the FP assay of 50%, between 20% and 80%, and of 90%, respectively (*28*).

Assay Optimization. Several physicochemical factors influencing the immunological reaction were studied in the FPIA, including pH, salt concentration, and organic solvent. Modification of mP_{max} (mP_{max} is the maximum FP value of the inhibition curve) and IC_{50} parameters of the standard curves was evaluated under different conditions.

Antibody Dilution Curve. Mabs were serially diluted 1/100, 1/400, ..., 1/819 200 in BB. Then 1 mL of diluted antibody solution was added to 1 mL of tracer in BB. Tracer concentration was selected such that the total final fluorescence intensity was 10 times higher than the background of BB (the excitation and emission slits were set at 5 nm for these measurements). The reaction mixtures were incubated for 2 min, and then polarization readings were made.

Competitive Fluorescence Polarization Immunoassay (FPIA) Procedure. FPIA calibration curves were obtained by adding to a cuvette 700 μL of standard solutions or sample, 700 μL of tracer in BB, and 700 μL of the optimal dilution of antibody. The reaction mixture was vortex-mixed, allowed to stand for 5 min, and then measured in the fluorescence spectrophotometer.

To normalize the FP value, the ratio mP/mP_{max} (where mP_{max} is the maximum FP value of the inhibition curve and mP is the current value) resulting in relative units was used.

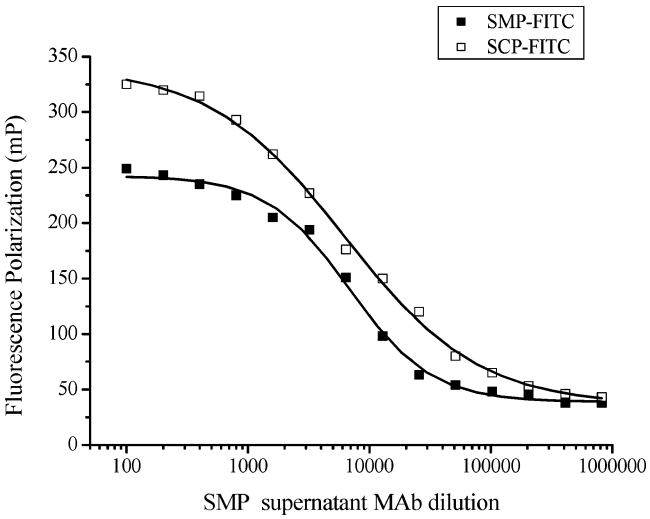


Figure 2. Dilution curves for SMP monoclonal antibody with different tracers SMP–FITC and SCP–FITC.

Cross-reactivity (CR) was calculated according to the equation

$$CR = \frac{IC_{50}(SMP)}{IC_{50}(sulfonamide)} \times 100\%$$

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

Dissociation constants were calculated with Scatchard plot analysis using FP data according to Hatzidakis et al. (*33*).

Fortification of SMP in Milk. Cow whole milk samples were purchased from a local market and simply defatted by centrifugation for 15 min (5000*g* at 4 °C). Aliquots (4 mL) were fortified with SMP to at 50, 100, and 500 ng/mL and then mixed with an equal volume of 1.25% trichloroacetic acid (TCA). The mixtures were agitated on a shaker for 1 min and then deproteinized through centrifugation for 10 min (5000 × *g* at 4 °C). The supernatants were diluted 1/5 in BB and used in FPIA.

RESULTS AND DISCUSSION

Selection of Reagent (Tracer) Concentration. Limiting concentrations of antibody and tracer immunoreactants are required for a competitive immunoassay to obtain the required analytical sensitivity. In particular, tracer concentration dictates the extent of competition allowed to be measured by the analyte and therefore the ultimate assay sensitivity. The lowest possible tracer concentration, which allows the reliable detection of label and does not affect the competition, is desirable for highest sensitivity. The lowest tracer concentration giving a signal must be approximately 10 times higher than the background signal from BB (total fluorescence intensity) (*30*). The antibody titer is usually assessed by FPIA using serially diluted antibody with a fixed concentration of the fluorescence-labeled tracer. In this study, antibody titer was determined using SMP−FITC and SCP−FITC. Antibody titration curves with these two tracers, both of which gave satisfactory binding, are shown in **Figure 2**. Here, sufficient antibody concentration was used to observe binding to both SMP−FITC and SCP−FITC. The highest possible sensitivity for the SMP analyte was experimentally determined in the competitive FPIA by then varying the antibody concentration within the optimal range, to give the lowest IC_{50} value. This effect of antibody concentration on the immunoassay sensitivity (IC_{50}) was studied. Different concentrations of anti-SMP antibody were used to reach the more optimal IC_{50} using SMP−FITC and SCP−FITC tracers. The relationship between the antibody concentration and the IC_{50} is shown in **Figure 3**,

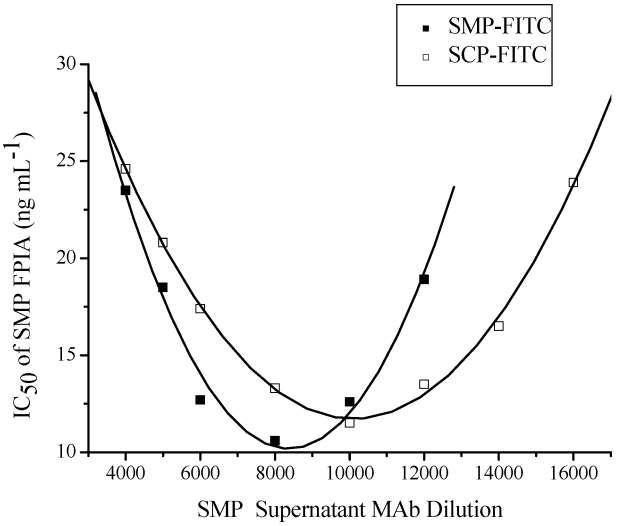


Figure 3. Relationship curve between IC_{50} and antibody dilution.

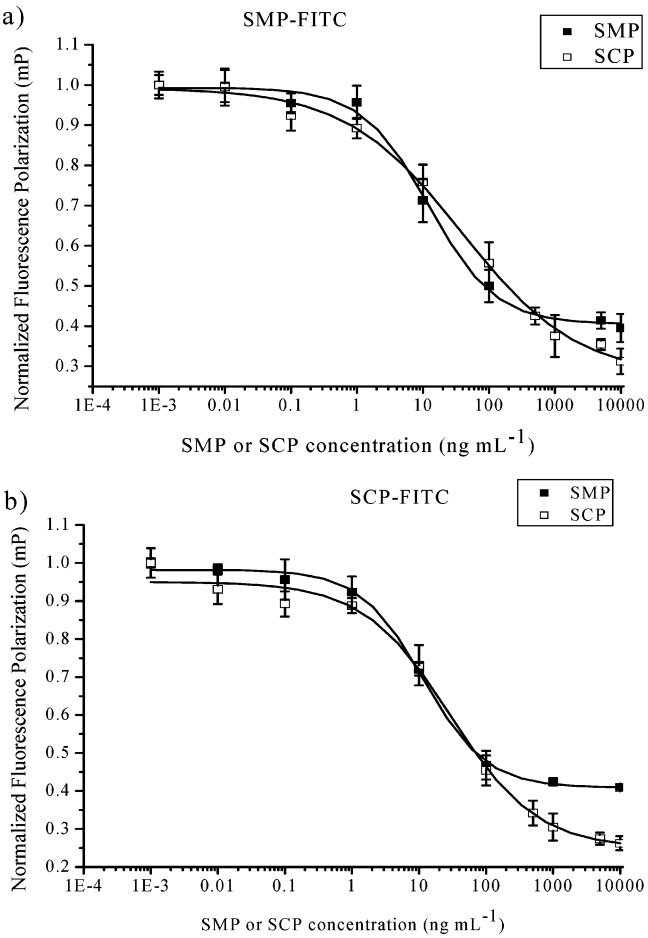


Figure 4. FPIA calibration curves for SMP and SCP using the same Mab and two different tracers: (**a**) SMP–FITC and (**b**) SCP–FITC. FPIA calibration curve and the SCP and SMP standard drug were dissolved in BB buffer. FP values, which are means of three replicates, were plotted against analyte concentration.

where we observed that 1/8000 would provide the best IC_{50} value. Calibration curves obtained under these optimal conditions are presented in **Figure 4**. The analytical characteristics of the FPIA developed are listed in **Table 2**.

Competitive FPIA. The antibody is a key determinant of both analytical specificity and analytical sensitivity in immunoassay methods. However, the structure of the tracer also affects these assay characteristics. Theoretically, a sensitive competitive immunoassay for a small molecule, such as a veterinary drug, can be achieved when the antibody affinities for tracer and analyte are comparable. However, in practice the affinity for the labeled antigen is generally higher than for analyte (*34*). Both the structural features of the tracer hapten itself and the structure and length of the bridge between the hapten molecule and the fluorescein label markedly influence recognition of the tracer by the antibody, and under conditions of competitive interaction with the analyte, assay sensitivity is modified (*35*). Labeled antigens that are either structurally similar or minorly different from the primary target analytes were investigated in this study. In agreement with previous studies, some FPIA systems were found to be more sensitive when structurally more different hapten tracers were used (*36*). To study the influence of tracer structure on assay sensitivity, both SMP−FITC and SCP−FITC tracers were used in the following investigation. These were selected because SMP and SCP are similar molecules, both containing a pyridazine group in the N1 position of the aromatic sulfonamido nucleus which is different from other sulfonamides. The structures of the two tracers are show in **Figure 1** and **Table 1**. As can been seen in **Table 2**, more slightly satisfactory IC_{50} values (11.5 and 29 ng/mL for SMP and SCP) and LOD (0.7 and 0.25 ng/mL for SMP and SCP) were observed when SCP−FITC was used, although SMP−FITC also presented with satisfactory analytical parameters (IC_{50} of 10.6 and 38.7 ng/mL, LOD values of 0.8 and 0.3 ng/mL for SMP and SCP).

Table 2. Analytical Parameters of Standard Curves for SMP and SCP by FPIA Using the Same MAb and Two Different Tracers SMP–FITC and SCP–FITC

		IC_{50} (ng/mL)		working range (ng/mL)		LOD (ng/mL)	
tracer	dilution	SMP	SCP	SMP	SCP	SMP	SCP
SMP–FITC	1/8000	10.6	38.7	2.3–82	1.8–364	0.8	0.3
SCP–FITC	1/10000	11.5	29	2–49	2–179	0.7	0.25

The developed FPIA was specific for SMP with cross-reactivity for SCP of 40% and 27%, using SCP−FITC and SMP−FITC tracers, and sulfamethizole of 1% using SCP−FITC, respectively. No significant cross-reactivity was observed with other sulfonamides (sulfamerazine, sulfamethazine, sulfadiazine sulfamethoxazole, sulfaphenazole, sulfadimethoxine, sulfamonomethoxine, sulfisoxazole, sulfaquinoxaline, sulfameter, sulfamoxol, sulfamethizole, sulfapyridine, and sulfanilamide). Standard curves for SMP and SCP obtained using both the SMP−FITC and the SCP−FITC tracers are shown in **Figure 4**. The tracer SCP−FITC was used in all subsequent studies. Martlbauer et al. (*22*) produced polyclonal antibodies to SMP and homologous tracer SMP−enzyme using glutaraldehyde as the coupling reagent. Their ELISA showed an LOD of 2.8 ng/mL for SMP and a cross-reactivity below 1% for SCP. Spinks et al. (*6*) synthesized an SCP-immunogen conjugate to raise SCP antisera using 2-fluoro-1-methypyridinium-*p*-toluenesulfonate (FMP) as a cross-linking reagent and a heterologous coating antigen using hemisuccinate bridge, having the same chemical structure as with the immunogen in our studies (**Figure 1**). The developed ELISA was highly specific for SCP and had a LOD of 0.65 ng/mL in assay buffer and showed cross-reactivity with SMP and sulfamethizole 3.1% and 1.9%, respectively. With the aim of obtaining sulfonamide group-specific antibodies, several authors have described the production of polyclonal antibodies and the isolation of one monoclonal antibody using different

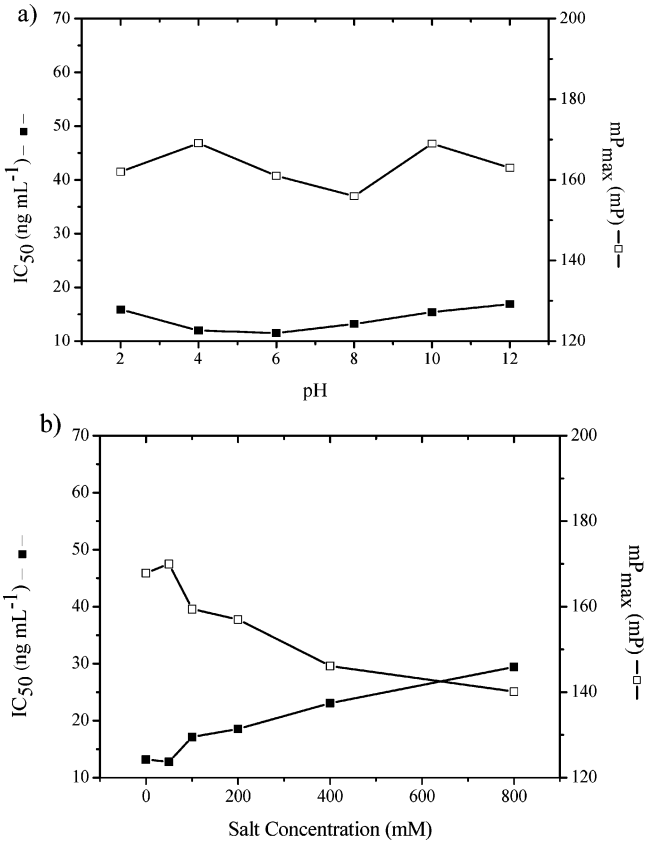


Figure 5. Influence of the (**a**) pH and (**b**) salt concentration of assay buffer on the analytical characteristics of SMP competitive standard curve: (■) value of IC_{50} for SMP and (□) mP value in the absence of SMP (mP_{max}). All reagents were prepared in BB at the appropriate pH and salt concentration. Each point represents the mean of three replicates.

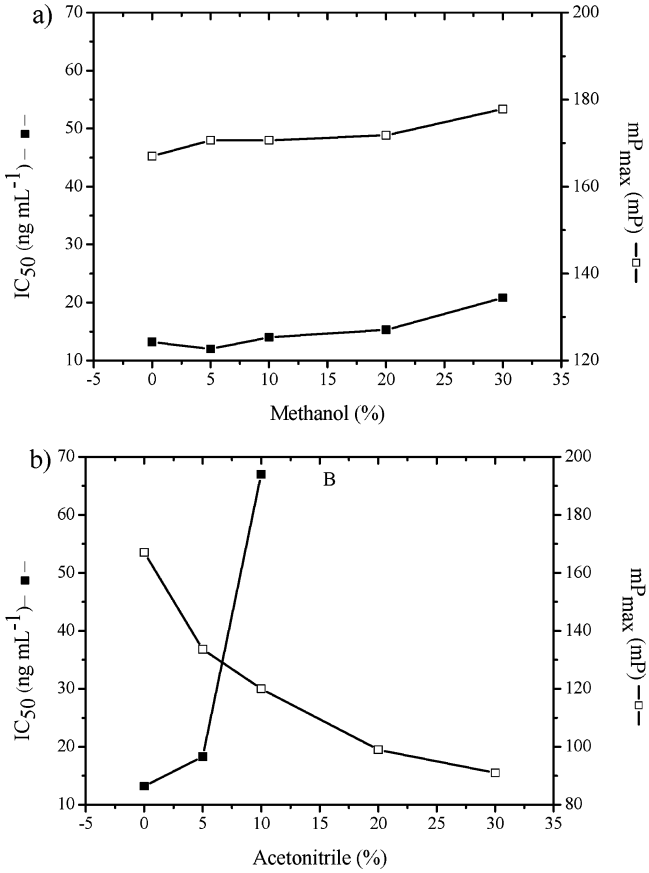


Figure 6. Effect of (**a**) methanol and (**b**) acetonitrile on SMP FPIA performance: (■) value of IC_{50} for SMP and (□) mP value in the absence of SMP (mP_{max}). Values refer to the final concentrations of solvents (v/v) in the competitive assay solution. Each point represents the mean of three replicates.

group-specific haptens in the past few years (*31*, *37*, *38*). The group-specific polyclonal antibodies obtained following immunization with *N*-sulfanil-4-aminobutyric acid (SAB) linked to soybean trypsin inhibitor (STI) showed sufficiently high cross-reaction to SMP and SCP (75% for SMP and 28% for SCP) and the group-specific monoclonal raised from *N*-sulfanilyl-4-aminobenzoic acid (SUL) linked to keyhole limpet hemocyanin (KLH) exhibited little or no ability to recognize SMP or SCP (1.55% for SCP) (*36*, *37*). Similarly, polyclonal antibodies against sulfacetamide (SAM) linked to bovine thyroglobulin (BTG) showed no binding with any sulfonamides (*31*).

Effects of Physicochemical Conditions on Assay Performance. Immunoassay performance is often influenced by chemical parameters such as salt concentration, pH, and organic solvent concentration. The effects of these parameters were assessed by comparing IC_{50} and mP_{max} obtained under various conditions: the maximum polarization (mP_{max}), reflecting the Mab recognition of the tracer in the absence of analyte, and the IC_{50} for SMP, reflecting the Mab affinity for the analyte itself.

To study the influence of pH in the assay system, competition curves for SMP using SCP−FITC as the tracer were obtained at different pH values from 2 to 12. The relationship of these parameters as a function of pH is shown in **Figure 5**. Deleterious effects on IC_{50} were observed at lower or higher pH, but no significant changes were observed in the range pH 4−8. The results indicated that the assay sensitivity decreased at a more acidic or basic pH value.

Because the ionic strength of the assay system can affect antibody binding, the salt concentration of working buffer was varied from 0 to 800 mM (**Figure 5**). For the FPIA method, higher salt concentration greatly reduced mP_{max}, whereas the IC_{50} rose dramatically. An almost threefold increase in the IC_{50} value occurred as a result of 800 mM salt concentration, while a 20% decrease in the mP_{max} value was seen. Because the primary effect of increased salt ion concentration is to reduce hydrophobic bond formation and stability, the interaction of antibody−analyte will decline under conditions of increasing salt concentration. The results obtained were consistent with the study of the polar pesticide TCP by ELISA (*39*), in contrast to the case of the polar 4-nitrophenol ELISA, wherein increasing salt concentration increased the antibody affinity for 4-nitrophenol and improved the assay sensitivity (*40*). Our studies indicated that the ability of the antibody to recognize both the tracer (mP_{max}) and the analyte (IC_{50}) follows a similar trend; that is, when antibody recognition of analyte increases, the value of mP_{max} will decrease accordingly because of the analyte and tracer possessing almost the same chemical immuno-recognition moiety.

Table 3. Constant of Affinity for Monoclonal Antibody

tracer	K_{aff} for SCP ($\times 10^9$ M^{-1})	K_{aff} for SMP ($\times 10^9$ M^{-1})
SCP–FITC	2.1	1.1
SMP–FITC	2.3	1.1

In addition, the effects of methanol and acetonitrile were studied because these solvents are water-miscible and commonly used in sample extraction procedures. The mP_{max} and IC_{50} changes were recorded, and results are shown in **Figure 6**. In general, IC_{50} increased and mP_{max} also slightly increased

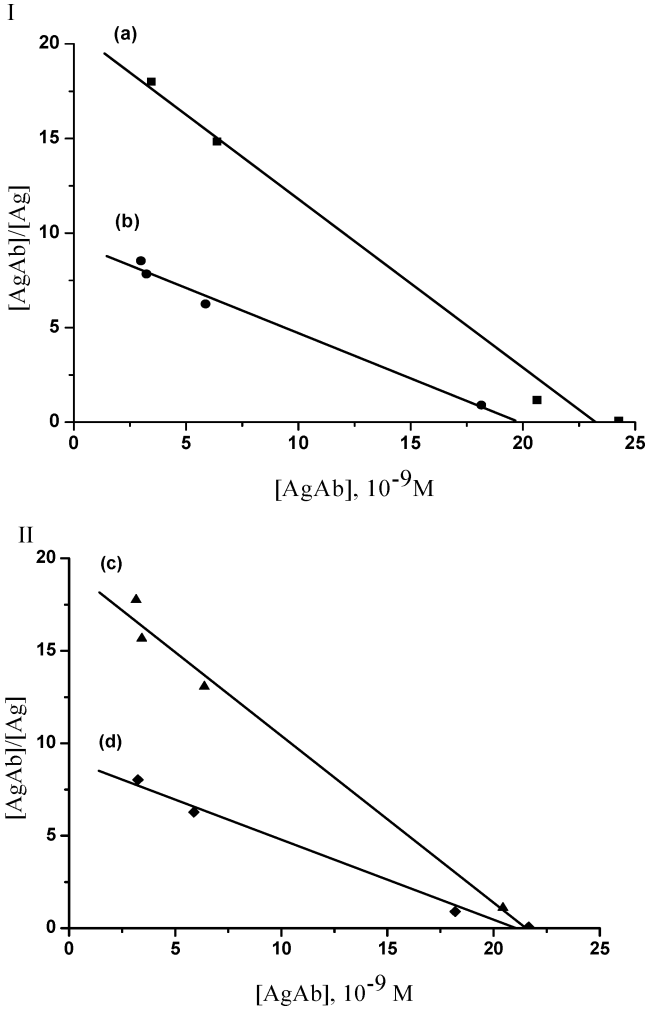


Figure 7. Scatchard plot for K_{aff} calculation. **I**: (**a**) tracer SCP–FITC, antigen SMP, $Y = -0.903X + 19.442$; (**b**) tracer SCP–FITC, antigen SCP, $Y = -0.433X + 9.121$. **II**: (**c**) tracer SMP–FITC, antigen SMP, $Y = -0.891X + 20.716$; (**d**) tracer SMP–FITC, antigen SCP, $Y = -0.478X + 9.488$.

gradually as the concentration of methanol and acetonitrile increased. It can be observed that tolerance of methanol was greater than that of acetonitrile. Acetonitrile concentrations exceeding 5% (final concentration) were not tolerated, whereas for methanol a concentration of 20% was still tolerated under the conditions used for the FPIA, because in this situation, no significant increase of IC_{50} and decrease of mP_{max} were observed. The presence of 30% methanol resulted in a 37% rise in IC_{50} value, but the mP_{max}, however, did not change appreciably. Therefore, solvent type and concentration should be carefully controlled for a reproducible FPIA procedure.

Measurement of Affinity Constant (K_{aff}). To explain the difference in sensitivity, the K_{aff} values of monoclonal antibody with two tracers were calculated. The results from Scatchard plots are summarized in **Table 3**. The Scatchard graphs are shown in **Figure 7**. From **Table 3** and **Figure 7** we observed that the antibody affinity for SMP is the similar when SCP−FITC and SMP−FITC were used; however, antibody affinity for SCP using SMP−FITC (2.3×10^9 M^{-1}) is slightly higher than that of SCP−FITC used (2.1×10^9 M^{-1}). This difference in affinity maybe result in that the FPIA presents slightly better sensitivity by SCP−FITC than SMP−FITC for SCP. The competition of SCP was more effective in the case of tracer SCP−FITC giving thus 25% better IC_{50} and consequently better sensitivity in the assay.

Table 4. Percentage Recovery of SMP and SCP Fortified Milk Samples by FPIA Using Tracer SCP–FITC ($n = 3$)

		found (ng/mL)		mean recovery (%)	
type of deproteinization	added (ng/mL)	SMP	SCP	SMP	SCP
acetonitrile	50	34 ± 2.7	30 ± 3.5	68	60
	100	87 ± 9.6	96 ± 8.3	87	96
	500	726 ± 65	600 ± 62	145	120
1.25% TCA	50	32 ± 3.6	32 ± 4.1	64	64
	100	70 ± 7.5	78 ± 7.5	70	78
	500	489 ± 43	513 ± 46	98	103

Table 5. Influence of the Milk Sample Matrix on the FPIA Standard Curves for SMP and SCP Using SCP–FITC as Tracer after Protein Precipitation

		IC_{50} (ng/mL)		mP_{max}	
sample	precipitate content	SMP	SCP	SMP	SCP
buffer		11.5	29	167	165
milk	acetonitrile	17	44	148	143
	1.25% TCA	15	39	151	153
	2.5% TCA	30	74		

Sample Preparation. The optimized FPIA method was used to detect SMP and SCP spiked in milk. The analytical characteristics of an immunochemical technique can be significantly influenced by the organic solvent and the various components existing in complicated matrices, such as protein and fat (*41*). FPIA is susceptible to interference by different components existing in milk. As some papers have reported previously, the most common ways to reduce such matrix effects are solid-phase extraction (SPE) techniques or dilution after selective extraction (cleanup) to bring the interfering substances below a concentration that would affect the assay. The SPE approach is relatively time-consuming and expensive and would counteract the advantage of the FPIA method in being a rapid and simple screening application. Thus, in the present study, the extracts were diluted to reduce matrix influences before analysis.

The impact on FPIA of several common organic solvents has been reported (*42*, *43*). In these studies, methanol had been selected as the preferred extraction reagent, not only because of the efficiency of drug extraction and precipitation properties but also because the methanol extract has the least influence on the immunoassay. In the present recovery studies, negative milk spiked with SMP and SCP (50, 100, and 500 ng/mL) was extracted using three organic solvents, methanol, acetonitrile, and 1.25% TCA with the same sample preparation protocol, and recoveries of SMP and SCP from the milk were determined. Samples for the recovery studies were prepared by extraction of 4 mL of spiked milk with an equal volume of three extraction reagents, followed by centrifugation to remove the protein and dilution of 1 mL of supernatant in 4 mL of BB. The results are represented in **Table 4**. The data obtained from methanol were not presented because the extract was white (containing a lot of protein) and could not be measured by the FPIA machine. In a separate experiment for evaluation of matrix interference, 4 mL of unspiked milk was treated similarly, and the protein was precipitated by 4 mL of methanol, acetonitrile, and 1, 1.25, 2.5, and 5% TCA, respectively. SMP standards were prepared in the diluted extract to obtain standard curves. The mP_{max} and IC_{50} for each standard curve are presented in **Table 5**. The mP_{max} and IC_{50} values were significantly affected by methanol and

TCA concentrations above 2.5 or below 1% in the final extract (data not shown). Although successfully applied to extract some sulfonamides from milk and other drugs in food samples by ELISA or FPIA (*6*, *29*), methanol was not an effective protein precipitation reagent without further purification processes in this experiment. The TCA proved to be a strong protein precipitation reagent (*29*). A 1.25% solution was an effective concentration of TCA to completely remove the protein in milk with only a slight effect on mP_{max} and IC_{50}. The mean recoveries of three spiked concentrations at 50, 100, and 500 ng/mL were 60−98% for SMP and 64−103% for SCP, respectively. A concentration of TCA below 1% was ineffective in removing interfering protein from the milk sample, and conversely, a concentration of TCA exceeding 2.5% reduced antibody interactions and showed an increase in IC_{50} and a decrease in mP_{max}. At 5% TCA, there was a 12-fold increase in IC_{50} and 33% decrease in mP_{max}, compared to that of the FPIA test result obtained using BB. Similar recovery results were observed using acetonitrile for interfering protein precipitation; however, the presence of acetonitrile in the final extract solution resulted in an 11% and 13% drop in mP_{max} and 34 and 37% rise in IC_{50} for SMP and SCP, respectively. Recoveries using acetonitrile were 68−145% for SMP and 60−120% for SCP, and the mean inter-assay coefficients of variation found for the assay for SMP and SCP were 9.3 and 10.1%.

Conclusion. A sensitive, specific FPIA based on a Mab for determination of SMP and SCP was developed. The Mab was generated using an SMP hapten with a hemisuccinate bridge spacer arm at the N4 position and conjugated to BSA. The IC_{50} values of the optimized FPIA were 11.5 and 29 ng/mL with a detection limit of 0.7 and 0.25 ng/mL for SMP and SCP, and the cross-reactivities with SMP and SCP were 100% and 40%, respectively. The antibody showed negligible cross-reactivity to other sulfonamides. SMP and SCP spiked in milk were analyzed with satisfactory recovery. It was found that acetonitrile and 1.25% TCA were optimal regents to remove the protein in milk, and dilution of the extract was an effective means to reduce the matrix and organic reagent interference. However, further work will be needed to validate this assay for other applications. The major advantages of FPIA are rapidity, simplicity, and convenience of use in routine screening analysis.

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