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Development of an Enzyme-Linked Immunosorbent Assay for Seven Sulfonamide Residues and Investigation of Matrix Effects from Different Food Samples

HONGYAN ZHANG,† LEI WANG,† YAN ZHANG,† GUOZHEN FANG,† WENJIE ZHENG,‡ AND SHUO WANG*,†

Tianjin Key Laboratory of Food Nutrition and Safety, Faculty of Food Engineering and Biotechnology, Tianjin University of Science and Technology, Tianjin 300222, People's Republic of China, and Tianjin Entry-Exit Inspection and Quarantine Bureau, Tianjin, 300201, People's Republic of China

Direct competitive enzyme-linked immunosorbent assays (ELISA) were developed to detect a broad range of sulfonamides in various matrices. Screening for this class of antibiotics in pig muscle, chicken muscle, fish, and egg extracts was accomplished by simple, rapid extraction methods carried out with only phosphate-buffered saline (PBS) buffer. Twenty milliliters of extract solution was added to 4 g of sample to extract the sulfonamide residues, and sample extracts diluted with assay buffer were directly analyzed by ELISA; matrix effects could be avoided with 1:5 dilution of pig muscle, chicken muscle, and egg extracts with PBS and 1:5 dilution of fish extract with 1% bovine serum albumin (BSA)−PBS. For liver sample, the extraction method was a little more complicated; 2 g of sample was added to 20 mL of ethanol, mixed, and then centrifuged. The solvent of 10 mL of the upper liquid was removed, and the residues were dissolved in 10 mL of PBS and then filtered; the filtrate was diluted two-fold with 0.5% BSA−PBS for ELISA. These common methods were able to detect seven sulfonamide residues such as sulfisozole, sulfathiazole, sufameter, sulfamethoxypyridazine, sulfapyridine, sulfamethizole, and sulfachlorpyridazine in pig muscle, liver, chicken muscle, egg, and fish. The assay's detection limits for these compounds were less than 100 μg kg^{-1}. Various extraction methods were tested, and the average recovery ($n = 3$) of 100 μg kg^{-1} for the matrices was found to range from 77.3 to 123.7%.

KEYWORDS: Sulfonamides; ELISA; detection; residues

INTRODUCTION

The sulfonamides are a group of antimicrobial agents applied in veterinary as well as human medicine for the treatment and prophylaxis of bacterial infections (*1*). Widespread use of sulfonamide drugs in factory farming without proper withdrawal periods has led to accumulation of sulfonamides in meat, eggs, and milk as well as in fish (*2*). Because of the possible risk of resistance development in human pathogens, the maximum residue limits (MRLs) for sulfonamides were set to 100 μg kg^{-1} in edible animal tissue (valid for the European Union and the United States) (*3*, *4*). The current sulfonamide detection technologies are mainly based on bacteriological growth inhibition (*5*, *6*) or chromatographic methods (*7*, *8*). These methods are either laborious or slow for mass screening. During the past decades, a variety of immunoassays were developed, each specific for an individual sulfonamide (*9*−*15*). However, it would be more efficient to have an immunoassay able to detect all sulfonamides instead of an immunoassay for each individual sulfonamide.

The sulfonamides share a common *p*-aminobenzoyl ring moiety with an aromatic amino group at the N_4-position and differ in the substitution at the N_1-position. The backbone and the structures of sulfonamides studied in this work are shown in **Figure 1**. Polyclonal antibodies (pAbs) were generated by methods using immunogens in which the sulfonamides of interest were linked to a carrier protein via the N_1-position, resulting in a general assay for the class of sulfonamides. Sheth and Sporns (*16*) were the first to report the development of sulfonamide-selective antibodies. The pAbs that they obtained recognized nine sulfonamides showing 50% inhibition at a concentration of less than 5000 μg L^{-1} (*16*). The pAbs obtained by Assil et al. showed 50% inhibition with seven sulfonamides at concentrations less than 10000 μg L^{-1} (*17*). The first published study about sulfonamide-selective monoclonal antibodies (mAbs) was from Muldoon et al. (*18*). Only one mAb was obtained that recognized eight sulfonamides at concentration

* To whom correspondence should be addressed. Tel: (86 22)6060 1456. Fax: (86 22)6060 1332. E-mail: s.wang@tust.edu.cn.
† Tianjin University of Science and Technology.
‡ Tianjin Entry-Exit Inspection and Quarantine Bureau.

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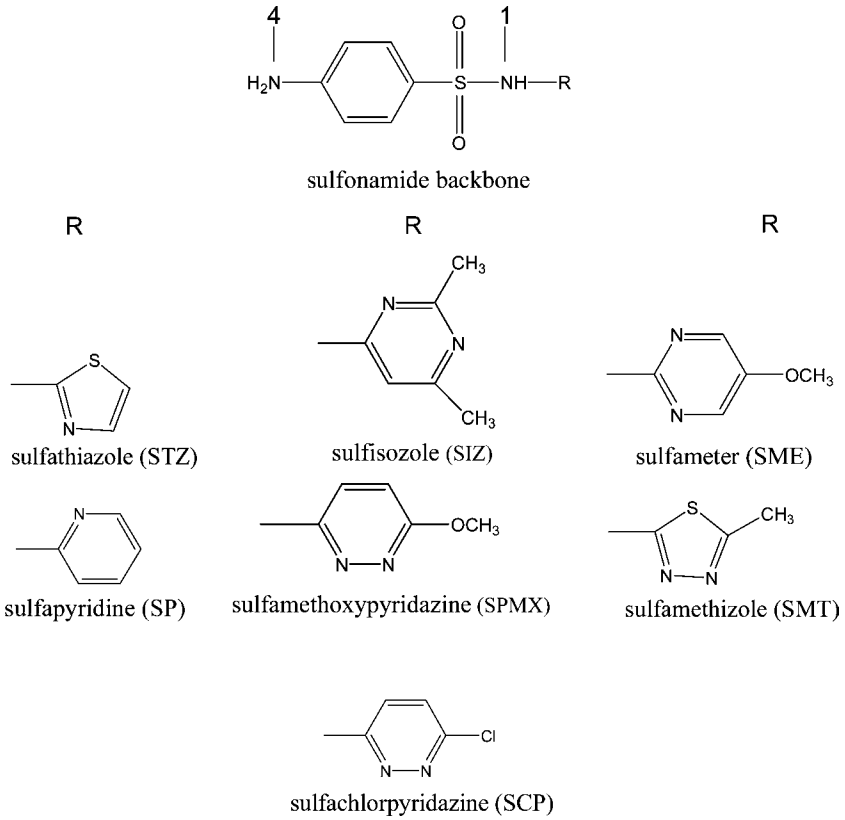

Figure 1. Structures of the sulfonamides studied in this work.

levels less than 10000 μg L^{-1} (*18*). Haasnoot et al. (*19*, *20*) used the same sulfonamide derivatives that Sheth and Sporns (*16*) and Assil et al. (*17*) had used to obtain mAbs. The best mAb showed 50% inhibition with 18 tested sulfonamides at values less than 10000 μg L^{-1} and with eight at concentrations of less than 1000 μg L^{-1} (*19*, *20*). Spinks et al. (*21*) carried out a molecular modeling study on the sulfonamide structure and deduced that cross-reactive antibodies could possibly be obtained using a sulfonamide as a hapten with a more planar structure (sulfacetamide) or a greater bend (sulfachlorpyridazine). Despite this interesting hypothesis, immunization with such conjugates did not lead to antisera with a broader specificity for sulfonamides (*21*). Different strategies for the development of sulfonamide-selective mouse antibodies were compared by Cliquet et al. (*22*). With the best mAb (3B5B10E3), five sulfonamides were detected at their MRLs (100 μg L^{-1}) in buffer solution (*22*). Korpimäki et al. (*25*) used protein engineering to improve the broad specificity of sulfonamide antibody 27G3 and improved the best mutant of the previous studies with site-directed mutagenesis. The new mutants recognized different sulfonamides with affinities sufficient for detection of all 13 tested sulfonamides at levels below the MRLs. They also demonstrated the functionality of one mutant in real sample matrices (*23*−*25*).

None of the reported pAbs or mAbs could detect all of the relevant sulfonamides at the level of the MRL. Furthermore, because of the low sensitivity, there have been a few reports about the studies on sample matrices. Previously, we reported the synthesis of five sulfonamide derivatives, the production of broad specificity pAbs, and the development of an enzyme-linked immunosorbent assay (ELISA) suitable for the determination of 11 of the tested 16 sulfonamides within a concentration range of 1.3−90 ng mL^{-1} in assay buffer (*26*). In the present study, we applied the developed ELISA assay to determine seven sulfonamide residues such as sulfisozole, sulfathiazole, sufameter, sulfamethoxypyridazine, sulfapyridine, sulfamethizole, and sulfachlorpyridazine in pig muscle, liver, chicken muscle, egg, and fish at or below the level of the MRL (100 μg kg^{-1}).

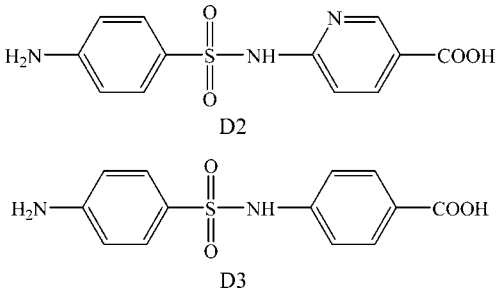

Figure 2. Chemical structures of haptens studied in this work.

MATERIALS AND METHODS

Reagents. Bovine serum albumin (BSA), reagent grade 3,3′,5,5′-tetramethylbenzidine (TMB), and fish skin gelation (FG) were purchased from Sigma (United States). Protein-A Sepharose 4B was purchased from Amersham (Sweden). High-performance liquid chromatography (HPLC) grade methanol was obtained from Merck (Germany). Reagent grade hydrogen peroxide was from Sigma.

Solutions. Phosphate-buffered saline (PBS, 10 mmol L^{-1} sodium phosphate, 137 mmol L^{-1} NaCl, and 2.7 mmol L^{-1} KCl, pH 7.5), phosphate-buffered saline with 0.05% Tween 20 (PBST), coating buffer (CB, 50 mmol L^{-1} sodium carbonate buffer, pH 9.6), and TMB substrate solution [prepared by adding 3.3 mg of TMB in 250 μL of DMSO to 25 mL of phosphate−citrate buffer (0.1 mol L^{-1} citric acid + 0.2 mol L^{-1} Na_2HPO_4; pH 4.3) containing 3.25 μL of a 30% H_2O_2 solution] were used.

Haptens Synthesis and Antibody Production. Chemical structures of haptens D2 and D3 used in this work are shown in **Figure 2**. Hapten synthesis and the preparation of enzyme and protein conjugates were according to Zhang's report (*26*). Antibodies used in this paper were

produced by haptens D3 coupled to keyhole limpet hemocyanin (KLH) as immunogens. Hapten D2 was coupled to horseradish peroxidase (HRP) as enzyme tracers. Antibodies were produced in rabbits as described by Wang et al. (*27*). IgG from the antisera was purified by protein A-Sepharose 4B affinity chromatography. The optimization and evaluation of the developed ELISA assays were described by Zhang et al. (*26*).

Immunoassay Procedure. Maxisorp polystyrene 96 well microwell plates were from Nunc (Rockilde, Denmark), and plates were washed with a 96 PW microplate washer from Bio-Rad (Hercules, CA). Immunoassay absorbance was read with a Multiscan Spectrum purchased from Thermo (Labsystems, Vantaa, Finland).

Direct competitive ELISA was applied in this study to analysize the sulfonamide residues in pig muscle, chicken muscle, egg, and fish. Direct and indirect competitive ELISA were applied to the analysis of sulfonamides in liver.

Direct ELISA. The microwell plates were coated with purified antibodies at 1 μg per well in 100 μL of CB and incubated overnight at room temperature. Plates were then washed three times with 10 mmol L^{-1} PBST, and unbound active sites were blocked with 200 μL of 1% BSA/PBS per well for 1 h. After the plates were washed four times, for competitive assays, 100 μL standards in PBS (or diluted sample solution) and 100 μL of HRP-haptens in PBS were then added to each well and incubated for 1 h at room temperature. Following washing five times, the HRP tracer activity was then measured by adding 150 μL per well of TMB substrate solution. The enzymatic reaction was stopped after 30 min by adding 2.5 mol L^{-1} H_2SO_4 (50 μL per well), and the absorbances were then read in dual-wavelength mode (450 nm as test and 650 nm as reference).

Sample Preparation. For use in the ELISA performance evaluation, five different matrices such as pig muscle, liver, chicken muscle, egg, and fish were chosen according to several criteria, one of which is their presence in the table of established MRLs. Negative samples were bought from local markets. Before the spiking and recovery studies, each test sample was verified to contain sulfonamides at less than 5 μg kg^{-1} by HPLC. The sample tissues were minced with a conventional kitchen mixer. For a spiking study of pig muscle, chicken muscle, egg, and fish, 4 g of sample was spiked with single sulfonamide at three different levels (50, 100, and 200 μg kg^{-1}); 20 mL of PBS was added, then thoroughly mixed for 2 min, and then filtered by using filter paper or centrifuged at 4000*g* for 20 min. The tubes containing egg samples were gently mixed for 30 min to avoid foam and emulsion and then diluted with PBS directly. For a spiking study of pig liver, 2 g samples were spiked with single sulfonamide at three different levels (50, 100, and 200 μg kg^{-1}); 20 mL of ethanol was added, then thoroughly mixed for 2 min, and centrifuged at 4000*g* for 20 min. The solvent (10 mL of the upper liquid) was removed under reduced pressure. The residues were dissolved in 10 mL of PBS and then filtered, and the filtrate was diluted with appropriate diluent before ELISA analysis. Three samples were prepared per concentration, and the extracts were analyzed in triplicate. The sample extracts were appropriately diluted before ELISA analysis, and 1% BSA, 0.5% FG, or 0.05% Tween 20 was added to the PBS to avoid the sample matrix effect.

Instrumentation for HPLC Analysis. The test samples were verified using a Shimadzu (Japan) HPLC equipped with a LC-10AT vp. pump with Hamilton injector (25 μL loop), a DGU-12A online degasser, and a CTO-10AS vp column oven. A C_{18} reversed-phase column (15 cm $\times$ 4.6 mm i.d., 5 μm) was used. The analysis was performed at 270 nm, and the mobile phase was methanol−water (28:72) (the water pH value was adjusted to 3.5 before it was mixed with methanol) at a flow rate of 1.0 mL min^{-1}. The temperature of the column oven was 35 °C.

RESULTS AND DISCUSSION

Immunoassays are rapid and convenient for food sample analysis primarily because they usually do not require sample preconcentration and cleanup steps. However, ELISA methods often have a high potential for nonspecific binding between nontarget analytes and antibodies and are consequently prone to matrix effects. Chemical compounds present in animal product samples or sample extracts, such as protein, fat, solvents, and others, might affect the binding of antibody and analytes, and they also might affect other aspects of the assay. This "matrix effect" is a common problem for the immunoassay, which could reduce the sensitivity and reliability of the competitive immunoassay and cause false positives by lowering the color development. There are several methods available for the quantitative evaluation the matrix effect. Typically, interferences are quantified by comparing a standard curve produced in a control with a calibration curve generated in the sample matrix. If the two curves are superposable, the effect of the matrix is not significant, and then, the samples can be analyzed using the standard curve prepared in the control solution (*28*).

Pig muscle, liver, chicken muscle, egg, and fish were chosen as test samples to study the matrix effect in this study. Dilution is a commonly used procedure to reduce the interference from matrices, but this procedure would also reduce the sensitivity. The ELISA methods established here still maintain enough sensitivity after dilution to reduce the matrix interference.

In our study, five-fold dilution of extracts with PBS alone could reduce matrix interfence from pig muscle, chicken muscle, and egg extract to insignificant levels. The addition of Teleostean FG, BSA, and Tween 20 to dilute PBS extract was examined to reduce nonspecific interactions from liver and fish; matrix interference could be overcome after two-fold dilution with 0.5% BSA for liver and five-fold dilution with 1% BSA for fish samples. The BSA in the diluent seemed to act like a stabilizer to protect the enzyme from the interfering materials or to stabilize the antibody−antigen interaction. The MRLs for sulfonamides formulated by the Food and Agriculture Organization (FAO) in pig muscle, liver, chicken muscle, egg, and fish are 100 μg kg^{-1}. Therefore, simple dilution with PBS or BSA in PBS still maintained the detection limit at legal requirements and is applicable to determination of sulfonamide residues with the currently developed ELISA.

ELISA for Sulfonamides in Pig Muscle. Two different extraction procedures such as filtration using filter paper or centrifuging at 4000*g* for 20 min were tested, and the effects of these two methods were compared. Each kind of sample was fortified with three levels of a single sulfonamide and then analyzed by ELISA. Each sample was evaluated with three replicates to verify repeatability. The results of recovery studies are shown in **Table 1**. Both of the methods gave recoveries between 80 and 120% at 100 μg kg^{-1}(MRL) in each sample, but the centrifuge procedure is more complicated. Filtration using filter paper could conform to this requirement, since it was efficient, extracting over 95% of the residue, and convenient enough to suit on-site residue analysis.

The effect of adding teleostean FG, BSA, and Tween 20 to PBS extracts was examined, and there was no obvious improvement other than dilution with PBS directly. The effects of dilution were also investigated, and 10- and 20-fold dilutions were found that matrix effects caused a large decrease in the observed absorbance than five-fold dilution; however, the five-fold dilution can maintain the error less than 10%, and considering the sensitivity of the ELISA, the five-fold dilution with PBS was selected to reduce the matrix interference in this study. **Figure 3** showed the standard curves of seven sulfonamides in pork sample extracts after appropriate dilutions.

ELISA for Sulfonamides in Liver. Sulfathiazole was applied to evaluate the elimination of matrix effect, and pig liver samples were extracted with PBS as the method for pig muscle; considering the sensitivity, the extracts were diluted five-fold in PBS, 1% BSA in PBS, 0.5% FG in PBS, or 0.05% Tween

Table 1. Recovery Studies from Pig Muscle Spiked with Single Sulfonamide Using Filtration or Centrifuge Operation at Three Levels by ELISA ($n = 3$ Replicates)

		different extraction methods			
		thoroughly mixed for 2 min and then filtered using filter paper		thoroughly mixed for 2 min and then centrifuged at 4000*g* for 20 min	
analytes	fortification level (μg kg^{-1})	recovery (%)	CV (%)	recovery (%)	CV (%)
sulfisozole	50	76.6	15.7	99.8	19.0
	100	81.3	9.3	91.2	8.4
	200	81.6	11.8	74.0	8.2
sulfathiazole	50	120.0	13.1	112.2	17.4
	100	103.1	12.9	96.4	9.6
	200	108.8	16.0	101.3	10.7
sufameter	50	82.5	9.2	104.3	17.9
	100	80.0	6.2	97.6	9.5
	200	77.9	11.1	66.5	9.1
sulfamethoxypyridazine	50	99.5	16.0	84.3	14.4
	100	79.3	12.7	81.2	11.8
	200	68.2	10.9	76.5	11.2
sulfapyridine	50	89.9	10.3	126.5	15.2
	100	87.4	11.8	98.4	9.7
	200	88.7	8.6	94.2	7.0
sulfamethizole	50	84.9	19.9	72.1	20.1
	100	87.6	13.4	77.3	15.0
	200	74.1	16.7	69.8	12.6
sulfachlorpyridazine	50	137.6	21.4	129.3	23.8
	100	113.0	13.1	108.8	12.7
	200	119.8	11.8	112.4	12.3

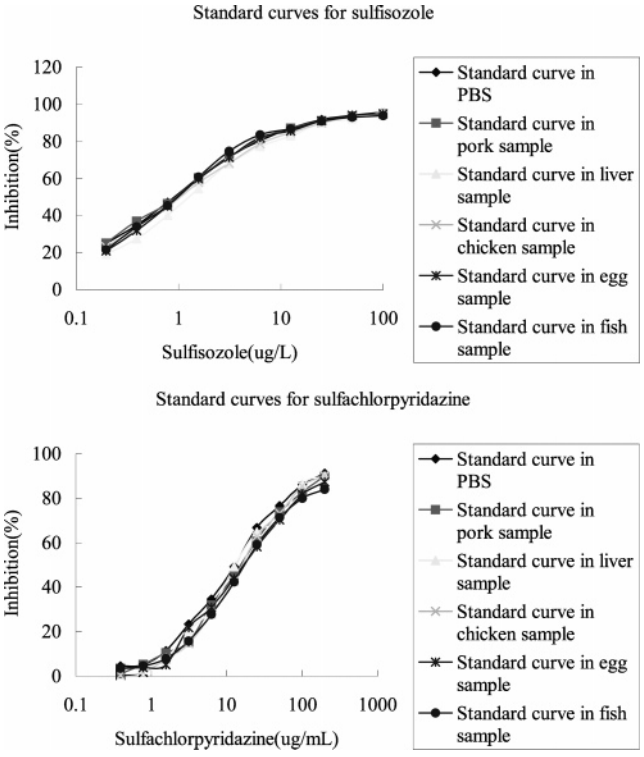

Figure 3. Standard curves for two sulfonamides in five samples.

20 in PBS. These four inhibition curves were all superposable with a standard curve, but the absorbency values of control were all lower than the control of standard. So organic solvent extraction was investigated. It was found that if the proportion of the solvent was higher than 10% in the final determination solution, it would denature the antibody or enzyme. Considering the final proportion of solvent, 10, 25, and 50% ethanol were selected for this study. Although the recovery of the residues was higher with a higher proportion of ethanol, more impure materials were extracted at the same time. The recovery of the sulfonamide residues could satisfy the need, and the matrix effect could be controlled in the acceptance range when extraction with 25% ethanol was used. Balancing the sensitivity and the matrix effect, 25% ethanol was chosen as the extraction solvent. The matrix effect and the recovery of SME, STZ, SIZ, SP, SPMX, SMT, and SCP were also investigated, and it was found that the matrix effect and the recovery of STZ and SME were satisfactory to meet regulatory needs, but the matrix effect could not be eliminated for SIZ, SP, SPMX, SMT, or SCP and the apparent recoveries were high due to the matrix effect.

Table 2. Recovery Studies from Pig Liver and Chicken Muscle Spiked with Single Sulfonamide Using Filtration Operation at Three Levels by ELISA ($n = 3$ Replicates)

		samples			
		pig liver		chicken muscle	
analytes	fortification level (μg kg^{-1})	recovery (%)	CV (%)	recovery (%)	CV (%)
sulfisozole	50	131.5	14.1	99.8	10.5
	100	98.8	6.0	92.3	6.1
	200	84.9	8.2	90.0	5.5
sulfathiazole	50	65.0	10.9	102.0	8.0
	100	82.4	11.4	89.6	9.6
	200	88.5	17.3	101.5	7.3
sufameter	50	139.9	8.8	135.8	13.8
	100	113.1	5.0	118.0	9.9
	200	131.3	4.6	95.6	6.0
sulfamethoxypyridazine	50	134.7	19.3	132.4	16.5
	100	107.8	13.7	115.5	13.4
	200	109.9	9.6	122.5	11.1
sulfapyridine	50	125.3	9.6	133.3	14.8
	100	113.0	4.2	115.8	9.5
	200	103.1	11.5	104.2	12.2
sulfamethizole	50	112.4	6.9	122.8	20.9
	100	96.6	10.7	103.7	14.4
	200	102.4	15.9	91.4	16.1
sulfachlorpyridazine	50	125.3	10.0	118.1	18.8
	100	104.3	7.7	92.7	11.9
	200	105.7	8.2	98.2	13.7

Because of the above reasons, a more complicated extract method was chosen as indicated in the Sample Preparation section for liver in which the extract solvent was removed under reduced pressure, the residues were dissolved with PBS and then filtered, and the filtrate was diluted two-fold with 0.5% BSA in PBS before ELISA analysis. Using this method, the matrix effect was eliminated and the recovery for seven sulfonamides at 100 μg kg^{-1} in five samples was 82.4−113.1%. The results of recovery studies are shown in **Table 2**. The standard curves of seven sulfonamides in liver sample extracts are shown in **Figure 3**.

ELISA for Sulfonamides in Chicken Muscle. As in the case of the ELISA for chicken muscle, we used filtration with filter paper to deal with the chicken muscle followed by a five-fold dilution of the filtrate with PBS directly. We found that the standard curves for sulfonamide standards prepared in PBS were almost superposable with those obtained when standards were prepared in PBS extracts of chicken muscle diluted five-fold in PBS. The matrix interference was fairly diminished. **Figure 3** shows the standard curves of sulfonamides in chicken sample extracts. The results of recovery studies are shown in **Table 2**.

ELISA for Sulfonamides in Egg. Because of the abundant protein in egg, initially, we used the ethanol or acetone as a protein-depositing solvent, and the clear filtrate was diluted with

Table 3. Recovery Studies from Egg and Fish Muscle Spiked with Single Sulfonamide Using Filtration Operation at Three Levels by ELISA ($n = 3$ Replicates)

	samples				
		egg		fish muscle	
analytes	fortification level (μg kg^{-1})	recovery (%)	CV (%)	recovery (%)	CV (%)
sulfisozole	50	103.7	8.6	81.5	12.2
	100	96.7	5.2	95.7	9.2
	200	113.6	9.3	80.3	7.5
sulfathiazole	50	110.5	10.7	98.5	11.0
	100	105.8	11.4	105.2	8.8
	200	122.0	8.5	86.0	9.1
sufameter	50	125.4	16.9	77.6	9.5
	100	114.7	13.7	83.6	12.7
	200	121.5	15.0	75.7	7.4
sulfamethoxypyridazine	50	122.1	14.4	119.5	13.9
	100	101.3	12.9	99.2	12.0
	200	116.8	9.7	81.0	15.8
sulfapyridine	50	130.6	17.1	72.9	11.7
	100	114.4	14.4	84.9	13.2
	200	108.1	15.2	101.7	9.0
sulfamethizole	50	139.3	15.5	139.4	17.9
	100	123.7	13.1	118.8	14.8
	200	117.2	16.3	95.5	12.6
sulfachlorpyridazine	50	128.5	19.4	142.2	16.3
	100	120.6	12.9	118.9	14.5
	200	119.1	17.7	120.6	14.9

PBS before ELISA analysis. We also compared the effects of the above methods with that of extraction using PBS and found that the standard curves prepared in PBS were almost superposable with those obtained when standards were prepared in PBS extracts of egg diluted five-fold in PBS, but the standard curves prepared in PBS (containing 5% ethanol or acetone) were not superposable with those obtained when standards were prepared in ethanol or acetone extracts of chicken muscle diluted five-fold in PBS. The above results indicate that although the ethanol or acetone could precipitate protein in egg, they also effected the results of ELISA, so we selected the PBS as an extraction and diluent solvent. **Figure 3** shows the standard curves of seven sulfonamides in egg sample extracts after appropriate dilutions. The results of recovery studies are shown in **Table 3**.

ELISA for Sulfonamides in Fish. A significant effect of the fish matrix was seen for sulfonamide standards in PBS extracts with depression of absorbance by about 40%. The addition of Teleostean FG, BSA, and Tween 20 to dilute PBS extract was examined to reduce nonspecific interactions from fish sample, and it was found that the matrix effect almost disappeared when it was diluted five-fold with 1% BSA in PBS. **Figure 3** shows the seven standard curves of dilutions with 1% BSA in PBS for fish. The results of recovery studies are shown in **Table 3**.

In conclusion, the competitive direct ELISA presented here successfully determined seven sulfonamides in pig muscle, liver, chicken muscle, egg, and fish at or below the level of MRL (100 μg kg^{-1}). Sulfonamides were analyzed by simple extraction and dilution using PBS or BSA in PBS without any concentration or cleanup steps except pig liver. The five-fold dilution with PBS for pig muscle, chicken muscle, and egg, two-fold dilution with 0.5% BSA in PBS, and five-fold dilution with 1% BSA in PBS for fish samples used in this experiment resulted in no effect from the matrix. For the liver sample, the extraction method was a little more complicated; 20 mL of ethanol was added to 2 g of sample, mixed, and then centrifuged. The solvent of 10 mL of the upper liquid was removed, and the residues were dissolved in 10 mL of PBS and then filtered; the filtrate was diluted two-fold with 0.5% BSA−PBS for ELISA. The ELISA method described in this paper can be used for quantitative analysis; however, it is more suitable for a screening facility complementary to the conventional instrument methods. This approach results in savings in terms of both time and costs, making it useful for users without access to sophisticated equipment to meet the requirements of the rapidly evolving legislation.

ABBREVIATIONS USED

ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; MRL, maximum residue limit; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; TMB, 3,3′,5,5′-tetramethylbenzidine; IC_{50}, concentration of analyte giving 50% inhibition of color development; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 0.05% Tween 20; CB, coating buffer.

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