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ARTICLE

Validation of an Enzyme-Linked Immunosorbent Assay for Detecting Sulfonamides in Feed Resources

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An enzyme-linked immunosorbent assay (ELISA) to screen sulfadiazine and sulfamethazine residues in feeds has been developed and validated according to Commission Decision 2002/657/EC criteria. Sulfonamides are easily extracted with a 95:5 acetonitrile/water mixture, obtaining recoveries between 80 and 100%. Accuracy, precision, selectivity, robustness, limit of detection (LOD), and detection capability (CCβ) of the assay have been assessed during the validation process. LOD values in pig feed samples were 0.2 μg/g for sulfadiazine and 0.04 μg/g for sulfamethazine without any sample treatment other than extraction, dilution with the assay buffer, and filtering of the resulting solution. Furthermore, a new strategy for the determination of CCβ in an ELISA screening method is proposed; this gave CCβ values of 0.8 μg/g for sulfadiazine and 0.1 μg/g for sulfamethazine. Besides sulfadiazine and sulfamethazine, other sulfonamides can be detected with this immunoassay; this has been verified calculating their LOD values and cross-reactivities. Finally, real feed samples were analyzed with the ELISA methodology and a previously developed liquid chromatography (LC) method, and results confirmed the utility of this new immunoassay for screening purposes.

KEYWORDS: Sulfonamides; feed analysis; enzyme-linked immunosorbent assay; liquid chromatography; method validation

INTRODUCTION

Social concern about the threats to food safety has increased as a consequence of several foodstuff crises in the past few years. In 2002, the European Union (EU) created the European Food Safety Authority (EFSA) and implemented a farm-to-table approach in the EU food legislation. Within this context, several debates on the use of antibiotics as growth promoters have taken place (*1*). This led to the banning of these compounds (except coccidiostats and histomonastats) for this specific use from January of 2006, because they were considered to be one of the main causes of the detected increase of resistance to antimicrobial drugs employed in human medicine (*2*, *3*).

Contamination of animal products (i.e., meat, milk, and egg) with antimicrobial residues is potentially harmful to the consumers. For this reason, the Committee for Medicinal Products for Veterinary Use (CVMP) of the European Medicines Agency (EMEA), according to the provisions of Council Regulation 470/2009/EC, establishes maximum residue limits (MRLs) for the veterinary drugs authorized for the treatment of food-producing animals. On an international basis, the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA), designated by the Codex Committee on Residues of Veterinary Drugs, is responsible for the assessment of veterinary drugs in relation to MRLs.

Only a few studies have investigated the relationship between the contamination of animal feeds and the presence of residues in animal products, but awareness that this can be a significant problem is increasing. Feed contamination depends upon several factors, such as human error and production practices in feed mills. Sometimes, feed mills and silos are not fully free of drugs after preparation of a medicated feed, and these drugs are carried over to non-medicated feeds, leading to cross-contamination (usually in the 2−10 mg/kg concentration range (*4*)); moreover, fraudulent use of medicated feeds as growth promoters cannot be discarded.

Sulfonamide chemotherapeutics are among the most widely used veterinary drugs in the EU, and in 2004, they represented about 15−20% of the total amount (*5*); in the U.S.A., however, only 5% of veterinary drugs used in the period 2005−2007 were sulfonamides (*6*). As a result, sulfonamides are among the most frequently identified contaminating antimicrobials in feed resources in the EU (*7*). In Spain, only two sulfonamides, sulfadiazine and sulfamethazine, can be legally used in medicated premixes, and they are mainly used for pig rearing (*8*).

Suitable analytical methodology is required for an efficient control of residues, contaminants, and unauthorized substances in animal feed and, thus, ensures safety in this first stage of the

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food chain. Detection methods for sulfonamide residues are usually based on liquid chromatography (LC) with ultraviolet, fluorimetric, or mass spectrometry detection (*9*−*17*). Most of these confirmatory methods are time-consuming, require qualified staff, and consequently, are not suitable for screening large amounts of samples. Therefore, alternative screening techniques, able to detect positive samples, later to be analyzed by a confirmatory method, are necessary.

Several immunochemical techniques have been developed and used in the past few years to determine sulfonamides in many kinds of matrices, such as pig and chicken muscle, liver, eggs, milk, honey, fish, hair (*18*−*25*), and to a lower extent, feeds. The present paper reports an immunochemical screening method for the detection of sulfonamides in contaminated feed samples that has been validated according to the provisions of Council Decision 2002/657. Performance characteristics [specificity, accuracy, robustness, and detection capability (CCβ)] were determined. Pig feed was selected as the matrix for the validation because, according to data from the European Feed Manufacturers Federation (FEFAC) (*26*), in 2007, these feeds represented 35% of the total production in Europe. Moreover, the applicability of the method to other kinds of feeds (feeds for chicken, calves, hens, piglets, rabbits, and sheep) has also been assessed.

MATERIALS AND METHODS

Apparatus. Microtiter polystyrene plates were purchased from Nunc Maxisorp (Roskilde, Denmark). Washing steps were performed on a SLT Labinstruments GmbH (Salzburg, Austria) SLY96 PW microplate washer. Absorbances were read on a Molecular Devices (Sunnyvale, CA) SpectramaxPlus spectrometer with SoftmaxPro v4.7 software. Competitive curves were analyzed with a four-parameter equation using GraphPad Software, Inc. (San Diego, CA) GraphPad Prism 4 software.

HPLC analyses were performed with a Shimadzu (Kyoto, Japan) system, equipped with a LC-10AD VP quaternary pump, a SIL-10AD VP automatic injector, a SPD-M10A VP diode array detector with deuterium and tungsten lamps, and a RF-10A XL fluorescence detector with a 150 W xenon lamp. The column used was a 150 × 4.6 mm inner diameter, 5 μm, Supelcosil LC-PAH RP-18, with a 2 cm guard column of the same material (Supelco, Bellefonte, PA).

Chemicals and Immunoreagents. Immunochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Preparation of the immunoreagents required for the enzyme-linked immunosorbent assay (ELISA) development, such as the polyclonal antiserum (As155) and the coating antigen (SA2-OVA), is described elsewhere (*24*).

Sulfadiazine, sulfamethazine, sulfachloropyridazine, sulfapyridine, sulfathiazole, sulfamethoxipyridazine, sulfamethoxazole, and sulfamerazine were supplied by Riedel-de Haën (Buchs, Switzerland). Ultrapure water (Milli-Q, Millipore, Molsheim, France) of 18.2 mΩ/cm resistivity was used. All other chemicals and solvents were supplied by Merck (Darmstadt, Germany). Stock solutions of each sulfonamide (10 mmol/L) were prepared in dimethyl sulfoxide and stored at 4 °C.

The pH 7.5 phosphate-buffered saline solution (PBS) contained 2 mmol/L KH_2PO_4 and 8 mmol/L Na_2HPO_4 in a 0.8% saline solution (137 mmol/L NaCl and 2.7 mmol/L KCl). PBST is PBS with 0.05% Tween 20, while 2× PBST is 20 mmol/L PBS with 0.10% Tween 20. The pH 9.6 coating buffer contained 15 mmol/L Na_2CO_3 and 35 mmol/L $NaHCO_3$. The pH 5.5 citrate buffer was prepared from a 40 mmol/L sodium citrate solution, adjusted to the correct pH with acetic acid. The substrate solution contained 0.01% 3,3′,5,5′-tetramethylbenzidine (TMB) and 0.004% H_2O_2 in pH 5.5 citrate buffer.

Feed Samples. Feed samples were provided by the "Associació Catalana de Fabricants de Pinsos" (ASFAC). Some samples were milled, but others were granulated; these were chopped with a domestic chopper. Blank samples were tested to be free of sulfonamides by the LC method described below. Samples spiked with sulfadiazine and sulfamethazine at three concentration levels (500, 50, and 5 μg/g) were prepared as described elsewhere (*14*). Feed samples affected by carry-over contamination were obtained from production lines, where feeds medicated with sulfadiazine had been recently prepared.

Feed Extraction Procedure. Feed samples (1 g) were extracted with 10 mL of a 95:5 acetonitrile (MeCN)/ultrapure water (v/v) mixture by hand-shaking for 1 min in a 25 mL centrifuge tube. The resulting mixture was centrifuged at 3500 rpm for 10 min. In all extraction optimization experiments, the analytes were determined by LC with UV detection.

LC Analysis. Two LC methods for the analysis of sulfadiazine and sulfamethazine in feeds were developed and validated in a previous study (*14*). UV detection was used for samples containing more than 5 μg/g of sulfadiazine and sulfamethazine, while fluorimetric detection (after derivatization with fluorescamine) was used for lower concentration samples. LC analyses with UV detection were carried out after a 1:10 dilution of the extracts with MeCN and 0.01 mol/L acetic acid/acetate buffer (13:87) mobile phase. The resulting solutions were filtered through a 0.45 μm nylon membrane and injected into the chromatographic system. The gradient elution program was as follows: a 13:87 mixture of MeCN and 0.01 mol/L acetic acid/sodium acetate buffer solution was run for 4 min; the proportion was then switched to 10:90; this new mixture was run up to min 10; finally, the eluting solution was returned to its original composition; and the system was re-equilibrated for 5 min. The mobile-phase flow rate was 1 mL/min; the injection volume was 20 μL; and the diode array detector was set at 268 nm. For LC with fluorimetric detection, sulfadiazine and sulfamethazine were derivatized by mixing 1 mL of feed extract or 1 mL of a standard solution in MeCN with 2 mL of formic acid/sodium formiate aqueous buffer solution (pH 3.4) and 1 mL of 0.2% fluorescamine solution in MeCN. The mixture was allowed to stand at least 2 h at room temperature, filtered through a 0.45 μm nylon membrane, and 50 μL was injected into the chromatographic system. An isocratic elution with a 29:71 mixture of MeCN and 0.01 mol/L formic acid/sodium formiate buffer at pH 3.4 was used. The mobile-phase flow rate was 1.2 mL/min. The fluorescence detector was set at 405 nm for λ_{exc} and 485 nm for λ_{em}. All sample extracts were injected in triplicate.

Immunoassay Method. The following steps were required for the ELISA test. Coating step: 100 μL of SA2-OVA (0.625 μg/mL in coating buffer) were placed in each well of a microtiter plate. After the wells were covered with adhesive sealers, the plates were kept overnight at 4 °C and then washed 4 times with PBST (300 μL/well). Competition step: 50 μL of either standard sulfonamide solutions (from 50 000 to 0.0256 nmol/L in ultrapure water containing 6.3% MeCN) or feed extracts (diluted 1:15 with ultrapure water and filtered through a 0.45 μm membrane) were added to each well. The As155 antiserum (diluted 1:8000 with 2× PBST) was then added (50 μL/well), and plates were incubated for 30 min at room temperature, with shaking. Finally, the plates were washed as in the coating step. Second antibody step: An anti-IgG-HRP solution (diluted 1:6000 in 10 mmol/L PBST) was added to the wells (100 μL/well), and the plates incubated for 30 min at room temperature. Finally, the plates were washed 4 times with PBST (300 μL/well). Substrate step: 100 μL of substrate solution were added to each well. Stopping enzymatic reaction step: 50 μL of 2 mol/L H_2SO_4 was added to each well after 30 min of reaction at room temperature to stop color development. Absorbance measurement and data treatment step: Absorbances were measured at 450 nm. Standard curves were fitted to a four-parameter equation: $y = [(A - B)/1 - (x/C)^D] + B$, where A is the maximum absorbance, B is the minimum absorbance, and C is the concentration at the inflection point of the sigmoidal curve, which in many cases corresponds to the IC_{50} value (concentration providing 50% of the maximum signal). For practical reasons, parameter C has been used as the IC_{50} value through the text for comparison of the detection capability reached under the different conditions. Finally, D is the slope at the inflection point of the sigmoid curve. Unless otherwise indicated, all data correspond to the average of at least two replicates.

Cross-Reactivity Study. Only sulfadiazine and sulfamethazine are approved for use in feed premixes in Spain, but fraudulent use of other sulfonamides cannot be discarded. Previous studies with this ELISA (*27*) had shown that it could detect several sulfonamides, and consequently, calibration curves were prepared with structurally related compounds in feed extracts to assess the cross-reactivity in this kind of matrix.

The limit of detection (LOD) for each sulfonamide was calculated as the concentration producing 90% of the maximal absorbance (IC_{90}). The cross-reactivity values (% CR) were calculated according to the equation [IC_{50}(sulfamethazine)/IC_{50}(cross-reactant)] × 100.

Method Validation. Blank feed extracts were spiked in duplicate at six different concentration levels, within the working range for each validated

sulfonamide, and measured in three consecutive days to assess accuracy and precision.

A threshold playing the same role as a decision limit was defined with the criterion provided by Decision 2002/657 to obtain this limit for quantitative assays. Consequently, threshold (abs.) = A − (2.33SD), where A was the average signal of 20 blank feed samples and SD was the standard deviation of 20 blank feed sample signals. CCβ was then calculated as CCβ (abs.) = threshold (abs.) − (1.64SD). Minus signs were required in these equations because, in competitive immunoassays, the higher the analyte concentration, the lower the absorbance of a sample. Both the threshold value and CCβ were obtained in terms of absorbance (or signal) with these equations, and these values were later interpolated in the appropriate calibration curves. The signal was used instead of the concentration, because any blank value higher than the maximum signal of the calibration curve would give a negative concentration. Analyses were performed 4 times on different days.

To check that all blank absorbances were higher than the threshold value, thus ensuring 0% of false non-compliant results, and that β error was lower than or equal to 5% (percentage of false complaints), assays were performed on different days using 20 blank feed extracts, 6 extracts spiked at the threshold values for sulfadiazine and sulfamethazine, and 20 blank feed extracts spiked at the CCβ levels for sulfadiazine and sulfamethazine.

The robustness of the method was tested introducing minor variations in optimal conditions and observing the effects on the results (*28*). This was performed using the Youden approach (*29*,*30*). The MeCN concentration in the extracting mixture, the shaking time in the extraction step, the dilution factor of sample extracts, the incubation time in the competition step, the pH of the buffer in the competition step, and the incubation temperature in the competition step are likely to affect the robustness of the method and were selected for testing. Each selected factor was slightly modified around the optimal value. For each factor, the averages of the results for high and low levels were calculated and the difference of these averages was considered to be the effect for each factor. The influence of each factor in the final result was assessed by comparing the calculated effect to a critical effect value (*31*).

RESULTS AND DISCUSSION

The ELISA described in this study uses immunoreagents (As155/SA2-OVA) previously developed and optimized for the analysis of sulfonamides in milk (*24*) and hair (*27*,*32*) on different detection platforms. Checkerboard titration experiments were used to select the appropriate concentrations of the immunoreagents to be employed in the competitive experiments; optimal concentrations were 0.625 μg/mL SA2-OVA and 1:8000 dilution for As155 antiserum.

Feed Extraction Procedure. In a previous study (*14*), a microwave-assisted extraction (MAE) with MeCN was used to extract sulfonamides from pig feeds; recoveries of 104% for sulfadiazine and 89% for sulfamethazine were obtained. For a rapid screening method, however, a simpler and faster extraction procedure is desirable and mechanical shaking was tested as an alternative. Feed fractions (1 g), spiked with sulfadiazine and sulfamethazine at 50 μg/g, were treated with 10 mL of the extracting solution for 60 min in a rotary mixer. Pure MeCN was initially used, because it had given good results in MAE, but it was observed that the addition of small percentages of water (1, 5, and 10%) improved sulfonamide recoveries. Best results were obtained with 10 mL of 95:5 or 90:10 MeCN/ultrapure water, because recoveries were high and only small amounts of matrix components were extracted (**Figure 1**); increasing water contents beyond 10% only increased extraction of matrix components. Consequently, a 95:5 MeCN/ultrapure water mixture was selected as the extracting solvent; the effect of shaking time was also evaluated, and good recoveries were obtained after shaking for only 1 min, which made the rotary mixer unnecessary. The final extraction procedure consisted of manually shaking 1 g of feed with 10 mL of 95:5 MeCN/ultrapure water for 1 min in a 25 mL centrifuge tube;

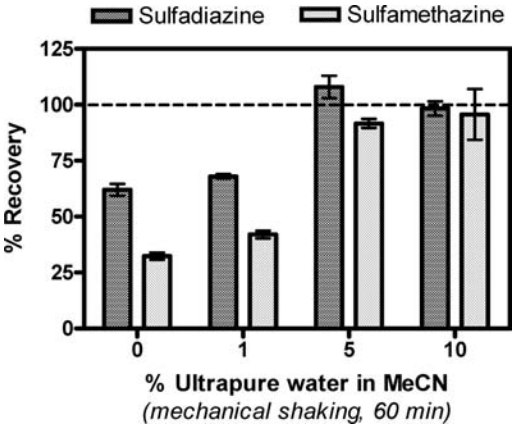

Figure 1. Effect of the percentage of water added to MeCN on recoveries for sulfadiazine and sulfamethazine in spiked feed samples (50 μg/g).

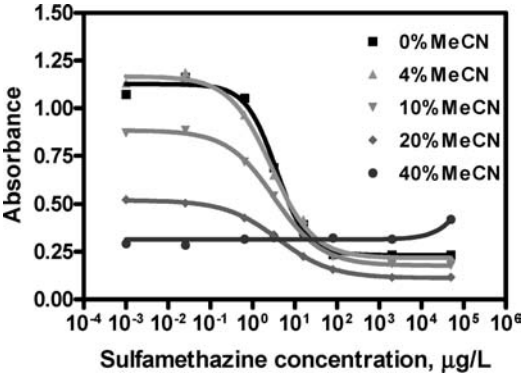

Figure 2. Effect of the MeCN percentage on the As155/SA2-OVA immunoassay for sulfamethazine analysis.

recoveries in these conditions were 102% for sulfadiazine and 80% for sulfamethazine.

Effect of the Organic Solvent and Matrix Components on ELISA. ELISA is usually inhibited by organic solvents. For this reason, the effect of different MeCN concentrations on the immunoassay performance was evaluated by preparing and running sulfadiazine and sulfamethazine calibration curves containing different percentages of MeCN (0, 4, 10, 20, and 40%). Results shown in **Figure 2** indicate that even relatively low percentages of MeCN adversely affected the assay for sulfamethazine, and the same behavior was observed for sulfadiazine. A 10% MeCN percentage was accepted as the maximum permissible concentration; this percentage still had a non-negligible influence, but this could be corrected by calibration curves obtained with standards containing the same percentage of MeCN as the samples. Feed extracts contained a much higher percentage of MeCN, and they had to be diluted with ultrapure water (1:10) prior to ELISA analysis.

To assess the influence of co-extracted matrix components, sulfamethazine calibration curves were prepared with blank feed extracts diluted 1:10 with ultrapure water and compared to curves obtained from standards in PBST buffer containing the same MeCN percentage. A slight matrix effect was observed in these conditions, but it was removed by filtration of the diluted feed extract through a 0.45 μm nylon membrane before preparation of the calibration standards. In these conditions, calibration curves prepared with feed extracts and 90:10 PBST/MeCN mixtures were virtually identical. These results, however, were not reproducible in sets of assays performed on different days. For this reason, higher dilutions (1:15 and 1:20) were tested. No matrix

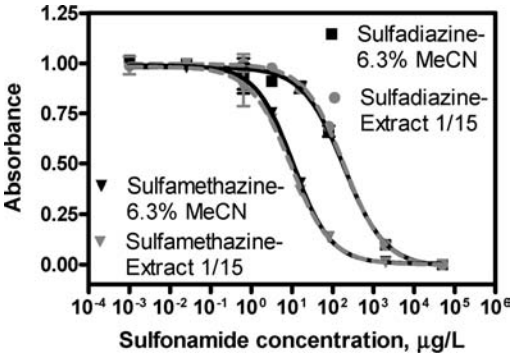

Figure 3. Calibration curves for the detection of sulfadiazine and sulfamethazine in feed extracts compared to a calibration curve run in a buffer containing the same MeCN percentage.

Table 1. Cross-Reactivity (% CR) and LOD of Related Sulfonamide Compounds in the Developed Assay in a Pig-Feed Matrix

compound	LOD (μg/g)	% CR
sulfamethazine	0.03	100
sulfadiazine	0.12	12
sulfachloropyridazine	0.21	5
sulfapyridine	0.004	180
sulfathiazole	0.02	128
sulfamethoxipyridazine	0.01	54
sulfamethoxazole	ND	0.1
sulfamerazine	0.05	44

effects were observed when sulfadiazine and sulfamethazine calibration curves prepared with extracts diluted 1:15 or 1:20 with ultrapure water and filtered were run on different days. Consequently, a feed extract diluted 1:15 with ultrapure water and filtered through a 0.45 μm nylon membrane was considered to be suitable, because it avoided matrix effects and organic solvent influence. **Figure 3** shows the standard calibration curves for sulfadiazine and sulfamethazine obtained with standards prepared in pig feed extracts and PBST/MeCN buffer. Comparable results were obtained when feeds for chicken, calves, hens, piglets, rabbits, and sheep were tested. IC_{50} values, corresponding to the average of six assays performed on different days using data from two microplate wells, were 4.4 μg/g for sulfadiazine and 0.54 μg/g for sulfamethazine. From these assays, working ranges obtained for sulfadiazine and sulfamethazine were, respectively, 0.7−28 and 0.11−2.6 μg/g.

Cross-Reactivity Study. Calibration curves in feed extract were prepared with eight sulfonamides to assess the cross-reactivity in this kind of matrix. Only one of the eight sulfonamides tested, sulfamethoxazole, was not detected. Sulfapyridine showed the maximum sensitivity, with a LOD of 0.004 μg/g in feed, while sulfachloropyridazine had the highest LOD (0.21 μg/g). **Table 1** shows the LOD in feed for all of the sulfonamides tested and also the cross-reactivity values. Low concentration levels of sulfonamides could be detected with this assay, making use of the sulfamethazine reference calibration curve; minimum detectable concentration values in feed would be 0.24 μg/g for sulfadiazine, 0.66 μg/g for sulfachloropyridazine, 0.02 μg/g for sulfapyridine, 0.02 μg/g for sulfathiazole, 0.06 μg/g for sulfamethoxipyridazine, 29 μg/g for sulfamethozaxole, and 0.07 μg/g for sulfamerazine.

The method is highly selective against other veterinary drugs, because fluoroquinolones, tetracyclines, β-lactams, and trimetoprim do not interfere.

Method Validation. Blank feed extracts were spiked in duplicate at six different concentration levels to assess accuracy and precision. Measured concentrations on different days were plotted versus spiked sulfadiazine and sulfamethazine concentrations. The obtained correlation equations were $y = 0.991x + 3.861$ ($R = 0.992$) for sulfadiazine and $y = 1.003x + 0.149$ ($R = 0.991$) for sulfamethazine, indicating good accuracy of the method. Between-day precisions at concentrations about IC_{50} were 25%, and repeatability precisions were 10%.

Commission Decision 2002/657/EC does not require the evaluation of the decision limit (CCα) for the validation of a screening method, because any non-compliant sample will be verified by a confirmatory methodology. Consequently, only the CCβ must be determined. A literature survey provided only a few papers where CCβ for an ELISA was calculated (*29*, *33*−*37*), and moreover, several different approaches were proposed. Furthermore, MRLs have been set for sulfonamide residues in tissues (100 μg/g), but no limits have been established for them in feeds. In fact, sulfadiazine and sulfamethazine are unauthorized substances in non-medicated feeds, and consequently, concentrations should be zero. A threshold above which a sample is considered to be non-compliant is necessary, and therefore, a threshold concentration (the term CCα is usually avoided in these cases) is used to decide the samples requiring confirmatory analysis. Several criteria have been used for the definition of the threshold concentration: the LOD (average of 20 blanks plus 3 standard deviations) (*34*), the concentration corresponding to the highest blank signal (*36*, *37*), and the concentration corresponding to the lowest signal obtained with samples spiked at an arbitrary concentration level below the established minimum required performance limit (MRPL) (*33*); CCβ is then set as a concentration above the threshold for which the β error is lower than 5%.

In this study, a new and suitable procedure to obtain CCβ for an ELISA was developed. The threshold was first evaluated by performing analyses of 20 blank feed samples 4 times on different days; threshold values of 0.4 μg/g for sulfadiazine and 0.06 μg/g for sulfamethazine were obtained. These values were slightly higher than the corresponding LOD of the method, calculated as the concentration giving 90% of a blank absorbance. The absence of false non-compliant results was assured, as shown in **Figure 4A** for sulfadiazine, performing assays of 20 blank feed extracts and 6 extracts spiked at the threshold values for sulfadiazine and sulfamethazine on different days.

These threshold values were then used to obtain CCβ by means of the approach proposed in this paper. Calculated CCβ values were 0.8 μg/g for sulfadiazine and 0.1 μg/g for sulfamethazine; these values were similar to the corresponding IC_{80} values of the ELISA method. No false compliants were obtained for sulfadiazine (**Figure 4B**) and sulfamethazine when 20 blank feed extracts were spiked at the CCβ levels for each sulfonamide and another 6 extracts at the corresponding threshold levels and the assay was performed on different days.

The reliability of this new strategy for the determination of CCβ of ELISA methods for unauthorized compounds with no MRPL was demonstrated, because no false non-compliant results were found at the threshold level and no false compliant results were found at the calculated CCβ level, after 20 replicate measurements. In these cases, as low as possible, a CCβ must be reached. In contrast, the only requirement for compounds with a MRPL is that the ratio of false compliants at an arbitrarily chosen concentration below the MRPL must be lower than the limit fixed by European legislation.

The study of the method robustness, in which the Youden approach was used, concluded that the buffer pH and the incubation temperature in the competition step had a significant influence on the final result, and therefore, these factors must be strictly controlled.

Analysis of Feed Samples. Analysis of real samples was carried out in triplicate with the newly developed ELISA methodology.

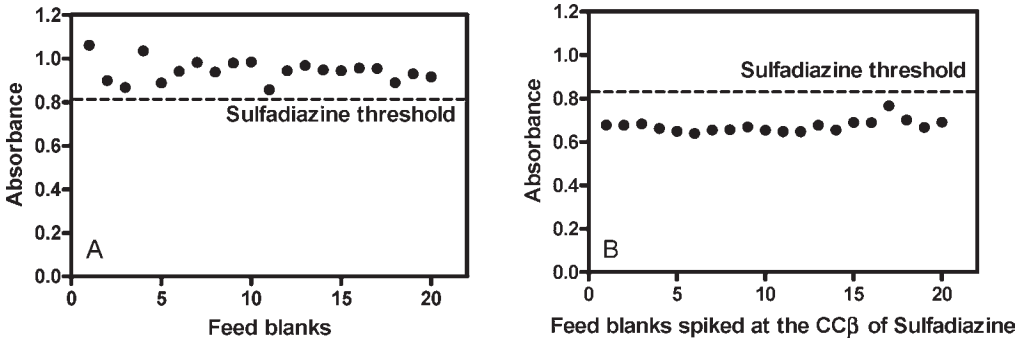

Figure 4. (A) No false non-compliant verification for sulfadiazine. (B) No false compliant verification for sulfadiazine.

Table 2. Results Obtained by LC and ELISA for Feed Samples

	LC		ELISA	
sample	μg of sulfadiazine/g of feed	% RSD	μg of sulfadiazine equiv/g of feed	% RSD
S1	3.3	4	4.2	25
S2	0.82	8	0.84	22
S3	8.7	5	7.7	12
S4	0.85	8	0.77	14
S5	53	9	45	7
S6	415	5	498	19
S7	5559	5	5177	24

Samples S1, S2, S3, and S4 were provided by a local Association of Feed Producers (ASFAC); they consisted of feeds for pigs, piglets, sheep, and calves, respectively, cross-contaminated with sulfadiazine. Concentrations of sulfadiazine in samples S1, S2, S3, and S4 (determined by the LC method with fluorimetric detection) were between 0.8 and 9 μg/g. As shown in **Table 2**, there was a good agreement between the results obtained with the ELISA test and the LC method for these samples.

Samples S5, S6, and S7 were prepared with a sulfonamide-free pig feed spiked with sulfadiazine and sulfamethazine at different concentration levels (5, 50, and 500 μg/g, respectively). ELISA results were given as sulfadiazine immunoreactivity equivalents, and immunochemical response of the sulfonamide present in the sample expressed in relation to the response of sulfadiazine was used as a reference for quantization in this assay. Results obtained with the LC method were converted to equivalents of sulfadiazine per gram of feed by means of the cross-reactivity value. No significant differences were observed between ELISA and LC results (**Table 2**).

Therefore, analyses of real samples demonstrate the good performance of this new immunoassay method.

In conclusion, results presented here show that this ELISA method is able to detect different sulfonamides in feed resources with good accuracy and reliability, which makes it a useful tool for screening purposes. Only a few ELISA methods have been proposed for the detection of antimicrobials in feeds (*35*, *38*). The method proposed in the present paper can be used for seven sulfonamides and is very simple and sensitive. Extraction by manually shaking for 1 min gives high recoveries. Additionally, no cleanup is required, because a simple dilution of the extracts suffices to avoid matrix interferences and to make the detection of the analytes possible; this is a significant advantage for a rapid screening method.

Supporting Information Available: Factorial design for robustness evaluation and features of sulfadiazine and sulfamethazine ELISA methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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