JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2009,** *57,* 385–394 **385**

Generation of Broad Specificity Antibodies for Sulfonamide Antibiotics and Development of an Enzyme-Linked Immunosorbent Assay (ELISA) for the Analysis of Milk Samples

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Immunoreagents appropriately produced to detect a wide range of sulfonamide antibiotic congeners have been used to develop a highly sensitive enzyme-linked immunosorbent assay (ELISA). The selectivity has been achieved by combining antibodies raised against 5-[6-(4-aminobenzenesulfonylamino)pyridin-3-yl]-2-methylpentanoic acid (SA1), covalently coupled to horseshoe crab hemocyanin (HCH), and 5-[4-(amino)phenylsulfonamide]-5-oxopentanoic acid (SA2), coupled to ovalbumin (OVA), on an indirect ELISA format. The immunizing hapten has been designed to address selectivity against the common aminobenzenesulfonylamino moieties, using theoretical calculations and molecular modeling tools. Hapten SA1 has been synthesized in four steps from methyl 5-(4-amino-3-pyridinyl)-2-methyl-4-pentenoate through a Heck reaction, under Jeffery conditions, to avoid introduction of additional epitopes in the linker. The microplate immunoassay developed is able to reach the necessary detectability for the determination of the sulfonamide antibiotics most frequently used in the veterinary field, in compliance with the EC Regulation 2377/90. As an example, the IC_{50} and LOD values accomplished for sulfapyridine are 2.86 ± 0.24 and 0.13 ± 0.03 $\mu g\ L^{-1}$, respectively. Studies performed with different types of milk samples demonstrate that direct and accurate measurements can be performed in this type of matrix without any previous sample cleanup method.

KEYWORDS: Sulfonamide antibiotics; hapten; molecular modeling; immunoassay; milk; class-selective antibodies

INTRODUCTION

Antibiotics are chemical substances extremely active at low doses that kill or slow the growth of bacteria. In the past decade, the irresponsible use of antibiotics in human medicine added to their inappropriate use in the veterinary field to prevent diseases and to improve productivity has favored the growth of bacterial resistance (*1*). Antibiotic-resistant bacteria may arrive in humans through the food chain or by contact with animals, causing diseases that can no longer be treated with presently known antibiotics (*2*). Moreover, antibiotic resistance causes an important impact on the ecosystem, water, and soil-dwelling organisms, producing adverse effects in the ecosystem. Actually, actions such as wastewater treatment plant effluents and confined animal feeding operations represent important sources of antibiotics to the environment (*3*). According to some environmental studies reported, as much as 90% of the sulfonamides are excreted after consumption, contaminating the subsoil, ground, and superficial water resources (*4*).

Governmental agencies have set limitations on the levels of residues attending to toxicological data. In Europe, EC Regulation 2377/90 sets maximum residue limits (MRL) for the majority of antibiotics in different sample tissues, and Directive 96/23/CE establishes procedures for inspection, dictating the frequency and number of substances that have to be monitored. However, the analytical procedures today available cannot respond to the requirements of these regulations because of their low throughput capabilities. Most of the traditional tests based on bacteria growth inhibition are slow and do not have the necessary detectability to comply with the legislation (*5*). Chromatographic techniques such as HPLC-UV (*6*) and/or HPLC-MS detector (*7*) are in contrast highly specific and can reach an excellent detectability. However, they usually require extensive sample preparation, sophisticated equipment, and skilled laboratory personnel. With this panorama, the need to develop alternative analytical technologies becomes clear, and

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10.1021/jf8027655 CCC: $40.75
Published on Web 01/07/2009

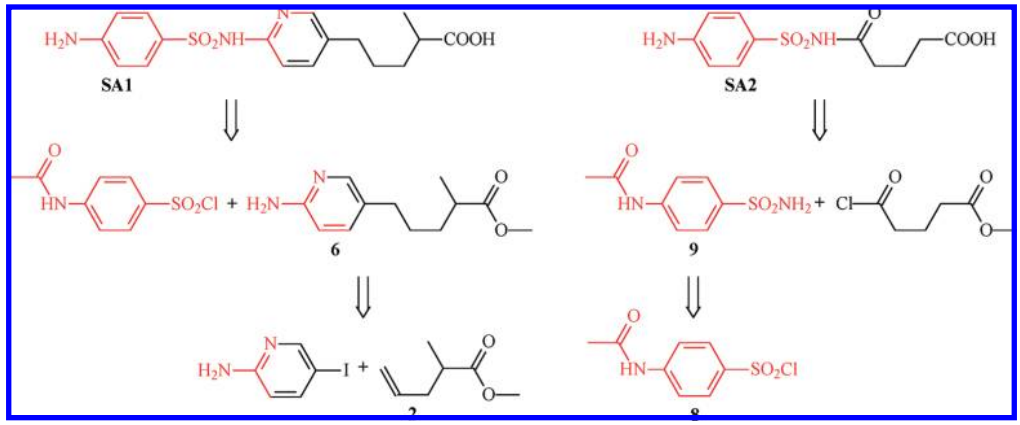


Figure 1. Structure and retrosynthetic analysis of sulfonamide **SA1** and **SA2** haptens.

for this reason, the EC and other governmental agencies are strongly supporting research in this area.

The milk and dairy products industries are between the sectors most affected by the presence of antibiotic residues, not only because of the adverse effects caused to the public health and ecosystems but also because of the economical losses derived by the effect of these biocides in the fermentation processes. Sulfonamides are among the four antibiotic families most frequently detected in these products. This antibiotic family is a wide group of synthetic antimicrobial agents that contain the sulfonamide group. A generic receptor able to interact with most of these congeners would allow development of new efficient and reliable biosensors or friendly user detection platforms. However, despite the efforts made by many research groups to produce class-selective antibodies (*8*−*13*), immunoassays recognizing a wide list of compounds of the same family with sufficient sensitivity to comply with the regulations have only seldom been reported (i.e., ref *14*). Similarly, most of the marketed immunoassay kits for sulfonamides detect only a few sulfonamide specimens below the MRLs (i.e., SulfaMRL by Charm Sci.; sulfamethazine sulfadimethoxine, sulfadiazine, and sulfathiazole; SNAP by IDEXX Laboratories is mainly addressed to sulfamethazine, etc.).

On the basis of our previous experience with molecular modeling tools to assist in hapten design and immunoassay development (*15*, *16*), this paper reports the development of an immunochemical analytical method with a broad specificity for this antibiotic family. The approach consisted of raising polyclonal antibodies against an appropriately designed hapten and their use on an indirect competitive ELISA under heterologous conditions (different haptens as immunogen and as competitor). Moreover, we demonstrate the excellent performance of this ELISA to analyze these antibiotics in milk samples after just simple dilution treatment.

EXPERIMENTAL PROCEDURES

Chemistry. *General Methods and Instruments.* Thin-layer chromatography (TLC) was performed on 0.25 mm precoated silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany). Unless otherwise indicated, purification of the reaction mixtures was accomplished by "flash" chromatography using silica gel as the stationary phase. ^{1}H and ^{13}C NMR spectra were obtained with a Varian Inova-500 (Varian Inc., Palo Alto, CA) spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). Liquid chromatography−electrospray ionization/mass spectrometry (LC-ESI/MS) was performed in a Waters (Milford, MA) model composed of an Acquity UPLC system directly interfaced to a Micromass LCT Premier XE MS system equipped with an ESI LockSpray source for monitoring positive ions. Data were processed with MassLynx (v 4.1) software (Milford, MA).

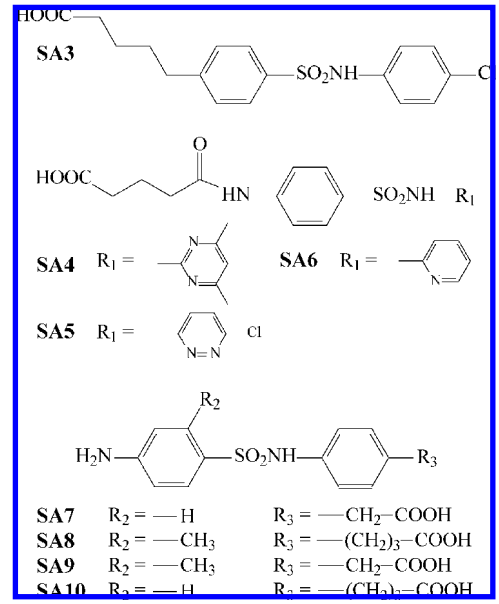


Figure 2. Chemical structures of the competitor haptens.

Molecular Modeling and Theoretical Calculations. Computational methodology was carried out by running the Hyperchem 6.03 software package (Hypercube Inc., Gainsville, FL) to compare physical−chemical features of the haptens with 12 of the most common sulfonamides. Minimum energy conformation, geometry, and molecular charge distribution were calculated using a semiempirical quantum model (PM3).

Preparation of Sulfonamide Haptens. Immunizing and competitor haptens **SA1** and **SA2** were synthesized following the retrosynthetic scheme shown in **Figure 1**. Experimental details on the synthetic and purification procedures together with their spectroscopic and spectrometric characterization can be found below. Preparation of haptens **SA3**−**SA10** (see **Figure 2**) also tested as competitors is described in the Supporting Information (see Figure A).

Synthesis of the Immunizing Hapten SA1. Methyl 2-methyl-4-pentenoate (2). Dimethyl sulfate (5 mL, 53 mmol) was added to a freshly prepared solution of 2-methyl-4-pentenoic acid **1** (5 mL, 44 mmol) in 44 N NaOH (40 mL), and the mixture was vigorously stirred at room temperature. After 4 h, a new equivalent of dimethyl sulfate was added (5 mL, 44 mmol), and the mixture stirred for 8 h more. The pH of the reaction was constantly controlled and adjusted to pH 8 with 1 N NaOH. When reaction was complete, the organic layer was separated by liquid−liquid extraction and washed with 1 N NaOH to afford a pale yellow liquid corresponding to the pure ester **2** (3.9 g, 66%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.13 (d, J = 7 Hz, 3H, CH_3), 2.15 (m, 1H, CH_2), 2.38 (m, 1H, CH_2), 2.50 (m, 1H, CH), 3.64 (s, 3H, $COOCH_3$), 4.99 (d, J = 11 Hz, 1H, H_{cis}), 5.02 (d, J = 18 Hz, 1H, H_{trans}), 5.71 (ddt, J = 18 Hz, 11 Hz, 7 Hz, 1H, =CH−); ^{13}C NMR

(125 MHz, $CDCl_3$) δ 16.4 ($-\mathbf{C}H_3$), 37.7 ($-\mathbf{C}HCOO$), 39.1 ($-\mathbf{C}H_2$), 51.5 ($-O\mathbf{C}H_3$), 116.8 ($\mathbf{C}H_2=$), 135.4 ($=\mathbf{C}H-$), 176.5 ($-\mathbf{C}OO^-$).

*Methyl 5-(4-Amino-3-pyridinyl)-2-methyl-4-pentenoate (**3**).* A solution of tetrabutylammonium chloride (160 mg, 0.57 mmol) and $Pd(AcO)_2$ (13 mg, 0.06 mmol) in anhydrous dimethylformamide (anh DMF, 3 mL) was slowly added to a solution of 2-amino-5-iodopyridine (500 mg, 2.27 mmol) and potassium formate (153 mg, 1.82 mmol) in the same solvent (3 mL). Subsequently, a solution of the ester **2** (1.3 g, 11.4 mmol) in anh DMF (2 mL) was added to the mixture, and the reaction was kept at 65 °C under Ar atmosphere for 6 h. Additional equivalents of potassium formate (50 mg, 0.59 mmol) and the ester **2** (300 mg, 2.63 mmol) were added, and the progress of the reaction was monitored by TLC using ethyl acetate (AcOEt) as mobile phase. After the reaction was finished, the crude material was cooled and the solvent evaporated. The oil obtained was redissolved in MeOH (25 mL) and filtered through Celite to remove all of the palladium formed, and the solvent was evaporated. The residue obtained was dissolved again with AcOEt (50 mL) and washed with saturated $NaHCO_3$ (3 × 25 mL). The organic layer was separated, dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure. The red oil obtained was purified by silica gel column chromatography using a 1:1 to 1:3 polarity gradient of hexane/AcOEt as mobile phase to obtain a yellow oil (250 mg) of a 4:1 mixture of the methyl 5-(4-amino-3-pyridinyl)-2-methyl-4-*trans*-pentenoate **3** (186 mg, 36% yield) and methyl 4-(4-amino-3-pyridinyl)-2-methyl-4-methylene-butanoate **4** (62 mg, 12% yield). **3**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.19 (d, J = 7 Hz, 3H, CH_3), 2.31 (m, 1H, CH_2), 2.53 (m, 1H, CH_2), 2.58 (m, 1H, CH), 3.67 (s, 3H, $COOCH_3$), 5.95 (dt, J = 16 Hz, J = 7 Hz, 1H, $=CH-$), 6.28 (d, J = 16 Hz, 1H, $-CH=$), 6.45 (d, J = 8.5 Hz, 1H, Ar_{meta}), 7.49 (dd, J = 8.5 Hz, J = 2.5 Hz, 1H, Ar_{ortho}), 7.99 (d, J = 2.5 Hz, 1H, Ar_{ortho}). **4**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.12 (d, J = 7 Hz, 3H, CH_3), 2.45 (m, 1H, CH_2), 2.90 (m, 1H, CH_2), 2.56 (m, 1H, CH), 3.63 (s, 3H, $COOCH_3$), 4.99 (d, J = 1 Hz, 1H, $CH_2=$), 5.20 (d, J = 1 Hz, 1H, $CH_2=$), 6.50 (d, J = 8.5 Hz, 1H, Ar_{meta}), 7.48 (dd, J = 8.5 Hz, J = 2 Hz, 1H, Ar_{ortho}), 8.12 (d, J = 2 Hz, 1H, Ar_{ortho}).

*Methyl 5-(4-Amino-3-pyridinyl)-2-methylpentanoate (**6**).* The mixture of esters **3** and **4** (250 mg) in MeOH (12 mL) was reduced under H_2 at atmospheric pressure in the presence of Pd/C (23 mg, 10% Pd, 0.018 mmol of Pd) for 12 h at room temperature. The reaction was passed through Celite, and the solvent was evaporated until dryness. The yellow oil obtained was purified by column chromatography using a 1:1 to 1:3 polarity gradient of hexane/AcOEt as mobile phase to isolate 180 mg of a yellow oil containing a 5:1 mixture of the desired ester **6** and methyl 4-(4-amino-3-pyridinyl)-2,4-dimethylbutanoate. Further purification of the mixture by preparative HPLC using a C_{18} column (25 × 1 cm, 5 μm, Kromasil 100) as stationary phase and ACN/H_2O (Et_3N/CH_3COOH, 0.2 M, pH 7.6) 30:70 as mobile phase allowed us to isolate the pure ester **6** (81 mg, 40% yield): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.13 (d, J = 7 Hz, 3H, CH_3), 1.42 (m, 1H, CH_2), 1.53 (m, 2H, CH_2), 1.65 (m, 1H, CH_2), 2.44 (m, 1H, CH), 2.45 (t, J = 8 Hz, 2H, CH_2), 3.65 (s, 3H, $COOCH_3$), 6.44 (d, J = 8.5 Hz, 1H, Ar_{meta}), 7.24 (dd, J = 8.5 Hz, J = 2.5 Hz, 1H, Ar_{ortho}), 7.85 (d, J = 2.5 Hz, 1H, Ar_{ortho}); ^{13}C NMR (125 MHz, $CDCl_3$) δ 17.4 ($\mathbf{C}H_3CH$), 29.3 ($\mathbf{C}H_2CH_2COO$), 32.2 ($\mathbf{C}H_2CH_2Pyr$), 33.9 ($\mathbf{C}H_2Pyr$), 39.6 ($\mathbf{C}HCOO$), 51.5 ($COO\mathbf{C}H_3$), 108.7 (C_3), 127.9 (C_5), 138.2 (C_4), 147 (C_6), 156.9 (C_2), 176.9 ($\mathbf{C}OOCH_2$).

*Methyl 5-[6-(4-Acetylaminobenzenesulfonylamino)pyridin-3-yl]-2-methylpentanoate (**7**).* Ester **6** (250 mg, 1.13 mmol) in anhydrous dioxane (2 mL) was added to a solution of *N*-acetyl-*p*-aminobenzenechlorosulfonic acid (290 mg, 1.24 mmol) in the same solvent (8 mL). Subsequently, triethylamine (175 μL, 1.24 mmol) was added slowly, and the mixture was kept under argon atmosphere for 15 h at room temperature. When the reaction was finished, the solvent was evaporated, and the oil obtained was redissolved with AcOEt (10 mL) and washed with saturated $NaHCO_3$ (3 × 5 mL). The organic layer was dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure to obtain yellow oil. Purification of the product was performed using a silica gel column, with 1:4 hexane/AcOEt as mobile phase to obtain the desired sulfonamide **7** (239 mg, 35% yield): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.13 (d, J = 7 Hz, 3H, CH_3), 1.41 (m, 1H, CH_2), 1.53 (m, 2H, CH_2), 1.65 (m, 1H, CH_2), 2.20 (s, 3H, CH_3CO), 2.43 (m, 1H, CH), 2.49 (t, J = 8 Hz, 2H, CH_2), 3.64 (s, 3H, $COOCH_3$), 7.52 (s, 1H), 7.57 (d, J = 9 Hz, 2H), 7.68 (d, J = 9 Hz, 2H), 8.13 (s, 1H), 8.28 (s, 1H).

*5-[6-(4-Aminobenzenesulfonylamino)pyridin-3-yl]-2-methylpentanoic acid (**SA1**).* A solution of the sulfonamide **7** (165 mg, 0.39 mmol) in 1 N NaOH (5 mL, 5 mmol) was kept under reflux for 4 h under argon atmosphere. The crude was cooled, acidified with concentrated HCl to pH 3, and extracted with AcOEt (3 × 5 mL). The organic layer was dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure to obtain the desired hapten **SA1** (105 mg, 73% yield) as a pale yellow oil: ^{1}H NMR (500 MHz, CD_3OD) δ 1.13 (d, J = 7 Hz, 3H, CH_3), 1.40 (m, 1H, CH_2), 1.58 (m, 2H, CH_2), 1.62 (m, 1H, CH_2), 2.41 (m, 1H, CH), 2.52 (t, J = 7.5 Hz, 2H, CH_2), 6.61 (d, J = 8.5 Hz, 2H), 7.12 (d, J = 8.5 Hz, 1H), 7.53 (dd, J = 8.5 Hz, J = 2.5 Hz, 1H), 7.56 (d, J = 8.5 Hz, 2H), 7.88 (d, J = 2.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 17.6 ($CH(\mathbf{C}H_3)$), 29.7 ($\mathbf{C}H_2CH_2COOH$), 32.6 ($\mathbf{C}H_2CH_2Pyr$), 34.2 ($\mathbf{C}H_2COOH$), 40.4 ($\mathbf{C}H_2Pyr$), 114.2 (C_3), 114.7 ($C_{meta\ SO_2NH_2}$), 127.9 ($C_{ortho\ SO_2NH_2}$), 130.1 (C_5), 132.6 ($C_{SO_2NH_2}$), 140.9 (C_4), 145.5 (C_6), 152.2 (C_2), 154.1(C_{NH_2}), 180.5 (COOH). HRMS (+EI) calcd for $C_{17}H_{22}N_3O_4S$ (M+): 364.1331; found, 364.1319.

*Synthesis of the Competitor Hapten SA2. N-(4-(Aminosulfonyl)phenyl)acetamide (**9**).* A 30% aqueous solution of NH_3 (5.6 mL, 4.3 mmol) was added slowly to a freshly prepared solution of *N*-acetyl-*p*-aminobenzenechlorosulfonic acid **8** (2 g, 8.6 mmol) in anhydrous acetonitrile (10 mL), and the mixture was kept for 2 h until the complete disappearance of the starting material by TLC analysis (1:1 AcOEt/CH_2Cl_2). The solvent was removed under reduced pressure, and the residue redissolved in water (20 mL) and extracted with AcOEt (3 × 10 mL). The organic layer was dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness to obtain the desired compound **9** (1.5 g, 76% yield) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$/CD_3OD, 14:1) δ 2.17 (s, 3H, CH_3-), 7.71 (dd, J = 9.2 Hz, J = 2.1 Hz, 2H, Ar_{meta}), 7.84 (dd, J = 9.2 Hz, J = 2.1 Hz, 2H, Ar_{ortho}); ^{13}C NMR (125 MHz, $CDCl_3$/CD_3OD, 14:1) δ 24.6 (CH_3), 119.5 ($C_{meta\ SO_2Cl}$), 127.51 ($C_{ortho\ SO_2Cl}$), 139.6 ($C_{SO_2NH_2}$), 143.6 (C_{NHCOCH_3}), 171.9 (CO).

*Methyl 5-(4-(Acetylamino)phenylsulfonamide-5-oxopentatanoate (**10**).* Methyl 4-(chloroformyl) butyrate (0.65 mL, 4.69 mmol) was added slowly to a freshly prepared solution of **9** (1 g, 4.69 mmol) in pyridine (2 mL), and the mixture was kept under argon atmosphere for 2 h. Then, the reaction was stopped by adding concentrated HCl in ice (30 mL). The aqueous layer was extracted with AcOEt (3 × 15 mL), and the organic layer was dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure to obtain a pale yellow solid. Isolation of compound **10** was performed by crystallization with 9:1 2-propanol/water (0.85 g, 50% yield): ^{1}H NMR (500 MHz, $CDCl_3$/CD_3OD, 14:1) δ 1.79 (m, J = 7.2 Hz, 2H, $-CH_2-$), 2.17 (s, 3H, $-CH_3$), 2.25 (t, J = 7.2 Hz, 2H, CH_2CO), 2.27 (t, J = 7.2 Hz, 2H, $-CH_2Ph$), 3.65 (s, 3H, $COOCH_3$), 7.75 (dd, J = 9.2 Hz, J =2.1, Hz 2H, 2H, Ar_{meta}), 7.95 (dd, J = 9.2 Hz, J = 2.1 Hz, 2H, 2H, Ar_{ortho}); ^{13}C NMR (125 MHz, $CDCl_3$/CD_3OD, 14:1) δ 19.2 (CH_3), 23.6 ($\mathbf{C}H_2$), 32.4 ($NHCO\mathbf{C}H_2$), 34.5 ($\mathbf{C}H_2COOH$), 51.4 ($-OCH_3$), 119.1 ($C_{meta\ SO_2NH_2}$), 127.1 ($C_{ortho\ SO_2NH}$), 132.8 (C_{SO_2NH}), 143.4 (C_{NHAc}), 170.19 ($\mathbf{C}ONH$), 171.1 ($\mathbf{C}OOH$).

*Preparation of 5-[4-(Amino)phenylsulfonamide]-5-oxopentanoic Acid (**SA2**).* A solution of compound **10** (130 mg, 3.9 mmol) in 1 N NaOH (8 mL) was heated at 75 °C for 6 h until the total disappearance of the starting material was observed by TLC (9:1:1 EtAc/CH_2Cl_2/MeOH; 1% AcOH). The crude mixture was acidified with 1 N HCl to pH 2 and extracted with AcOEt (3 × 10 mL). The organic layer was finally washed with water, dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure. Isolation of **SA2** was performed using a silica gel column and 1:2 AcOEt/CH_2Cl_2 (1% acetic acid) as mobile phase to obtain **SA2** (71 mg, 65% yield): ^{1}H NMR (500 MHz, CD_3OD) δ 1.79 (m, J = 7.2 Hz, 2H, $-CH_2-$), 2.25 (t, J = 7.2 Hz, 2H, CH_2COOH), 2.27 (t, J = 7.2 Hz, 2H, $-CH_2Ph$), 6.67 (dd, J = 7.2, J = 2.1 Hz, 2H, $2H_{Ar\ meta}$), 7.66 (dd, J = 7.2, J = 2.1 Hz, 2H, 2H, Ar_{ortho}); ^{13}C NMR (125 MHz, $CDCl_3$/CD_3OD, 9:1) δ 20.74 ($\mathbf{C}H_2$), 32.4 ($NHCO\mathbf{C}H_2$), 34.5 ($\mathbf{C}H_2COOH$), 113.8 ($C_{meta\ SO_2NH_2}$), 125.8 (Cortho SO2NH2), 131.6 (CSO2NH2), 154.9 (CNH2), 172.9 (CO). HRMS (+EI) calcd for C11H15N2O5S (M+): 287.0702; found, 287.0693.

Table 1. Hapten Densities of the BSA Conjugates Calculated by MALDI-TOF-MS

immunoreagent	δ-hapten[a]	% conjugation[b]
SA1−BSA	22.2	63−74
SA2−BSA	18.0	51−60
SA3−BSA	13.5	39−45
SA4−BSA	10.9	31−36
SA5−BSA	6.5	19−22
SA6−BSA	8.4	24−28
SA7−BSA	7.5	21−25
SA8−BSA	9.7	28−32
SA9−BSA	18.0	51−60
SA10−BSA	5.7	16−19

[a] Moles of hapten per mole of protein. [b] The conjugation is calculated on the basis of the assumption that BSA has 30−35 free lysine groups.

Immunochemistry. *General Methods and Instruments.* 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 software Voyager-DE-RP (version 4.03) developed by Perspective Biosystems Inc. (Framingham, MA) and Grams/386 (for Microsoft Windows, version 3.04, level III) developed by Galactic Industries Corp. (Salem, NH). The pH and conductivity of all buffers and solutions were measured with a pH-meter pH 540 GLP and a conductimeter LF 340, respectively (WTW, Weilheim, Germany). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, Denmark). Washing steps were performed on an SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). A Heidolph Titramax 1000 vibrating platform shaker (Brinkmann Instruments, Westbury, NY) was used to shake the microplates at 900 rpm. Absorbances were read on a SpectramaxPlus (Molecular Devices, Sunnyvale, CA). The competitive curves were analyzed with a four-parameter logistic equation using the software SoftmaxPro v4.7 (Molecular Devices) and GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA). The chemical reagents used in the synthesis were obtained from Aldrich Chemical Co. (Milwaukee, WI). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The preparation of the immunoreagents used is described below. Sulfonamides used for cross-reactivity studies were kindly supplied by Riedel-de Haën.

Buffers. Phosphate-buffered saline (PBS) is 0.01 M phosphate buffer on an 0.8% saline solution (137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl), and the pH is 7.5. PBST is PBS with 0.05% Tween 20. For milk experiments, 2× PBST is 20 mM PBS with 0.10% of Tween 20. Borate buffer is 0.25 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% 3,3′,5,5′-tetramethylbenzidine (TMB) and 0.004% H_2O_2 in citrate buffer.

Immunoreagents. Protein conjugates were prepared following described procedures (*17*) by activating the haptens (10 μmol) with *N*-hydroxysuccinimide (NHS, 12.5 μmol) and dicyclohexylcarbodiimide (DCC, 25 μmol) in anhydrous DMF (200 μL) and reacting with the protein (HCH, BSA, CONA, or OVA, 10 mg) in 0.2 M borate buffer (1.8 mL). The protein conjugates were purified by dialysis against 0.5 mM PBS (4 × 5 L) and Milli-Q water (1 × 5 L) and stored freeze-dried at −40 °C. Unless otherwise indicated, working aliquots were stored at 4 °C in PBS at 1 mg mL^{-1}. Hapten densities of the bioconjugates were estimated by measuring the molecular weight of the native proteins to that of the conjugates by MALDI-TOF-MS. Thus, MALDI spectra were obtained by mixing 2 μL of the freshly prepared matrix (*trans*-3,5-dimethoxy-4-hydroxycinnamic acid, 10 mg mL^{-1} in CH_3CN/H_2O 70:30, 0.1% TFA) with 2 μL of a solution of the conjugates or proteins in CH_3CN/H_2O 70:30, 0.1% TFA (10 mg mL^{-1}). The hapten density (δ-hapten) was calculated according to the following equation: {MW(conjugate) − MW(protein)}/MW(hapten)}. The coupling efficiency evaluated by MALDI-TOF-MS of the corresponding haptenized BSA immunoreagents is shown in **Table 1**.

Table 2. Immunoassay Features of the Best Competitive ELISA[a]

immunogen	assay	A_{max}	A_{min}	IC_{50}[b]	slope	R^2
SA1−HCH	As154/**SA2**−BSA	1.02	0.16	2.51	−0.84	0.99
	As154/**SA2**−OVA	0.86	0.25	1.80	−1.03	0.98
	As155/**SA2**−BSA	1.03	0.02	2.95	−0.66	0.99
	As155/**SA2**−OVA	0.92	0.07	1.41	−0.82	0.98
	As156/**SA2**−BSA	1.00	0.12	11.40	−0.77	0.98
	As167/**SA8**−CONA	0.67	0.01	3.57	−0.61	0.99
	As167/**SA9**−BSA	0.58	0.01	2.90	−0.54	0.99
	As167/**SA2**−OVA	0.91	0.09	4.06	−0.74	0.99

[a] Only some assays showing reasonable parameters and IC_{50} values below 100 μg L^{-1} are shown. [b] IC_{50} values are expressed in μg L^{-1}.

Polyclonal Antisera. As154−156 and As167 were obtained by immunizing female white New Zealand rabbits weighting 1−2 kg with **SA1**−HCH following a protocol already described (*17*).

As157−159 were produced in the same way but using **SA2**−HCH as immunogen. The evolution of the antibody titer was assessed using a noncompetitive indirect ELISA, by measuring the binding of serial dilutions of each antiserum to microtiter plates coated with **SA1**−BSA or **SA2**−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. Antiserum were obtained by centrifugation and stored at −80 °C in the presence of 0.02% NaN_3.

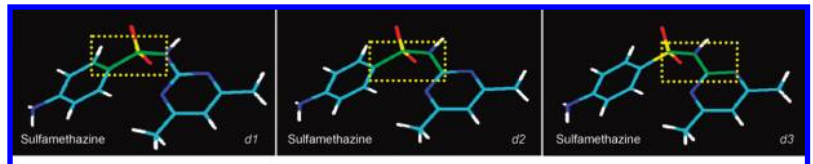
This research has the approval of the Ethical Committee of the CSIC, and all of the experiments have been performed following Animal Research Advisory Committee (ARAC) guidelines.

*Indirect As155/***SA2***−OVA ELISA. General Protocol.* The detectability and specificity of the antibodies raised were assessed through competitive ELISA experiments following a general protocol. Previously, the concentrations of the immunoreagents were selected by two-dimensional checkerboard titration experiments, where the avidity of the antisera for the better recognized coating antigens (CAs) was assessed (*15*). The features of best combinations able to provide competitive assays within the studied concentration interval are included in **Table 2**. Finally, microtiter plates were coated with **SA2**−OVA (0.625 μg mL^{-1} in coating buffer, 100 μL/well) overnight at 4 °C and covered with adhesive plate sealers. The next day, plates were washed four times with PBST (300 μL/well), and solutions of the sulfapyridine (SPY) standards (from 50000 to 0.0256 nM) or cross-reactants (same concentration range) in PBST (50 μL/well) and the antiserum As155 (1/4000 diluted in PBST, 50 μL/well) were added and incubated for 30 min at room temperature, under shaking. The plates were washed as before, and a solution of anti-IgG−HRP (1/6000 in PBST) was added to the wells (100 μL/well) and incubated for 30 min more at room temperature. The plates were washed again, and the substrate solution was added (100 μL/well). Color development was stopped after 30 min at room temperature with 4 N H_2SO_4 (50 μL/well), and the absorbances were read at 450 nm. The standard curves were fitted to a four-parameter equation according to the following 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. Unless otherwise indicated, the data presented correspond to the average of at least two well replicates.

Specificity Studies. Stock solutions of different sulfonamides were prepared in DMSO at a concentration of 10 mM and kept at 4 °C. Standard curves were prepared in PBST and run in the ELISA following the protocol described before. The cross-reactivity (CR) values were calculated according to the equation {IC_{50} [nM] (SPY)/IC_{50} [nM] (cross-reactant)} × 100. Accuracy Studies.

This parameter was assessed by preparing different blind spiked samples in PBST buffer and measuring them in duplicate in the ELISA.

Immunochemical Analysis of Milk. Samples. Skimmed, semi-skimmed, and whole milk samples free of antibiotics were supplied by the Agencia Española para la Seguridad Alimentaria (AESA; Spanish Agency for Food Security, Madrid, Spain). Contaminated blind samples were prepared at the Nestlé Research Center (Lausanne, Switzerland) from fresh milk samples.



Structures	Dihedral Angle			Charge Distribution		pKa
	d1	d2	d3	SO_2NHC	SO_2NHC	
Sulfamethazine	94.0	-76.1	-161.3	-0.394	0.078	7.03
Sulfadiazine	97.2	-74.8	-162.4	-0.392	0.071	6.84
Sulfaquinoxaline	98.1	-72.7	17.2	-0.413	0.095	7.61
Sulfadoxine	-100.3	-69.8	13.2	-0.396	0.109	7.05
Sulfamerazine	-97.1	-75.4	-161.5	-0.393	0.079	6.93
Sulfamethoxydiazine	98.8	-77.8	158.4	-0.393	0.035	7.21
Sulfapyridine	81.5	-94.5	148.7	-0.410	0.044	7.99
Sulfathiazole	93.9	-73.0	18.8	0.376	0.110	7.83
Sulfamethiazole	95.2	-67.8	16.2	-0.367	-0.135	7.05
SA1 Hapten	*80.8*	*-95.4*	*-147.4*	*-0.409*	*0.039*	*8.20*
SA2 Hapten	*88.8*	*-99.5*	*-166.8*	*-0.530*	*0.322*	*5.62*

Figure 3. Results from the theoretical studies performed to assess the suitability of the immunizing hapten to produce class-selective antibodies. Comparison of dihedral sulfonamide angle, charge distribution, and calculated pK_a values for the most important commercial sulfonamides and the haptens proposed. Sulfamethazine dihedral angles are shown as an example.

Matrix Effect Studies. SPY standard curves were prepared in milk samples diluted with Milli-Q water in different proportions and used to study the parallelism with respect to the curves prepared in buffer.

Sample Measurements. Milk samples were analyzed in duplicate with the As155/**SA2**−OVA ELISA using SPY calibration curves and following the general protocol described above. Alternatively, milk samples were also measured using SPY calibration curves prepared in whole milk free of antibiotics diluted 5 times with Milli-Q. In this case, samples and standards were added to the coated plates (50 μL/well) followed by the antibody solution (1/2000 in 2× PBST, 50 μL/well). Subsequently, the plates were processed as described before. Concentration results were expressed as immunoreactivity equivalents of SPY (SPY IR equiv).

RESULTS AND DISCUSSION

Sulfonamide class-selective antibodies and immunoreagents have been developed with the objective of detecting a significant number of sulfonamide antibiotic congeners used in the veterinary field. The chemical structure of the immunizing hapten has been designed on the basis of chemical criteria and information extracted from theoretical models and calculations (*11*, *18*, *19*) to assess how the proposed immunizing hapten chemical structure mimics that of most of the sulfonamide congeners. On the basis of chemical criteria, generic recognition of the sulfonamide antibiotic family is required to maximize recognition of the common aniline moiety. Therefore, a chemical group for covalent attachment to the carrier had to be placed at the opposite site of the molecule. Moreover, it was considered to be relevant that most of the sulfonamide chemical structures show with the presence of one electron-withdrawing atom close to the carbon situated in the α-position to the sulfonamide bond. With this criteria was defined the fundamental chemical structure moiety that had to be present in the immunizing hapten (see area marked in red in **Figure 1**). Both haptens **SA1** and **SA2** possessed the mentioned area; however, whereas hapten **SA1** contained the whole structure, hapten **SA2** had just the common moiety of the sulfonamide antibiotics. The strategy of using just a fragment of the chemical structure has been used before (*11*, *20*, *21*), although it has not always provided the desired results with regard to antibody selectivity. For this reason and with the aim of obtaining additional information, we made use of theoretical and molecular modeling studies. **Figure 3** shows dihedral sulfonamide angles in the sulfonamide bridge of the haptens and the most important sulfonamide antibiotics, where it can be observed that with regard to geometry the differences between all of these chemical structures are not so great. Moreover, in the figure can also be found molecule charge distribution in the area close to the sulfonamide bridge, where it can be observed that the main difference is at the carbon α to the sulfonamide group of hapten **SA2**. Finally, the pK_a values calculated also allowed us to predict a distinct behavior of this hapten at the physiological or the usual assay pH of 7.5.

Hapten **SA1** was prepared in four steps from methyl 5-(4-amino-3-pyridinyl)-2-methyl-4-pentenoate **6** through a Heck reaction under Jeffery conditions (*22*, *23*). The reaction consists of an sp2−sp2 coupling between an iodoaromatic compound, in this case the 2-amino-5-iodopyridine, and an alkene group, the methyl 2-methy l-4-pentenoate, using palladium acetate as catalyst, tetrabutylammonium chloride, and potassium formate. The mixture obtained was hydrogenated and subsequently purified by HPLC to obtain the necessary synthon **6**. Subsequently, the sulfonamide was formed by reacting the amino group with *N*-acetylsulfanilyl chloride. The simultaneous hydrolysis of the acetyl group and the methyl ester, under basic conditions, allows us to obtain **SA1** (see **Figure 1**). On the other hand, the synthesis of **SA2** was accomplished by reacting *N*-4-(aminosulfonyl)phenyl)acetamide **9** with methyl 4-(chloroformyl)butyrate, followed by the hydrolysis of the ester and the acetyl group (see **Figure 1**).

Both haptens **SA1** and **SA2** were covalently coupled through their carboxylic groups to the lysine amino acid residues of the HCH and used to raise antibodies (As154−156 and As167 from **SA1**−HCH; As157−159 from **SA2**−HCH). Similarly, both haptens were conjugated to BSA, CONA, and OVA to use them as coating antigens to develop the competitive ELISA. As has been reported (*18*, *24*−*28*), the chemical structure of the competitor hapten plays a crucial role in the sensitivity and specificity of the competitive assays. The screening of all possible antiserum/coating antigen (As/CA) combinations was performed using SPY as analyte and the general ELISA protocol described under Experimental Procedures. In all cases, the antisera raised against **SA1**−HCH provided the best assays. In contrast, no usable assays (IC_{50} > 100 μg L^{-1}) were obtained with the As raised against **SA2**−HCH, demonstrating that the presence of the heterocycle, and the electron-withdrawing group next to the carbon atom in α to the sulfonamide group, played an important role in antibody recognition. On the other hand,

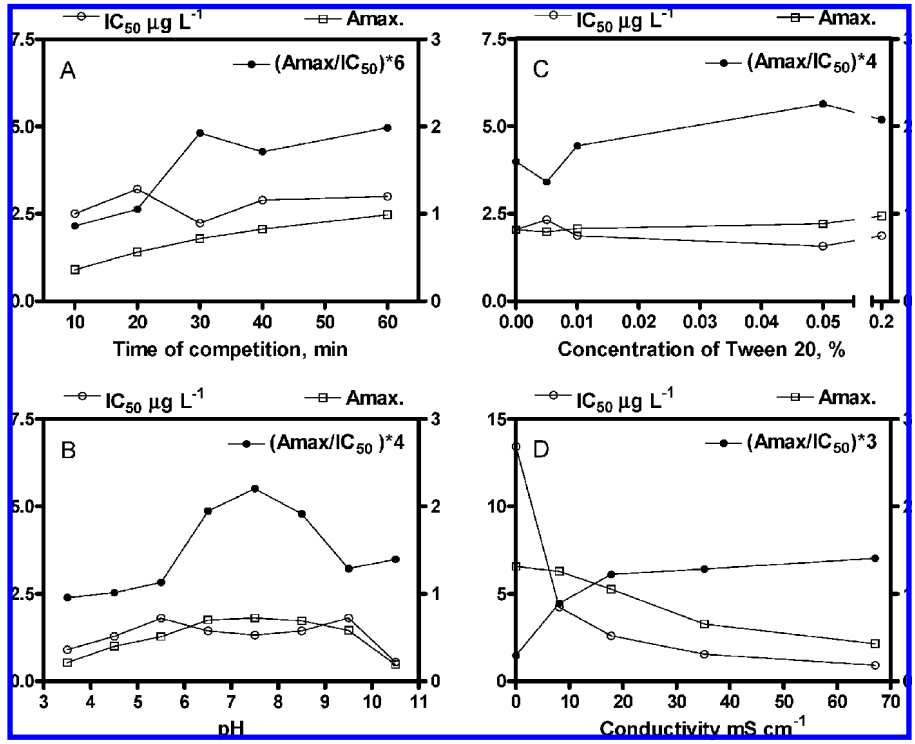


Figure 4. Effect of the length of the competition step, Tween 20, pH, and conductivity on the As155/**SA2**−OVA immunoassay features for the sulfapyridine (SPY) analysis.

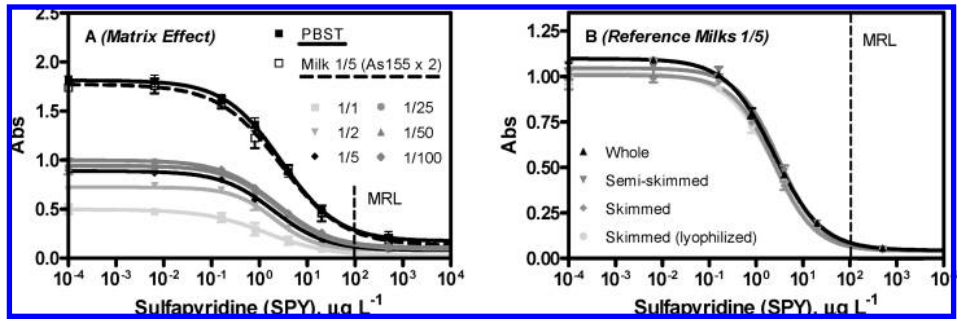


Figure 5. (**A**) Calibration curve of the As155/**SA2**−OVA immunoassay in PBST buffer. The data shown are the averages of six assays performed on different days. The parameters of the assay are shown in **Table 3**. Matrix effect was caused by milk in the As155/**SA2**−OVA ELISA. Whole milk and PBST diluted whole milk were used. Parallelism was found between the standard curve prepared in buffer and the one prepared in 1/5 PBST diluted milk using a double concentration of the antibody in the competition step. (**B**) Comparison of the immunoassay response when different types of milk samples were analyzed after being diluted 5 times with PBST.

as predicted by the theoretical studies, the charge of the carbon α to the sulfonamide group and the pK_a of hapten **SA2** may also have also been some of the reasons for the results obtained.

Table 2 shows the features of the best competitive indirect ELISAs obtained. As can be observed, the best antibody/coating antigen combinations are heterologous. The assays obtained under homologous conditions provided IC_{50} values far from the required detection limits. Although several combinations rendered usable assays, As155/**SA2**−OVA was finally selected for further studies because of the excellent features and reproducibility observed on repetitive experiments. Evaluation of the effect of different physicochemical parameters (time, pH, ionic strength, etc.) showed that no improvement in the detectability was obtained after an overnight preincubation of the antisera with the analyte before the competitive step, by varying the length of the competitive step or by changing the concentration of surfactant Tween 20 in the buffer, with respect to the standard conditions used while screening the different antisera/coating antigen combinations (see **Figure 4A**,**C**). Concerning pH, the assay tolerated quite well pH values between 6 and 9. Outside this pH range the assay is almost inhibited (see **Figure 4B**). With regard to ionic strength, in the absence of salts the detectability is very bad (high IC_{50} value), whereas a marked increase of the detectability was observed with the increase of the ionic strength. However, a slight decrease of the maximum absorbance is also produced, which makes the assay not usable after a certain ionic strength value (see **Figure 4D**).

Figure 5A shows a standard calibration curve corresponding to the average of six assays performed on different days using two-well replicates, and immunoassay features are shown in **Table 3**. The IC_{50} and limit of detection (LOD; 90% of the zero dose) values accomplished were 2.86 ± 0.24 and 0.13 ± 0.03 μg L^{-1}, respectively, with a working range between 0.42 ± 0.06 and 19.2 ± 4.35 μg L^{-1} (20−80% of the assay response at zero doses).

Specificity studies were performed by preparing the calibration curves with several structurally related compounds and measuring them with the assay. As can be observed, most of the sulfonamides tested were highly recognized in this assay, with LOD values far below the MRLs of the EC (see **Table 4**). Only

Table 3. Features of the SPY ELISAs in Buffer and Milk Samples after Evaluation[a]

parameter	PBST curve	milk curve
$signal_{min}$	0.17 ± 0.05	0.04 ± 0.01
$signal_{max}$	1.81 ± 0.05	1.03 ± 0.04
slope	0.74 ± 0.07	0.93 ± 0.07
R^2	0.991 ± 0.005	0.997 ± 0.002

sample measured	buffer	milk (1/5)[b]	milk (1/5)[b]
IC_{50}, $\mu g\ L^{-1}$	2.86 ± 0.24	14.3 ± 1.20	13.0 ± 1.45
working range, $\mu g\ L^{-1}$	0.42 ± 0.06 to 19.2 ± 4.35	2.10 ± 0.30 to 96.0 ± 21.75	2.75 ± 0.70 to 59.5 ± 7.00
LOD, $\mu g\ L^{-1}$	0.13 ± 0.03	0.65 ± 0.15	1.10 ± 0.45

[a] Values obtained correspond to the average and standard deviation of each parameter of at least six assays performed on different days. [b] Real assay parameters of both methods for the analysis of milk samples (5 times diluted).

Table 4. Cross-Reactivity of Related Sulfonamide Compounds in the As155/**SA2**−OVA ELISA

	ELISA		
compound	IC_{50} ($\mu g\ L^{-1}$)	LOD ($\mu g\ L^{-1}$)	% CR[a]
sulfapyridine	2.25	0.15	100
sulfaquinoxaline	79.15	1.17	4
sulfachloropyridazine	61.81	1.34	5
sulfisomidine	2.10	0.23	140
sulfathiazole	1.30	0.13	202
sulfadiazin	14.05	0.38	15
sulfadimethoxine	41.22	0.99	8
sulfamerazine	3.73	0.43	79
sulfamethoxypyridazine	4.95	0.33	61
sulfamethazine	1.78	0.15	119
sulfamethoxazole	>MRL	>MRL	<1
sulfadoxine	>MRL	>MRL	<1
N^4-acetylsulfamethazine	>MRL	>MRL	<1
sulfanilamide	>MRL	>MRL	<1

[a] Cross-reactivity is expressed as a percent of the IC_{50} (nM) of the sulfapyridine divided by the IC_{50} (nM) of the other compounds tested.

a few congeners such as sulfamethoxazole or sulfadoxin were not sufficiently recognized, probably due to the methoxy groups present in these chemicals. Similarly, the N^4-acetylsulfamethazine was not recognized, which demonstrated the relevance of the free amino group of the *p*-aminobenzenesulfonamide moiety. The heterologous indirect ELISA format increased significantly the number of sulfonamide congeners detected in comparison with the direct homologous ELISA reported before (*29*), where only SPY, sulfamethoxypyridazine (SMP), sulfathiazole (STZ), and sulfachloropyridazine (SPC) antibiotics were recognized. It should be noted that it has already been reported that homologous antibody/competitor combinations tend to render more specific immunoassays (*28*, *30*).

Due to the interest of controlling the presence of antibiotic residues in milk samples, studies were performed to assess potential nonspecific matrix effects. With this purpose, standard curves were prepared in milk diluted several times with Milli-Q water and run in the ELISA (see **Figure 5**). Surprisingly, under these conditions, where the pH and conductivity of the milk samples were close to those of the buffer, the assay response did not reach that of the assay run in buffer and remained inhibited independent of the dilution applied (see **Figure 5A**). This matrix effect observed was overcome with small modifications of the analytical protocol, such as the concentration of the immunoreagents or using a reference milk free of antibiotics to build the calibration curves. Thus, almost identical curves were obtained by just increasing the concentration of the antibody and diluting the milk 5 times with Milli-Q water, as can be observed in **Figure 5A**. Thus, by increasing the antibody concentration added to the sample we accomplish the same maximum absorbance as that of the assay run in buffer. Therefore, by using this strategy the concentration of sulfonamide antibiotics in milk samples can be quantified using the calibration curve run in buffer. With these conditions, the LOD achieved in milk was 0.65 ± 0.15 $\mu g\ L^{-1}$. The same procedure was applied to different kinds of milk samples (whole, semiskimmed, and skimmed), certified by AESA, to assess if the strategy was suitable to analyze any type of milk. As can be observed in **Figure 5B**, reproducible calibration curves with excellent features could be obtained. Moreover, the immunochemical response was very similar independent of the type of milk used, which pointed to the possibility of using a representative milk as a reference material to build standard calibration curve for subsequent milk measurements. The almost negligible matrix effect caused by the milk samples has been attributed to the excellent features of the antibodies produced, being also crucial the fact of shaking the microtiter plates during the competition step, which minimized the formation of fat/protein layers in the bottom of the microtiter wells, improving assay reproducibility and accuracy (*31*−*33*). Thus, although several monoclonal (*9*, *34*, *35*), polyclonal (*36*−*38*), and recombinant antibodies (*10*, *14*) against sulfonamide antibiotics with different recognition patterns versus the different congeners have been reported, on only a very few occasions has direct performance of these antibodies in complex biological samples 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 ELISA has also been attempted, but high dilution factors (100 times) had to be applied to avoid matrix interferences (*37*). Alternatively, solid-phase extraction methods have been introduced prior to the immunochemical analysis. With regard to milk, homogenization and protein removal by precipitation with trichloroacetic acid or acetone followed by neutralization or dilution have been as some of the sample preparation methods reported to analyze this sample by immunoassay (*13*, *14*).

The *accuracy* of the assay was first evaluated in buffer by measuring several blind samples prepared in PBST. The results shown in **Figure 6** (left) correspond to the correlation found between the measured and the spiked concentration values. As can be observed, results obtained matched very well the spiked values. A slope near 1 was obtained (0.96) with a coefficient of correlation of $R^2 = 0.997$. Similarly, the accuracy of the assay when measuring whole milk blind samples was excellent. The correlation studies provided slopes close to 1 independent of whether the PBST or the whole milk calibration curves were used as reference (slope values were 1.07 and 0.95, respectively). Moreover, the coefficients of correlation were very good (PBST, 0.996; milk, 0.993), indicating the good accuracy of both methods to analyze milk samples.

Finally, for a preliminary evaluation of the performance of the ELISA as screening method, a set of milk samples spiked with distinct sulfonamides at different concentration levels was prepared at the Nestlé Research Centre (Lausanne, Switzerland) and measured with ELISA using SPY as the reference analyte. According to the immunoassay response the samples were classified as positive ($\geq 100\ \mu g\ L^{-1}$) or negative ($<100\ \mu g\ L^{-1}$). Data were collected as SPY immunoreactivity equivalents (SPY IR equiv) because this sulfonamide was used as reference in the standard curve. Subsequent data treatment consisted of the

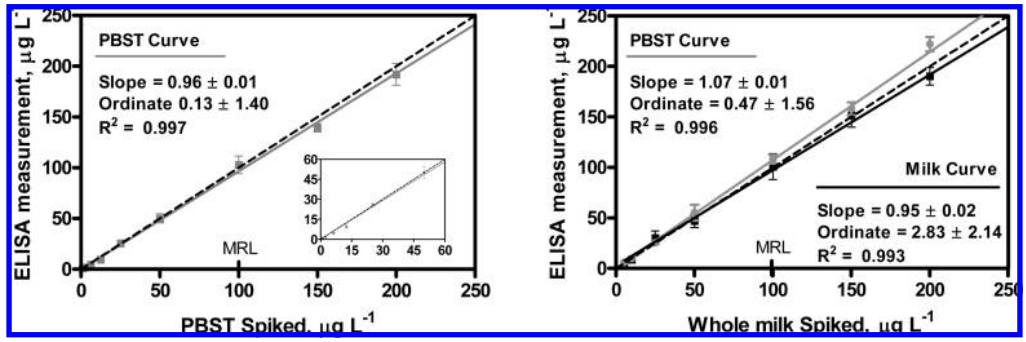


Figure 6. Results from the accuracy studies. The graphs show the correlation between the spiked and measured concentration values using the ELISA format to analyze buffer (left) and milk samples (right). Milk samples were measured both with the buffer and with reference milk calibration curves following the protocols described. The dotted line corresponds to a perfect correlation (slope = 1). The data correspond to the average of at least two replicates.

Table 5. Preliminary Evaluation of the ELISA as Screening Method

		calibration curve measurement[a]			
milk sample[b]		PBST (μg L^{-1})		milk (μg L^{-1})	
GF1 (SMZ, 10 ppb)	N	21 ± 2	N	11 ± 1	N
GF2 (STA, 10 ppb)	N	19 ± 1	N	12 ± 1	N
GF3 (SCP, 10 ppb)	N	75 ± 1	N	55 ± 9	N
GF4 (SMZ, 25 ppb)	N	35 ± 3	N	22 ± 2	N
GF5 (STA, 25 ppb)	N	44 ± 5	N	29 ± 3	N
GF6 (SCP, 25 ppb)	N	120 ± 9	FP	71 ± 6	N
GF7 (blank)	N		N		N
GF8 (SMZ, 50 ppb)	N	70 ± 4	N	69 ± 5	N
GF9 (STA, 50 ppb)	N	76 ± 6	N	56 ± 4	N
GF10 (SCP, 50 ppb)	N	137 ± 23	FP	106 ± 19	FP
GF11 (SMZ, 100 ppb)	P	127 ± 14	P	120 ± 11	P
GF12 (STA, 100 ppb)	P	146 ± 16	P	109 ± 15	P
GF13 (SCP, 100 ppb)	P	153 ± 32	P	165 ± 23	P

[a] Results are expressed as follows: first as equivalents of the sulfonamide spiked in the sample using sulfapyridine as the reference analyte to build the calibration curves (left) and as positive (P), negative (N), false positive (FP), and false negative (FN) according to MRLs established for sulfonamides by the EU (right). Antibiotics spiked in milk samples were sulfamethazine (SMZ), sulfathiazole (STA), and sulfachloropyridazine (SCP). [b] Blind samples were prepared at the Nestlé Research Center (Lausanne, Switzerland).

transformation of the equivalents of SPY detected in the milk samples to the respective sulfonamides using the assay cross-reactivity values shown in **Table 4**. The results obtained are summarized in **Table 5**. It is important to note the lack of false negatives for both protocols. The only false positives obtained corresponded to samples contaminated with sulfachloropyridazine (SPC); however, results indicated the presence of sulfonamides, although in reality they were below the MRLs. Confirmatory methods should in this case provide the final result to say if the levels of antibiotics in these samples are acceptable for consumption.

The analysis of the data obtained in this preliminary evaluation study of the use of the ELISA as a generic sulfonamide screening method evidence that the concentration of sulfonamides that cross-react far from the analyte selected to be used as the reference (SPY), like the already cited SPC, are more likely to be over- or underestimated, increasing the risk of false-positive or false-negative results. This situation could be overdriven by including on each microplate control samples of sulfonamides with different cross-reactivity patterns. This strategy, already suggested and demonstrated in a previous publication (*41*), would considerably increase the efficiency of the assay as screening method. Moreover, when the calibration curve prepared in milk is used as reference, the accuracy seems to be slightly better than if PBST is used as reference, which can be due to the particular properties of each type of milk samples. However, the approach of quantifying with a PBST curve provides acceptable results for use of this method with screening purposes.

In conclusion, high-quality class-selective antibodies have been produced using hapten **SA1** coupled to HCH as immunogen. The use of heterologous conditions using hapten **SA2** as competitor may have also played an important role in the recognition pattern obtained. The excellent features of these immunoreagents have allowed the setting up of a robust immunoassay that improves considerably, in terms of detectability and number of sulfonamides recognized, the direct ELISA format previously reported. The immunoassay performs very well in milk samples without any prior treatment other than dilution of the sample. Evaluation studies show that the immunoassay is able to detect 10 sulfonamides directly in milk samples at the MRL values. No false negatives have been observed, whereas false positives indicate the presence of sulfonamides, and these samples should always be followed by the use of confirmatory methods. However, further research has to be made for a complete validation in compliance with Commission Decision 2002/657/EC, which is still not completely clear for the evaluation of screening methods. Considering the successful analysis of sulfonamides in a complex biological sample, such as milk, there is great promise regarding the potential application of this immunoassay on new matrices such as water and soil samples, where antibiotics are considered to be emerging pollutants. Moreover, the immunoreagents produced in this work have been subsequently incorporated with success onto novel transducers with the objective of developing new biosensor devices for on-site measurements (*42*, *43*). On the other hand, investigations were also addressed to build a multiplexed device able to screen simultaneously for the presence of other relevant antibiotics that may contaminate milk or other dairy products.

Supporting Information Available: Preparation of haptens **SA3**−**SA10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review September 7, 2008. Revised manuscript received November 15, 2008. Accepted December 1, 2008. This work has been supported by the Ministry of Science and Technology (Contracts ALG2005-07700-C06-01, NAN2004-09195-C04-04, and TEC2004-0121-E) and by the European Community (IST-2003-508774 and KBBE2007-211326). 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).

JF8027655