JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Immunochemical Assays for Direct Sulfonamide Antibiotic Detection In Milk and Hair Samples Using Antibody Derivatized Magnetic Nanoparticles

HÉCTOR FONT,[†,§] JAVIER ADRIAN,[†,§] ROGER GALVE,[†] M.-CARMEN ESTÉVEZ,[†] MASSIMO CASTELLARI,[‡] MARTA GRATACÓS-CUBARSÍ,[‡] FRANCISCO SÁNCHEZ-BAEZA,[†] AND M.-PILAR MARCO*[,†]

Applied Molecular Receptors Group (AMRg), IIQAB-CSIC, CIBER of Bioengenieering, Biomaterials, and Nanomedicine, Jorge Girona, 18-26, 08034, Barcelona, Spain and Institute for Food and Agricultural Research and Technology, Meat Technology Centre (IRTA-CTC) - Food Chemistry Unit, Granja Camps i Armet s/n, Monells 17121, Girona, Spain

Two direct enzyme-linked immunosorbent assays (ELISAs) have been developed for detection of sulfonamide antibiotic residues in milk samples. One of them is using magnetic nanoparticles (MNP) for target capture/enrichment (Ab-MNP-ELISA), and the second is performed using microtiter plates. Selective polyclonal antibodies, raised against 5-[6-(4-amino-benzenesulfonylamino)-pyridin-3-yl]-2-methyl-pentanoic acid (SA1), used in combination with an enzyme tracer prepared with the same hapten, has allowed us to reach a limit of detection (LOD) lower than 0.5 μg L^{-1} for both ELISA formats. Sulfapyridine, sulfamethoxypyridazine, sulfathiazole, and sulfachloropyridazine are detected below the maximum residue limits established by the European Union for these antibiotics in milk (100 μg L^{-1}). Matrix effects and accuracy studies performed with full-cream milk and hair extracts indicated a lack of interference from these sample matrices and very good recovery values, especially when using the Ab-MNP format. Milk samples and hair extracts can be measured without any previous treatment. The results demonstrate the high potential of these methods as screening tools for food safety and inspection controls.

KEYWORDS: Sulfonamide antibiotics; sulfapyridine; immunoassay; magnetic particles; milk; hair

INTRODUCTION

Following their 20th century triumph in human medicine, antimicrobials have also been increasingly used for the treatment of bacterial diseases in animals, fish, and plants. In addition, they became an important element for intense animal husbandry because of their observed growth-enhancing effect, when added in subtherapeutic doses to animal feed. However, the widespread use of antimicrobials outside human medicine is the cause for the alarming emergence in humans of bacteria that have acquired resistance to antimicrobials. This situation is causing a serious threat to the public health, as more and more infections can no longer be treated with the presently known antidotes (*1, 2*). However, although the amount of antimicrobials used in food animals is not known precisely, it is estimated that about half of the total amount of antimicrobials produced globally is used in food animals. The increase in meat production in many developing countries is mainly due to intensified farming, which is often coupled with increased antimicrobial usage for both disease therapy and growth promotion. Some of the newly emerging resistant bacteria in animals are transmitted to humans, mainly via meat and other food of animal origin or through direct contact with farm animals. The best-known examples are the foodborne pathogenic bacteria *Salmonella* and *Campylobacter* and the commensal (harmless in healthy persons and animals) bacteria *Enterococcus*.

Sulfonamides are an important group of antimicrobials, whose chemical structure contains a 4-aminobenzensulfonamide functionality with different heterocycles attached to the N1-position of the sulfonamide bridge (see **Figure 1**). During the last decades, sulfonamide residues have been detected at significant concentrations levels in many biological samples, such cow's milk (*3–5*). To safeguard the public, maximum residues limits (MRL) of sulfonamides have been established in different matrices. The European Union, Canada, and the USA have stipulated MRLs of 100 μg Kg^{1-} for total sulfonamides in edible tissues (*6, 7*). On the other hand, antibiotic residues in milk samples is the cause of important economical losses in the dairy products industry because of the inhibition caused in the fermentation processes (*8*).

* To whom correspondence should be sent: Phone: 93 4006100Ext. 415; fax: 93 2045904; e-mail: mpmqob@iiqab.csic.es.
† Applied Molecular Receptors Group.
§ These authors contributed equally to this work.
‡ Institute for Food and Agricultural Research and Technology.

10.1021/jf072550n CCC: $40.75
Published on Web 01/05/2008

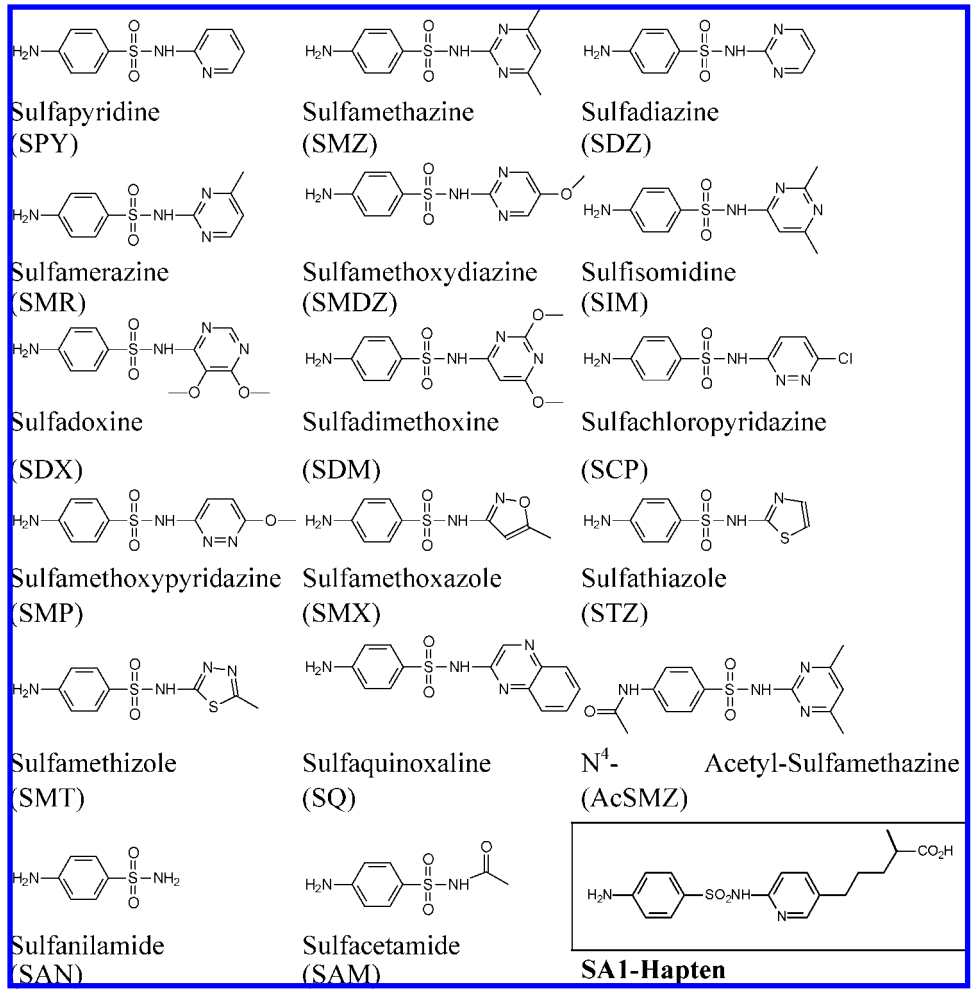

Figure 1. Chemical structures and abbreviations of some of the most common sulfonamides. The selected immunizing hapten (SA1) is also shown.

Table 1. Features of the Three Immunoassays Developed against Sulfapyridine[a]

	As154/SA1-HRP	As167/SA1-HRP	Ab167-MNP/SA1-HRP
A_{max}	1.04 ± 0.08	0.71 ± 0.23	0.86 ± 0.35
A_{min}	0.04 ± 0.01	0.05 ± 0.03	0.07 ± 0.01
IC_{50}, $\mu g\ L^{-1}$	11.21 ± 2.88	5.35 ± 2.40	8.37 ± 2.10
dynamic range, $\mu g\ L^{-1}$	1.6 ± 0.5 to 72.8 ± 23.7	0.7 ± 0.3 to 36.8 ± 16.5	1.2 ± 0.3 to 57.3 ± 21.1
LOD, $\mu g\ L^{-1}$	0.44 ± 0.13	0.22 ± 0.11	0.38 ± 0.12
slope	−0.75 ± 0.05	−0.69 ± 0.07	−0.76 ± 0.14
R^2	0.972	0.985	0.974
N	6	4	6

[a] Data correspond to the average and standard deviation of the parameters extracted from the logistic equation used to fit the standard curves. Assays were performed using three-well replicates on different days.

Usually, sulfonamide antibiotic residues are analyzed in samples such as milk, liver, meat, eggs, and feedstuff. However, recently, other animal target samples such as hair are being seriously considered to be taken and used to control misuse of contaminants in animals due to their lower metabolic and degradation activity as compared with conventional biological samples. This point could be very attractive for the official control organizations because hair analysis could increase the time window in retrospective detection of veterinary drug residues. Over the last 20 years, hair testing has gained increasing attention and recognition for the retrospective investigation of chronic drug abuse as well as intentional or unintentional poisoning (for reviews see refs *9–13*). Originally used to evaluate human exposure to heavy metals, hair analysis is used for criminal court proceedings (*9, 14*), clinical purposes (*15*), and doping control (*16–19*). Whereas urine analysis allows the detection of residues during a period ranging from several hours to 2–3 weeks, hair testing permits long-term detection, sometimes up to several months. Deposition of anabolic hormones (*16, 19*) and β-agonists (*20*) was confirmed in bovine hairs. The accumulation of different antibiotics, such as fluoroquinolones and sulfonamides, particularly in racehorse hair (*15, 21*), has also been reported. Recently, some of us have described that deposition of fluoroquinolones and sulfonamides antibiotics also occurs in cattle and pig hair after a veterinary administration of these drugs (*22, 23*).

Traditionally, most of the tests used to detect antibiotic residues have taken advantage of their antibacterial activity. Thus, growth inhibition tests have been used in different animal matrices; however, they are time-consuming and no conclusion may be drawn about the identity of the antibiotic or its concentration (*24*). Other methods for the analysis of sulfona-

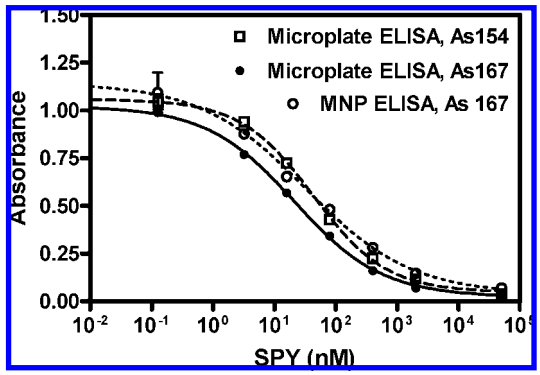

Figure 2. Standard curves of the microplate (As154/SA1-HRP and As167/SA1-HRP) and Ab-MNP-ELISAs (Ab167/SA1-HRP) using SPY as standard analyte. The curves were prepared using three-well replicate. The features of the assays are summarized in **Table 1**.

mides, such as HPLC (*25–27*) and GC (*28*), are highly specific techniques but require extensive sample preparation, sophisticated equipment, and skilled laboratory personnel, thereby limiting their use as fast and efficient high-throughput screening (HTS) methods. As an alternative, immunochemical techniques can be excellent HTS tools for residue analysis in different matrices because of their high detectability and specificity. Attending to it, the main objective of this work was the establishment of a rapid and reliable immunochemical method for the detection of sulfonamides in complex biological samples while avoiding sample preparation methods.

Several monoclonal (*29–32*), polyclonal (*33–35*), and recombinant (*36–38*) antibodies against sulfonamide antibiotics with different recognition patterns versus the different congeners have been reported. However, in very few occasions, direct performance of these antibodies in complex biological samples has been demonstrated. Thus, for tissue immunochemical analysis, several procedures involving different extraction/cleanup methods have been reported (i.e., refs *39 and 40*.). Direct analysis of honey samples by enzyme-linked immunosorbent assays (ELISAs) has also been attempted, but high dilution factors (100 times) had to be applied to avoid matrix interferences. Alternatively, solid-phase extraction methods have been introduced prior the immunochemical analysis. Regarding milk, homogenization and protein removal, by precipitation with trichloroacetic acid or acetone followed by neutralization or dilution, have been some of the sample preparation methods reported to analyze this sample by immunoassay (*36, 41*). Antibody features strongly determine the extent of these undesired effects. On the other hand, it has been reported that the use of magnetic beads may assist in minimizing matrix interferences, increasing efficiency of washing and separation steps, besides improving performance due to an increase in the surface area and the faster assay kinetics, because the immunoreaction takes place in a suspension. In this paper, we describe antibody production by using suitable immunizing hapten combined with the use magnetic nanoparticles with the objective to achieve direct immunochemical detection of sulfonamide antibiotics in milk and hair samples.

MATERIALS AND METHODS

Chemicals and Supplies. All the sulfonamides used in this work were from Riedel-de Haën (Seelze, Germany). The synthesis of the hapten SA1 (5-[6-(4-amino-benzenesulfonylamino)-pyridin-3-yl]-2-methyl-pentanoic acid), will be described elsewhere. Horseshoe crab hemocyanin (HCH), bovine serum albumin (BSA), horseradish peroxidase (HRP), and other biochemical reagents were from Sigma Chemical Co. (St. Louis, Missouri). Magnetic nanoparticles (MNP) modified with carboxyl groups (MP−COOH) with 196 nm of diameter and active chemical functionality of 0.155 mmol g^{-1} were purchased from Estapor (Product No. 00–39, Merck). The filters were Millex-GV 0.22 μm filters (Millipore Corp., Bedford, Massachusetts). Polystyrene microtiter plates used in the ELISA were purchased from Nunc (Maxisorp, Roskilde, Denmark). In the Ab-MNP-ELISA, round-bottom well low-binding microtiter plates were used (Product 900010, U-form individually wrapped, Daslab).

Buffers and Solutions. Unless otherwise indicated, phosphate-buffered saline (PBS) is 0.01 M phosphate buffer and 0.8% saline solution, pH 7.5. PBST is PBS with 0.05% Tween 20. Borate buffer is 0.2 M boric acid/sodium borate, pH 8.7. Coating buffer is 0.05 M carbonate/bicarbonate buffer, pH 9.6. Citrate buffer is a 0.04 M solution of sodium citrate, pH 5.5. The substrate solution contains 0.01% TMB (3,3′,5,5′-tetramethylbenzidine) and 0.004% H_2O_2 in citrate buffer.

Instrumentation. ^1H and ^{13}C NMR spectra were obtained with a Varian Inova 500 spectrometer (500 MHz for ^1H and 125 MHz for ^{13}C) (Varian Inc. Palo Alto, California). Thin-layer chromatography (TLC) was performed on 0.25 mm, precoated silica gel F_{254} on aluminum sheets (Merck, Darmstadt, Germany). HPLC analysis was performed with a Merck Hitachi pump L-7100, a diode array detector L-7455, an autosampler L-7200, and an interface D7000 (Merck, Darmstadt, Germany). The matrix-assisted laser desorption ionization-time-of-flight mass spectrometer (MALDI-TOF-MS) used for analyzing the protein conjugates was a Perspective BioSpectrometry Workstation provided with the Voyager-DE-RP software. Washing steps were performed on a SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). Absorbances were read on a SpectramaxPlus (Molecular Devices, Sunnyvale, California). For the Ab-MNP-ELISA, Dynal MPC-S (Product No. 120.20, Dynal Biotech ASA, Norway) or 96-well plate magnetic separation racks (CD1001, Cortex Biochem, CA, USA) were used. The oven employed for the hair alkaline digestion was from Digitronic (JPSelecta, Abrera, Spain).

Immunoreagents. The *haptenized proteins* (SA1-HCH, SA1-BSA, and SA1-HRP) were prepared by covalently coupling the hapten SA1 to the proteins using the active ester method. Briefly, SA1 (10 μmol), N-hydroxysuccinimide (NHS) (25 μmol), and 1,3 dicyclohexylcarbodiimide (DCC) (50 μmol) were left to react in 200 μL of dry DMF at room temperature (RT) for 3 h. The suspension was centrifuged for 10 min at 10 000 rpm, and the supernatant was slowly added to a solution of the protein (10 mg of protein or 2 mg of HRP in 1.8 mL of borate buffer. The mixture was left to react for 3 h at RT. The conjugates were purified using a Sephadex G-25 desalting column and were stored at 4 °C. Stock solutions of the conjugates (1 mg mL^{-1} in PBS) were stored at −20 °C and working aliquots at 4 °C. The hapten density of the conjugates was assessed by MALDI-TOF-MS by comparing the molecular weight of the bioconjugates with those intact proteins. *Antibodies* As155–157 and As167 were raised against SA1-HCH using the active ester method. Four female New Zealand white rabbits, weighting 1–2 Kg, were immunized following the immunizing protocol already described (*42*). The evolution of the antibody titer was assessed by measuring the binding of serial dilutions of the antisera to a microtiter plate coated with SA1-BSA. After an acceptable antibody titer was observed, the animals were exsanguinated and the blood was collected on vacutainer tubes provided with a serum separation gel. Antisera were obtained by centrifugation and stored at −40 °C in the presence of 0.02% NaN_3. *Antibody derivatized magnetic nanoparticles* (Ab-MNP) were prepared by coupling a purified fraction of immunoglobulins, prepared by ammonium sulfate precipitation (*43*) from As167, to magnetic beads. A solution (2.5 mL) of 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide (EDC, 45 mg, 0.23 mmol) and NHS (27 mg, 0.23 mmol) in PBS (3 mL, pH 6) was mixed with a suspension of MNP (250 μL, 50 mg mL^{-1}), previously washed twice with PBS (pH 6), and the mixture was shaken for 15 min at RT. The magnetic beads were then washed with HCl (2 mM, 1 mL) and resuspended in PBS (20 mM pH 7.5, 1.5 mL). Then, a PBS solution of the antibody (5 mg mL^{-1}, 71 μL) was added to the suspension of activated MNP, and the reaction was allowed to proceed for two hours at RT with gentle mechanical shaking to minimize the sedimentation of beads. The particles were washed again three times with PBS (20 mM, pH 7.5)

Table 2. Selectivity of As154/SA1-HRP and As167/SA1-HRP Direct ELISA[a]

	microplate ELISA				Ab-MNP ELISA	
	As154/ SA1-HRP		As167/SA1-HRP		Ab167/SA1-HRP	
sulfonamide	IC_{50}[b]	% CR	IC_{50}[b]	% CR	IC_{50}[b]	% CR
sulfapyridine (SPY)	11.7	**100**	4.6	**100**	6.3	**100**
sulfaquinoxaline (SQX)	933.7	2	547.5	1	≫MRL	<0.1
sulfachloropyridazine (SCP)	94.6	**14**	88.8	**6**	79.7	**8**
sulfamethoxazole (SMX)	1298.3	1	204.7	2	≫MRL	<0.1
sulfisomidine (SID)	972.5	1	>13916	<0.01	≫MRL	<0.1
sulfathiazole (STZ)	36.4	**33**	97.7	**5**	7.9	**80**
sulfadiazine (SDZ)	779.6	2	344.7	1	≫MRL	<0.1
sulfadimethoxine (SDM)	1752	1	646.8	1	390.9	2
sulfamerazine (SMR)	649.9	2	616.4	1	133.2	5
sulfadoxine (SDX)	>15516	<0.1	976.6	1	≫MRL	<0.1
sulfamethoxypyridazine (SMP)	26.8	**49**	8.5	**61**	15.7	**40**
sulfamethazine (SMZ)	>13916	<0.1	1623.2	0.3	≫MRL	<0.1
N^4-acetyl-sulfamethazine	>16005	<0.1	1132.8	1	≫MRL	<0.1
sulfanilamide (SIA)	>8600	2	401.2	1	≫MRL	<0.1

[a] The percentage of recognition has been expressed as cross reactivity (CR%) according to the expression [IC_{50}(SPY)/IC_{50}(crossreactant)] × 100. Chemical structures of the different sulfonamides are shown in **Figure 1**. [b] Concentrations are expressed in $\mu g\ L^{-1}$.

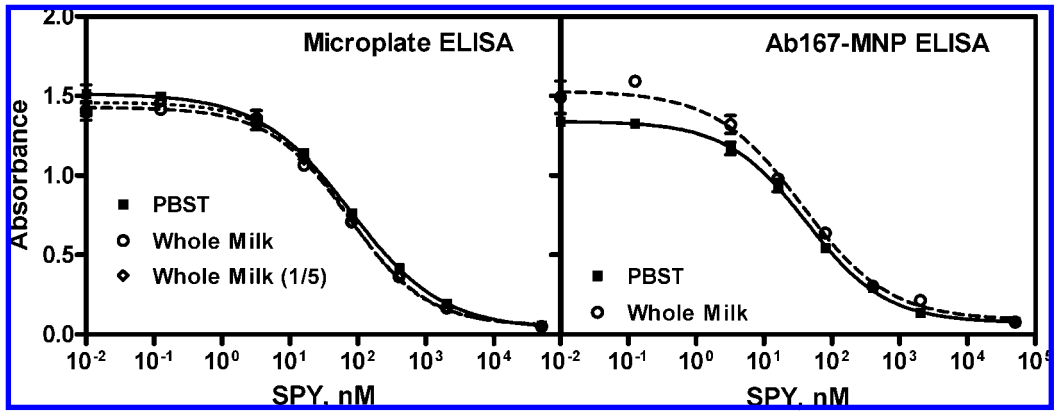

Figure 3. Matrix effect produced by full-cream fresh milk on the As167/SA1-HRP ELISA (left panel) and Ab-MNP-ELISA (right panel). Milk was directly used without including any previous pretreatment and was measured undiluted or after 5× dilution with the assay buffer. Curves were constructed in milk using SPY as standard. The points are the average of three-well replicates.

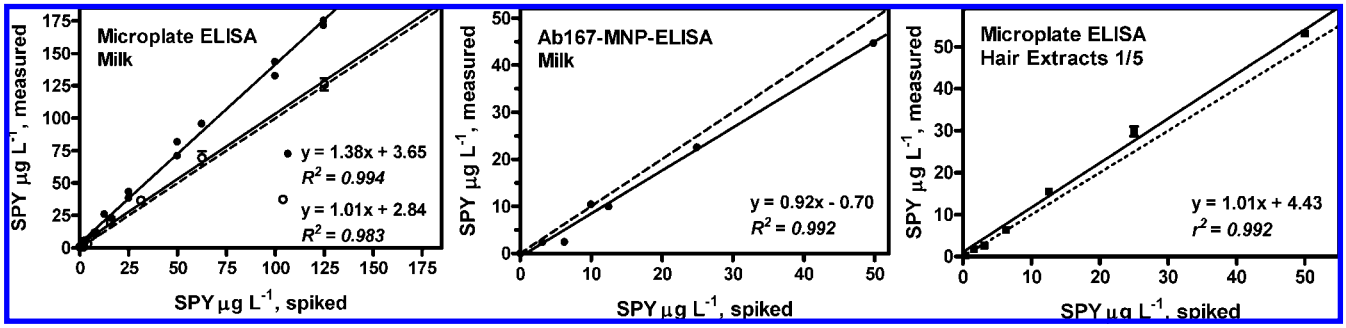

Figure 4. Accuracy studies performed using blind spiked samples prepared in full-cream fresh milk and used directly (solid symbols) in the ELISAs or after 5× dilution with the assay buffer (open symbols) with the As167/SA1-HRP microtiter-plate (left panel) and Ab-MNP (center panel) ELISAs. Accuracy of As167/SA1-HRP microtiter-plate ELISA measuring SPY in hair extracts is also shown (right panel). Blind spiked samples were prepared with the pigmented calf hair extracts. Before ELISA measurements, the extract was buffered and diluted 5× with the assay buffer. In all cases, graphics show the correlation between the spiked concentrations and the values measured. The data shown correspond to the average of at least two well replicates. The dotted line corresponds to a perfect correlation (slope = 1).

and finally resuspended in the same buffer containing 100 mM Glycine, 0.05% (w/v) BSA, and 0.02% NaN_3 to reach a 50 mg mL^{-1} stock solution of Ab167-MNP. The efficiency of the coupling was evaluated using the Bradford test (*44*), analyzing the protein concentration in the supernatant before and after the coupling reaction, using pure IgG to construct the standard curve.

Checkerboard Titration Experiments. The avidity of the antibodies for the enzyme tracer SA1-HRP was determined using two-dimensional (2D) checkerboard titration experiments by measuring the binding of serial dilutions of the enzyme tracer SA1-HRP (1−0.015 $\mu g\ mL^{-1}$ and zero in PBST) to microtiter plates coated with 12 different dilutions of the antisera (1/1000–1/1024000 in coating buffer). After 30 min of incubation at RT, the plates were washed four times with PBST, and the substrate solution (100 μL/well) was added and incubated for 30 min more before stopping the reaction with H_2SO_4 4N (50 μL/well) and reading the absorbances at 450 nm. For the Ab-MNP, a solution of Ab167-MNP (2 mg mL^{-1} in PBST) was prepared, sonicated, and washed three times with PBST. Solutions (50 μL/well) of Ab167-MNP

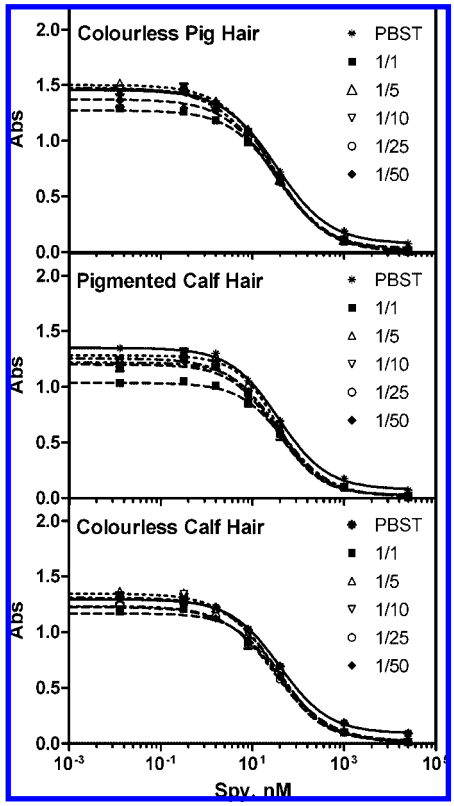

Figure 5. Matrix effect produced by extracts prepared using different types of hair (pigmented or colorless) from calf or pig in the microtiter plate ELISA. Samples were obtained from untreated controlled animals. Aqueous extracts were prepared with 0.1 M NaOH or 0.2 M NH_4OH. Before ELISA measurements, the extracts were buffered and diluted with the assay buffer several times. Curves were constructed using SPY as standard. The points are the average of three-well replicates.

(2–0.031 mg mL^{-1} in PBST) were added to wells of a 96 round-bottom well microtiter plate containing different concentrations of SA1-HRP (1–0.015 μg mL^{-1} in PBST, 50 μL/well) and incubated for 30 min at RT and under slight shaking. The Ab167-MNP were separated and washed three times with PBST (100 μL/well) using a magnetic separation rack. Then, the substrate was added, and the absorbances were read as described above. In both formats, optimal concentrations were chosen to produce absorbances around 0.7–1 units of absorbance under nonsaturating conditions.

Direct ELISA. *Microtiter Plate-ELISA.* The microtiter plates were coated with the antisera appropriately diluted in coating buffer (100 μL/well) overnight at 4 °C and covered with adhesive plate sealers. The day after, the plates were washed (four times with PBST 300 μL/well), and the sulfapyridine standard solutions (0.125 nM −50 μM and zero in PBST, 50 μL/well) followed by SA1-HRP appropriately diluted in PBST (50 μL/well) were added; the plates were then incubated for 30 min at RT. The plates were washed, and the substrate solution was added (100 μL/well). The plates were incubated for 30 min protected from light at RT before the enzymatic reaction was stopped by adding H_2SO_4 4N (50 μL/well). The absorbances were read at 450 nm. The standard curve was fitted to a four-parameter logistic equation according to the formula $y = (A - B/[1 - (X/C)D] + B$, where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve. To improve the immunoassay features, using the best antiserum/enzyme tracer combination, a set of experimental parameters (detergent concentration, ionic strength, length of the competition time, and preincubation effect) were sequentially studied as previously described (*45*).

Ab-MNP-ELISA. The competitive experiments were performed in a similar way as described above. The Ab167-MNP suspension (0.25 mg mL^{-1}, 50 μL/well) was first added to the microtiter plate, followed by the standard solutions of SPY (from 50 μM to 0.125 nM and zero, 25 μL/well) and the SA1-HRP solution (0.025 μmL^{-1}, 25 μL/well), and the mixture was incubated for 30 min at RT and under gentle shaking. After washing, the assay proceeded as described before.

Selective Studies. Stock solutions of thirteen sulfonamides (10 mM in DMSO) were prepared and stored at 4 °C. Standard curves (0.125 nM to 50 μM and zero) were prepared in PBST by serial dilution. Each IC_{50} was determined in the competitive experiments following the optimized protocol described above. The cross-reactivity (CR) values were calculated according to the equation [IC_{50}(SPY)/IC_{50}(cross-reactant) × 100].

Samples. *Milk samples* were obtained from a normal grocery store and were used directly for the analysis or after dilution with PBS.

Hair samples, finely cut, were obtained in the Centre de Tecnologia de la Carn (IRTA, Girona, Spain) from different untreated controlled farm animals. Thus, different types of animal hair (colorless pig, colorless calf, and pigmented calf hair, 50 mg) were taken and extracted with 0.1 M NaOH (4 mL, 1 h at 60 °C for pig hair) or 0.2 M NH_4OH (4 mL, 24 h at 60 °C, for calf hair). Subsequently, extracts were centrifuged and filtrated through a paper filter as described (*23*).

ELISA Matrix Effect Studies. *Full-cream fresh milk* was used, either directly of after dilution with PBS, to prepare SPY standard curves and to analyze them in both immunoassay formats as described previously. The sigmoidal curves obtained were compared to that prepared in the assay buffer to evaluate the extent of the interferences caused by the matrix.

Hair extracts prepared as described above were buffered by adding 10% (v/v) of 100 mM PBS and adjusting the pH to 7.5 with few drops of 5N HCl, if necessary. Then, the extracts were diluted with PBS and used to prepare SPY standard curves that were measured in the microplate ELISA as previously described. The sigmoidal curves obtained with hair extracts were compared to the buffer standard assay to evaluate the extent of the interferences caused by the matrix.

Accuracy Studies. *Milk.* Different blind spiked samples were prepared in full-cream fresh milk and measured directly using the microtiter plate ELISA (undiluted or diluted 5× in PBS) and the Ab-MNP-ELISA (undiluted). Analyses were done in triplicate. The correlation was evaluated by establishing a linear regression between the spiked and the measured values.

Hair. Blind spiked samples were prepared using extracts from pigmented calf finely cut hair. The samples were buffered with 10% (v/v) 100 mM PBS, diluted 5× with the same buffer and measured using the microtiter plate ELISA as described before. Analysis were performed in duplicate. Accuracy was evaluated by establishing a linear regression between the spiked and the measured values.

RESULTS AND DISCUSSION

Microplate and Ab-MNP ELISAs. Immunoreagents were prepared by covalently coupling hapten SA1 to HCH, BSA, and HRP with good yields (∼14 mols of hapten per mol of BSA according to MALDI-TOF-MS). SA1-HCH was used to raise antibodies (As154, As155, As156, and As167) in white New Zealand rabbits. All of these immunoreagents were used to establish competitive immunochemical protocols to detect sulfonamide antibiotics in milk samples, using the standard microtiter plate as solid phase or MNP. Ab-MNP were prepared using the IgG fraction obtained by ammonium sulfate precipitation.

In the microtiter plate ELISA format, As154 and As167 provided the assays with the best detectability using SPY as analyte, with IC_{50} values lower than 10 μg L^{-1}. Factors such as preincubating the analyte with the coated antibody or varying the length of the competitive step did not affect the assay, although incubation times shorter than 20 min led to a decrease in the maximum signal without improving the IC_{50} value. No significant effect on the immunoassay detectability was observed when varying the percentage of Tween 20 in the assay buffer. The effect on the ionic strength on the assay performance was evaluated in media with conductivity values ranging from 0 to

70 mS/cm (0− 50 mM PBS). The assay tolerated variations in the ionic strength of the media quite well, although the total absence of salts produced a drastic decrease of the maximum signal. According to these studies, a direct ELISA protocol was established consisting of coating the microtiter plates with the antibodies overnight at 4 °C, followed by a 30 min competition step between the enzyme tracer and the analyte for the antibody. The assay buffer was 10 mM PBS (15 mS cm^{-1}) with 0.05% of Tween 20.

The features of the As154/SA1-HRP and As167/SA1-HRP immunoassays summarized in **Table 1** are the average of four assays performed on different days. The LOD (0.49 and 0.22 $\mu g\ L^{-1}$, respectively) and IC_{50} values (8.25 and 5.35 $\mu g\ L^{-1}$, respectively) were considered sufficiently good to analyze milk samples, considering that the MRL established by the EC (Regulation 2377/90) in this matrix is 100 $\mu g\ Kg^{1-}$.

Antibody As167 was selected to further investigate performance of the assay using magnetic nanoparticles because, in combination with SA1-HRP, it had provided the ELISA with the best detectability. Partially purified antibodies (ammonium sulfate IgG fraction, Ab167) were covalently coupled through their amino groups to the magnetic particles (196 nm of diameter) functionalized with carboxylic groups (MP−COOH) following standard carbodiimide reaction conditions. The efficiency of the coupling was evaluated using the Bradford test, comparing the IgG concentration in the supernatant after the coupling procedure (20 $\mu g\ mL^{-1}$) with the initial value (98 $\mu g\ mL^{-1}$), and indicated that conjugation had taken place. Checkerboard titration experiments were used to select the appropriate concentrations of Ab167-MNP and SA1-HRP to be employed in the competitive experiments. All of the incubation steps were performed under a gentle shaking to avoid the deposition of the magnetic beads on the bottom of the wells and to facilitate the interaction with the other species. The experiments were performed using the same buffer media as that in the microplate ELISA format. As it can be observed in **Table 1**, the immunoassay detectability was slightly lower than that accomplished by the same immunoreagents in the microtiter plate format. However, with a LOD of 0.38 $\mu g\ L^{-1}$ and considering the excellent values of the rest immunoassay parameters (high maximum signal, low background and good slope, see **Table 1**), the Ab-MNP-ELISA was considered suitable to assess its performance in real matrices. **Figure 2** shows the calibration curves of the three immunoassays established.

Selective Studies. A set of 13 common sulfonamides were assessed using the two antibody/enzyme tracer combinations. As it can be observed in **Table 2**, SPY, sulfamethoxypyridazine (SMP), sulfathiazol (STZ), and sulfachlorpyridazine (SCP) were the antibiotics better recognized in both assays. This recognition pattern demonstrates the importance of the substitution group in the N1 position. Other sulfonamides containing a pyrimidine group, such as sulfadiazine, sulfamerazine, sulfamethazine, sulfadimethoxine, sulfadoxine, and sulfaisomidine were not recognized, probably because of the presence of two electroattractive groups close to the carbon linked to the sulfonamide bond, which affects the charges of nitrogen and the aromatic carbon. In spite of the chemical similarities between SMP and SCP (see **Figure 1** for chemical structures), the first one was recognized much better in both assays. The chlorine atom, at the para position in respect to N1, instead of the methoxy group, drastically reduced the recognition by both antibodies (from 49 to 14% and from 61 to 6% for As154 and As167, respectively). STZ was better recognized by As154 than by As167. Thus, in general, As 167 was more selective for SPY and SMP than As 154. It should be remarked that it has already been reported that homologous antibody/competitor combinations (same hapten as immunogen and as competitor) tend to rend more specific immunoassays (*46–48*).

Analysis of Milk Samples. The applicability of the developed immunoassays to measure milk samples was initially evaluated by determining the potential interference of this matrix on the parameters of both microplate and Ab-MNP- ELISAs. For this purpose, fresh full-cream milk was used to prepare standard curves with SPY. As can be observed in **Figure 3** the curve made in pure milk mimicked very well the standard curve in buffer, achieving almost a perfect superposition. Only a slight reduction of the maximum signal was observed in the Ab-MNP-ELISA, although the IC_{50} was not affected. The same results were observed when analyzing different brands of fresh milk samples. These results suggested the possibility of directly analyzing full-cream milk with both ELISA formats without any kind of sample pretreatment. The absence of significant interferences from the milk samples seemed independent from the format used (microplate or Ab-MNP), and it may be related to the affinity and selectivity of the antibodies used. To demonstrate performance of the ELISAs in milk, blind spiked samples were prepared and measured with both formats. **Figure 4** shows the correlation observed between the measured and the spiked values. The results demonstrate that the accuracy of the Ab-MNP-ELISA is better (center panel), whereas a slight overestimation was observed when the samples were measured undiluted with microtiter-plate ELISA (left panel, solid symbols). The greater accuracy of the Ab-MNP-ELISA could be related to the matrix effect caused by milk. Because detectability of these assays is high enough, same samples were measured after a 1/5 dilution with the assay, and as it can be observed in **Figure 4** (left panel, open symbols), this treatment completely reduced the undesired effect observed before with the microplate-based ELISA.

Analysis of Hair Samples. Similarly, colorless hair samples obtained from pig and pigmented and colorless hair samples from calf were finely cut and extracted under alkaline conditions. The extracts were buffered to adjust the pH and then diluted several times in PBS. SPY standard curves were prepared with these extracts and measured in the microplate ELISA to assess the parallelism with the standard curve prepared in buffer. **Figure 5** shows that just a 5× dilution of the buffered hair extracts was sufficient to avoid undesired matrix effects caused from the matrix, independent of the type of hair. Following this consideration, accuracy was evaluated by measuring blind spiked samples of the hair extracts. A very good correlation between the measured and the real values was obtained as shown in **Figure 4** (right panel). Results obtained demonstrate the suitability of the immunoassay for determination of SPY in hair samples.

In conclusion, antibodies have been raised against a hapten possessing a linker in the pyridine group of the sulfapyridine. The antibodies have been used to establish two ELISAs, one using microtiter plates as solid-phase, whereas the other uses magnetic nanoparticles. Both formats allow detection of two important sulfonamide antibiotics (SPY and SMP) with LODs values below 0.5 $\mu g\ L^{-1}$, which is much more below the MRLs set by the EC for residues of these antibiotics in food samples. Milk samples can directly be measured without any sample treatment with both formats, although the use of magnetic beads has demonstrated more accurate results. This lack of accuracy was solved in the microplate-based ELISA by just diluting the

sample 5× with the assay buffer. Similarly, sulfonamide residues can be easily analyzed in hair extracts with very good detectability. The results shown in this paper demonstrates that direct analysis of complex matrices is possible with the antibodies developed in this work. Both ELISA formats perform well, although the results obtained with the Ab-MNP-ELISA open the door to development of a variety of other immunochemical techniques (i.e.immunosensors) involving extraction steps with MNP, or microlfuidic components in which the Ab-MNP can be driven with a magnet to the detector, or just immunosensors based on magnetic principles.

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Received for review August 25, 2007. Revised manuscript received November 16, 2007. Accepted November 27, 2007. This work has been supported by the Ministry of Science and Education (Contract Nos. AGL2005-07700-C06-01 and NAN2004-09195-C04-04). The AMR group is a Grup de Recerca de la Generalitat de Catalunya and has support from the Departament d'Universitats, Recerca i Societat de la Informació la Generalitat de Catalunya (expedient 2005SGR 00207).

JF072550N