

## Engineering of a Broad Specificity Antibody for Simultaneous Detection of 13 Sulfonamides at the Maximum Residue Level

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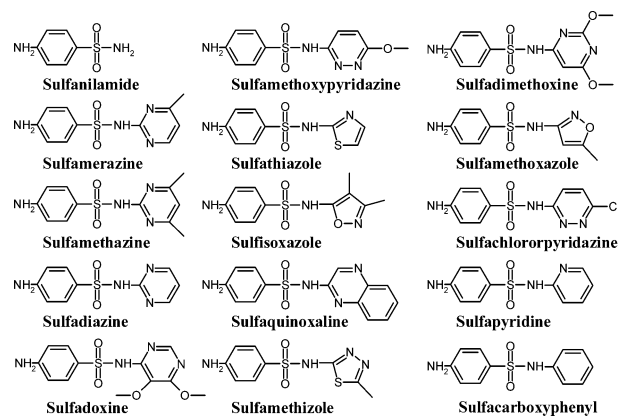
Sulfa antibiotics (sulfonamides) are a group of molecules sharing the *p*-aminobenzenesulfonamide moiety. Sulfonamides are used in veterinary and human medicine. Sometimes, the meat or milk of medicated animals is contaminated with residual sulfonamides. Current analytical methods for sulfonamides are unfit for screening of food, because they are either too laborious, insensitive, or specific for a few sulfa compounds only. A rapid immunoassay for detection of all sulfas in a single reaction would thus be useful. Previously, we used protein engineering to improve the broad specificity of sulfa antibody 27G3. In this study, we improved the best mutant of the previous studies with site-directed mutagenesis. The new mutants recognized different sulfonamides with affinities sufficient for detection of all 13 tested sulfonamides below the MRL level. We furthermore demonstrated the functionality of one mutant in some real sample matrices.

**KEYWORDS:** Food safety; drug residues; group specificity; phage display; sulfonamides; directed mutagenesis

## INTRODUCTION

Antibiotic molecules sharing the *p*-aminobenzenesulfonamide moiety (sulfa antibiotics or sulfonamides; **Figure 1**) are used in veterinary and human medicine for therapeutic and prophylactic purposes. Sulfonamides are also sometimes used as additives in animal feed, since prolonged ingestion of sulfas is thought to have growth-promoting effects. If the proper withdrawal periods are not observed before slaughtering or milking of the medicated animals, meat and milk from these animals may be contaminated with residual sulfonamides. It has been estimated that approximately 5% of the patients on sulfonamide medication receive unwanted symptoms from the drugs (*1*); thus, the presence of sulfonamide residues in food can be considered harmful to the consumers. A legal maximum residue limit (MRL) for sulfonamides has been set to 100 µg/kg in the United States and the European Union, whereas in Japan, for example, it has been set to 20 µg/kg (*2, 3*).

Current conventional sulfonamide detection methods capable of measuring a wide spectrum of different sulfonamides include bacteriological growth inhibition (*4–6*), chromatography (*7–13*), and capillary electrophoresis (*14, 15*). While these methods may be robust, immunochemical assays such as fluorescence immunoassay (FIA) have a number of advantages including speed, simplicity, and low costs over these methods. A number of immunochemical assays with these advantages have been developed demonstrating the suitability of immunochemical



**Figure 1.** Structures of selected sulfonamides.

assays for sulfonamide screening (*3, 16–21*), but unfortunately, each of these assays was capable of detecting only a single type of sulfonamide.

Immunochemical assays able to detect more than one type of sulfonamide with a sufficient sensitivity for the enforcement of the MRL have also been developed. Situ et al. (*22*) used a multichannel surface plasmon resonance biosensor approach and managed to build a fast assay for sulfamethazine (SHZ) and sulfadiazine (SDZ) in porcine bile, which they employed in an on-site study at an abattoir. Several authors (*1, 23–25*) managed to isolate monoclonal antibodies (Mab), which were capable of binding a few different sulfonamides with adequate affinity. Haasnoot et al. (*2*) isolated Mab 27G3, which bound a wide variety of sulfonamides with measurable affinity. Protein

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engineering of this antibody (26, 27) produced binders capable of detecting 10 of the tested 13 sulfonamides in a buffer system with an adequate sensitivity for screening of sulfonamide contamination below the EU MRL. Recently, using SHZ Mab 21C7 (19), which can bind a wide variety of sulfonamides, Haasnoot et al. (28) demonstrated the detection of eight sulfonamides below the MRL concentration from chicken serum with a surface plasmon resonance biosensor.

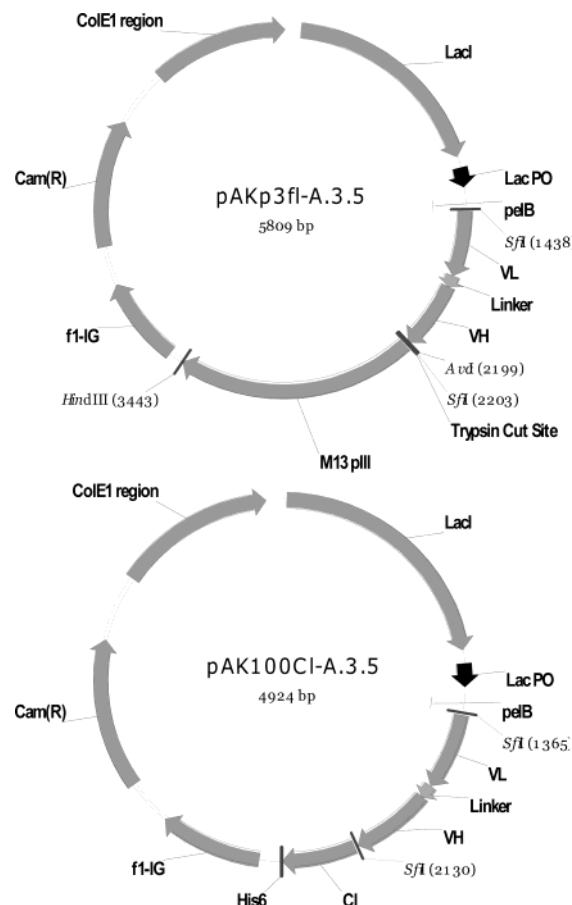
So far, there has not been an immunochemical assay that would be able to detect all sulfonamides with an adequate sensitivity so that all sulfas could be screened for in a single reaction. The main obstacle seems to be the development of an antibody capable of binding all of the different sulfonamides with affinities yielding sufficient assay sensitivity. In our previous studies (26, 27), we used protein engineering to improve Mab 27G3 (2) to recognize a wider range of structurally different sulfonamides with similar affinities. Several of the obtained mutants had significantly altered binding properties and improved broad specificity.

In this study, we aimed at improving the best mutant of the previous studies (mutant A.3.5) so that it would recognize all sulfonamides with an affinity sufficient for screening of foodstuffs. We used site-directed random mutagenesis to mutate selected residues of mutant A.3.5. The targeted residues were chosen based on their proximity to the assumed antigen binding site in a homology model of Mab 27G3 (27) and their tendency to mutate in the previous studies. The resulting new libraries were enriched with hyperphage display (29) using a SHZ derivative as the collection antigen. The binding properties of the new mutants were evaluated in a competitive time-resolved fluoroimmunoassay. The functionality of the best obtained mutant in different sample matrices was also briefly demonstrated by using it in the analysis of SHZ from semiskimmed milk, chicken serum, and minced beef samples.

## MATERIALS AND METHODS

**Strains, Plasmids, Reagents, and Instruments.** The bacterial host used throughout this work was *Escherichia coli* K12 strain XL1-Blue (Stratagene, La Jolla, CA). The vectors used (Figure 2) were constructed from the pAK series of vectors (30) in our lab. The original pAK vectors were obtained as gifts from the lab of Andreas Plückthun (Biochemisches Institut, Universität Zürich, Switzerland). The helper phage used in the phage production was hyperphage M13K07ΔpIII (*KanR*, Progen Biotechnik, Heidelberg, Germany). Recombinant single-chain antibody (scFv) A.3.5 was previously engineered in our lab (26, 27) from mouse antibody 27G3A9B10 (2), which was a gift from Willem Haasnoot (RIKILT-DLO, Wageningen, The Netherlands). The residue numbering of scFv A.3.5 follows the scheme devised by Kabat et al. (31) throughout this article.

All reagents used in the organic synthesis were commercially available and were of reagent grade or better. Rabbit Anti-mouse IgG-coated microtiter plates, dissociation-enhanced lanthanide fluoroimmunoassay (DELFI) assay buffer, DELFI wash solution, and DELFI enhancement solution were obtained from Perkin-Elmer Life Sciences (Turku, Finland). The biotinylation reagent Biotin-XX-NHS (catalog no. 203114) was purchased from Calbiochem (San Diego, CA). The 9dEu-chelate ( $\{[2,2',2'',2''']-[2-(4\text{-isothiocyanatophenyl})\text{ethylimino}]bis(\text{methylene})bis\{4-[[4-(\alpha\text{-galactopyranoxyl})\text{phenyl}]ethynyl\}pyridine-6,2\text{-diyl}\}bis(\text{methylenenitrilo})\}tetrakis(\text{acetato})\}europium(\text{III})$ ) for tracer labeling was synthesized in house according to protocols from Innorad Diagnostics (Turku, Finland). The LB agar plates, SB medium, and SOC medium were prepared as described previously (32). The antibiotics used in the cultures were obtained from Sigma-Aldrich (Helsinki, Finland). The concentrations of the antibiotics were as follows: ampicillin, 100 mg/L; chloramphenicol, 25 mg/L; tetracycline, 5 mg/L; and kanamycin, 50 mg/L. Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was purchased from Promega (Madison, WI). The various sulfa



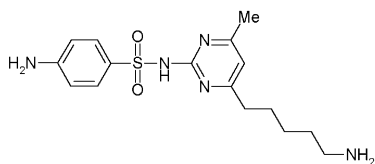
**Figure 2.** Plasmid vectors used. Two different plasmids constructed in our lab were used as the gene-carrying vectors in this work. The *E. coli* propagation signal (*ColE1*), the M13 phage packaging signal (*f1-IG*) and the chloramphenicol resistance selection marker [*Cam(R)*] are needed for the functioning of the vectors as phagemids. ScFv antibody ( $V_L$ ,  $V_H$ , and linker-domains are separately marked) expression from both plasmids is controlled by the *lac* promoter (*lac PO*) repressed by the *lac* inhibitor (*lacI*). The signal sequence (*pel B*) activates transport of scFv to periplasm for folding. The full length M13 phage protein III (*M13 pIII*) fused to the N terminus of the scFv by a short trypsin cut site facilitates the display of scFv antibodies on the surface of M13 hyperphage. The infectivity function of protein III is restored by trypsin digestion, which removes the fused scFv. The antibody constant light domain (*C*) enables scFv immobilization on rabbit anti-mouse IgG-coated plates.

antibiotics (Figure 1), fraction V bovine serum albumin (BSA), and aseptic trypsin from bovine pancreas were purchased from Sigma-Aldrich.

$^1\text{H}$  NMR spectra were recorded at 400 MHz on a JNM-GX-400 spectrometer (JEOL, Peabody, MA) or at 200 MHz on a AM200 spectrometer (Bruker, Täby, Sweden). The chemical shifts are given in ppm from internal tetramethylsilane. Time-resolved fluorescence was measured with a Victor 1420 Multilabel Counter (Perkin-Elmer Life Sciences).

**Synthesis of 6-Methyl-N1-[[4-(5-amino)pentyl]-2-pyrimidyl]-sulfanilamide Monohydrochloride.** (6-*N*-*tert*-Butoxycarbonylamino)-caproic Acid  $\alpha$ -*N*-Methoxy-*N*-methylamide (A). A was synthesized as previously described (26).

(9-*N*-*tert*-Butoxycarbonylamino)-non-2-yn-4-one (B). Propynylmagnesium bromide (12.4 mL, commercially available as 0.5 M in tetrahydrofuran) was added dropwise to a cooled ( $-78^\circ\text{C}$ ) solution of A (0.68 g) in dry ether (60 mL). The reaction mixture was stirred for 2 h, allowed to warm to room temperature, and stirred overnight. The reaction mixture was poured onto a vigorously stirred mixture of  $\text{Et}_2\text{O}$  (80 mL), 1 M  $\text{KH}_2\text{PO}_4$  (160 mL), and ice. The phases were separated, and the water phase was extracted with  $\text{Et}_2\text{O}$  ( $3 \times 60$  mL). The



**Figure 3.** SHZ derivative with an amino linker. 6-Methyl-N1-([4-(5-amino)pentyl]-2-pyrimidyl)sulfanilamide was synthesized for biotinylation and for labeling with an Eu-chelate.

combined organic phases were washed with aqueous 1 M  $\text{KH}_2\text{PO}_4$  ( $2 \times 60$  mL) and brine ( $2 \times 60$  mL), dried ( $\text{Na}_2\text{SO}_4$ ), and evaporated. The product was purified with flash chromatography (FC) using silica gel and 30% ethyl acetate in petroleum ether.

{2-Amino-6-methyl-[4-(5-*N*-*tert*-butyloxycarbonylamino)pentyl]-pyrimidine (C). A mixture of **B** (0.128 g) and guanidium nitrate (0.074 g) in ethanol (15 mL) was heated in an oil bath at 66–68 °C until the guanidium nitrate dissolved. A solution of NaOH in water (0.05 g/0.5 mL) was added. The reaction mixture was stirred at this temperature for 6 h and allowed to cool to room temperature before evaporation. The product was purified with FC (silica gel, gradient from 2% MeOH/dichloromethane to 8% MeOH/dichloromethane).

*N*-Acetyl-6-methyl-[4-(5-*N*-*tert*-butyloxycarbonylamino)pentyl]-2-pyrimidyl)sulfanilamide (D). **C** (0.06 g) was dissolved in dry pyridine and cooled in an ice bath. A cooled solution of *N*-acetylsulfanil chloride (0.055 g) in dry pyridine was added dropwise. The reaction mixture was stirred overnight at room temperature, evaporated, and dissolved in dichloromethane (10 mL). The solution was washed with brine (5 mL), dried ( $\text{Na}_2\text{SO}_4$ ), and evaporated. The product was purified with FC (silica gel, first 3/5/1 petroleum ether/ethyl acetate/triethylamine and finally 10% MeOH/dichloromethane).

6-Methyl-N1-([4-(5-*N*-*tert*-butyloxycarbonylamino)pentyl]-2-pyrimidyl)sulfanilamide (E). **D** (0.085 g) was dissolved in 2 M NaOH (20 mL) and stirred at room temperature overnight. NaCl was added to the reaction mixture followed by chloroform (30 mL). The phases were separated, and the water phase was extracted with chloroform ( $2 \times 35$  mL). The combined organic phases were dried ( $\text{Na}_2\text{SO}_4$ ) and evaporated. The product was purified with FC (silica gel, gradient from 2% MeOH/dichloromethane to 8% MeOH/dichloromethane).

6-Methyl-N1-([4-(5-amino)pentyl]-2-pyrimidyl)sulfanilamide. **E** (0.022 g) was dissolved in 1 M HCl and stirred overnight at room temperature. The reaction mixture was evaporated, and the product (**Figure 3**) was used without further purification.  $^1\text{H}$  NMR (200 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  7.87 (d, 2H,  $J = 8.6$  Hz), 7.37 (d, 2H,  $J = 8.6$  Hz), 6.59 (s, 1H), 2.75 (t, 2H,  $J = 7.5$  Hz), 2.43 (t, 2H,  $J = 7.5$  Hz), 2.18 (s, 3H), 1.50–1.29 (m, 4H), 1.22–1.00 (m, 2H). MS (EI)  $m/z$  (relative %): 193 (100), 291 (45), 349 (10)  $\text{M}^+$ .

**Biotinylation and Labeling of the Sulfonamide Derivatives.** Biotin-XX-NHS (8.2 mg) was added to the solution of 6-methyl-N1-([4-(5-amino)pentyl]-2-pyrimidyl)sulfanilamide (4.3 mg) in a mixture of pyridine, water, and triethylamine (90:15:1, 0.9 mL). The reaction mixture was stirred overnight at room temperature. The product was purified with reversed phase high-performance liquid chromatography (RP-HPLC) using a 150 mm  $\times$  4.6 mm, 5  $\mu\text{m}$  HyPurity Elite C18 column (Thermo Hypersil, Runcorn, U.K.) with a gradient from aqueous 0.1% TFA to acetonitrile in 30 min and a flow rate of 1.0 mL/min. The peak was detected at 265 nm,  $t = 23.55$  min. The product, from hereon referred to as biotinylated SHZ (Bio-SHZ), was dried and dissolved to 1 mL of dimethyl formamide (DMF). Because the final product mass was too low to be measured with our equipment, dilution of the stock solution used is given in the text instead.

6-Methyl-N1-([4-(5-amino)pentyl]-2-pyrimidyl)sulfanilamide (5.0 mg) was dissolved in aqueous 50 mM  $\text{CO}_3^{2-}$  buffer and stirred for 5 min before an equimolar amount of 9dEu-chelate was added. The reaction mixture was stirred overnight at room temperature. The product was purified with RP-HPLC using a 150 mm  $\times$  4.6 mm, 5  $\mu\text{m}$  Hypersil ODS column (Thermo Hypersil), a gradient from 95% A and 5% B to 100% B in 30 min, and a flow rate of 1.0 mL/min (A = aqueous 20 mM TEAA and B = 20 mM TEAA/50% acetonitrile). The peak was

**Table 1.** Site-Directed Mutagenesis of scFv A.3.5 Amino Acid Residues

residue	in library	residue	in library
L:D28	T, A, N, or D	L:K30	any
L:Y32	any	L:N34	F, L, I, M, V, S, P, T, A, Y, H, Q, N, K, D, E
L:R46	K, R	L:Y49	F, S, Y, C, I, T, N, S
L:Q50	any	L:G51	G, A, V
L:N53	any	L:T92	I, T, N, S
L:F94	F, Y, L, H, I, N	L:Q96	L, I, M, V, H, Q, N, K, D, E
H:Y32	any	H:G33	P, Q, R, A, E, G
H:M34	L, I, M	H:T35	T, N, S
H:N52	T, A, N, K, D, E	H:A52a	I, T, S, V, A, G
H:R94	K, R	H:R95	K, R
H:G97	A, G	H:G98	A, G
H:G99	A, G		

**Table 2.** Sequences of the PCR Primers

primer	sequence <sup>a</sup>
ScRev:	5'-GGAATTCGGCCCCGAGGCC-3'
ScFor:	5'-TTACTCGCGGCCAGCCGGCCATGGCG-3'
Mut1:	5'-GGAGACTGGCCTGGCCTCTGTAACATCCA-SDNCAASNNNTGTSNNGCCRKVACTATGTAA-GAGGCTCTGACTTGACTTGC-3'
Mut2:	5'-CAGAGGCCAGGCCAGCTCCAAAGARRCT-AATCNWYNNNSGBNTCTNNSCTGGACTCTGG-GGCCCTGAC-3'
Mut3:	5'-CTTGGAGCCTCTCCGAACGTNWBGGG-RWDATGRNTACCTTGCCAGCAATAATA-ACCC-3'
Mut4:	5'-ACGTTTCGGAGGAGGCTCCAAG-3'
Mut5:	5'-CTGTAACACCTTTCTGGAGCCTGCTTCAC-CCARBTYAKYBSSNNGTTTGTAAGGTATA-CCCAGAGGCC-3'
Mut6:	5'-CAGGCTCCAGGAAAGGGTTTACAGTGGATG-GGCTGGATARMNRBYTACACTGGGAGCCA-ACATATGCAG-3'
Mut7:	5'-GAGTCCCTTGCCCCAGTAAGCNSCNSCNS-CATCYTYTYYTGCACAGAAATATGTAGCCAT-GTCCTC-3'
Mut8:	5'-GCTTACTGGGGCCAAGGGACTC-3'
Mut9f:	5'-CAGAGGCCAGGCCAGTCTCC-3'
Mut9r:	5'-GGAGACTGGCCTGGCCTCTG-3'

<sup>a</sup> Degenerated bases are in bold, and *Sfi*I restriction sites are italicized.

detected at 320 nm,  $t = 20.52$  min. The product, from hereon referred to as Eu-labeled SHZ (Eu-SHZ), was dried and dissolved to 1 mL of DMF.

**Site-Directed Mutagenesis Libraries.** On the basis of the model of antibody 27G3 (27) and the mutation data of previous studies (26, 27), several amino acid residues of the CDR loops of scFv A.3.5 were selected for site-directed mutagenesis (**Table 1**). A selection of degenerated oligonucleotides (**Table 2**) was synthesized in order to construct libraries with the desired mutations in the gene of scFv A.3.5. A selection of scFv A.3.5 gene fragments was polymerase chain reaction (PCR) amplified with appropriate primer pairs, the resulting fragments were purified with agarose gel electrophoresis, and then, four different libraries were assembled with strand overlap extension PCR (SOE-PCR, 33). Lib 1 was assembled from PCR fragments given by primer pairs ScFor-Mut9r, Mut2-Mut7, and Mut8-ScRev; Lib 2 was built from PCR fragments amplified by primer pairs ScFor-Mut1, Mut9f-Mut3, and Mut4-ScRev; Lib 3 was put together from PCR fragments generated by primer pairs ScFor-Mut1 and Mut2-ScRev; and finally, Lib 4 was constructed from PCR fragments ScFor-Mut5 and Mut6-ScRev. A typical PCR reaction had 20 mM Tris-HCl (pH 8.8), 10 mM  $(\text{NH}_4)_2\text{SO}_4$ , 10 mM KCl, 0.1% Triton X-100, 0.1 mg/mL BSA, 2 mM  $\text{MgSO}_4$ , 200  $\mu\text{M}$  each dNTP, 40 ng each of appropriate template fragment(s),



1.25 U *Pfu*-polymerase, and 0.4  $\mu$ M of each primer in a total reaction volume of 50  $\mu$ L. The PCR program used was 95 °C for 2 min, 14 cycles of 95 °C for 1 min, 54–56 °C (depending on the primers) for 1 min and 72 °C for 35 s, and the final extension of 72 °C for 5 min.

The PCR products (about 2.5  $\mu$ g per library) were cloned to pAKp3fl phagemid vector (**Figure 2**) using the *Sfi*I sites. The ligation reactions were precipitated with ethanol and transformed to electrocompetent *E. coli* XL1-Blue cells with electroporation in six separate aliquots each, yielding approximately  $2 \times 10^7$  transformants per library (determined with platings on LB/chloramphenicol agar). Electroporated cells were diluted to 20 mL total volume in SOC medium, and they were incubated in shake flasks at 37 °C with 200 rpm for 1 h. Cultures were then diluted to 100 mL with SB medium containing tetracycline, chloramphenicol, and 0.2% glucose. Growth was continued at 37 °C with 240 rpm, until the optical density ( $OD_{600nm}$ ) of the cultures reached 0.5.

The cultures were infected with  $2.5 \times 10^{11}$  pfu of helper phage M13K07 $\Delta$ pIII each for 15 min at 37 °C without shaking. Cells were pelleted with centrifugation (3000g, 10 min, 4 °C) and resuspended in 20 mL of SB medium containing tetracycline, chloramphenicol, and 0.2% glucose. After 2 h of growth at 30 °C with 240 rpm, IPTG and kanamycin were added to the cultures to final concentrations of 500  $\mu$ M and 70 mg/L, respectively. Phage production was continued overnight (26 °C, 220 rpm). After the bacteria were removed by centrifugation, the phages were precipitated twice by adding poly(ethylene glycol) 8000 (Sigma-Aldrich) to 5% and NaCl to 4%. Phages were finally dissolved in 200  $\mu$ L of TBS/BSA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% BSA) and stored at 4 °C.

**Enrichment of the Libraries.** All four libraries were enriched with three rounds of hyperphage display (29). The wells of a streptavidin-coated microtiter plate (Innotrac Diagnostics) were coated by incubating an appropriate dilution of Bio-SHZ (panning round 1, dilution 1:3000; 2, 1:3000; and 3, 1:20 000) in 200  $\mu$ L of assay buffer each well for 1 h in slow shaking after which the wells were washed four times with 200  $\mu$ L aliquots of wash solution. Phage libraries were diluted in TBT-0.05 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Sigma-Aldrich fraction V BSA, and 0.05% Tween20) to final concentration of  $3 \times 10^{13}$  (for first round) or  $5 \times 10^{11}$  (for rounds 2 and 3) pfu/mL. Aliquots of 200  $\mu$ L of each diluted library were applied to one Bio-SHZ-coated microtiter well and one uncoated well. The microtiter plate was incubated in slow shaking for 1 h at 25 °C and washed 10 times as above. Phages were eluted by adding 200  $\mu$ L of TBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) with 14  $\mu$ g/mL of trypsin to each well and incubating the plate in 37 °C for 1 h without shaking.

One milliliter of fresh XL1-Blue cells was mixed with each batch of eluate. Cells were incubated for 30 min at 37 °C without agitation, and serial dilutions of each cell batch were plated on LB/chloramphenicol agar. Shake flasks containing 20 mL of SB medium with tetracycline, chloramphenicol, and 0.2% glucose were inoculated with 1 mL of infected cell suspension from each of the replicas that had been eluted from the Bio-SHZ-coated wells. The flasks were shaken at 37 °C with 300 rpm, until the  $OD_{600nm}$  of the cultures reached 0.4. The cells were then infected with  $5 \times 10^{10}$  pfu of helper phage M13K07 $\Delta$ pIII each, and the new recombinant phage preparations were produced as described above and used as the starting material of the next round of enrichment.

**Isolation of Active Clones.** The genes of 20 random clones from each enriched library were transferred to the pAK100Cl vector (**Figure 2**) using the *Sfi*I restriction sites, and cell lysates were prepared for antibody activity measurements as previously described (26). SHZ binding activity was determined using a time-resolved DELFIA (34). Rabbit anti-mouse IgG-coated microtiter plates were prewashed once with wash solution. A 100  $\mu$ L aliquot of assay buffer was applied to each well followed by 100  $\mu$ L of the cell lysate sample. The plates were incubated for 1 h at room temperature with gentle shaking and washed four times as above. To each well, 200  $\mu$ L of assay buffer containing 10 ng/mL of Eu-SHZ was applied. The plates were incubated for 1 h at room temperature with gentle shaking and washed four times as above. A 200  $\mu$ L aliquot of enhancement solution was added to each well. After 30 min of incubation in gentle shaking, time-resolved fluorescence was measured.

**Characterization of the Active Clones.** Active antibody mutants were sequenced in order to ascertain that each contained different mutations and that they were therefore different mutants. These mutants were also produced in 100 mL cultures as previously described (26), and their cross-reaction profiles for different sulfonamide antibiotics were determined. Rabbit anti-mouse IgG-coated microtiter plates were coated with the mutant antibodies as above. To each well, 100  $\mu$ L of assay buffer containing 6 ng/mL of Eu-SHZ was applied, followed by a 100  $\mu$ L aliquot of assay buffer containing varying concentrations of different sulfonamides (**Figure 1**). The plates were incubated for 1 h at room temperature with gentle shaking and washed four times. Time-resolved fluorescence was measured as above. Six different concentrations were measured for each sulfonamide in order to obtain data points for the whole range of inhibition, and sigmoidal curves were fitted to these data. The  $R^2$  values obtained from the comparison of the fitted curves to the raw data varied between 1 and 0.97, being almost always better than 0.99.

**Mutant Functionality in Real Sample Matrices.** To demonstrate that the new mutants of this study also work in real sample matrices, M.3.4 was applied to the measurement of SHZ from semiskimmed milk, minced beef, and chicken serum. Semiskimmed milk (pasteurized, unhomogenized, approximately 1.5% fat) was spiked with varying concentrations of SHZ and incubated for 24 h in 4 °C. The milk samples were then briefly vortexed and diluted 10-fold in assay buffer before the assay.

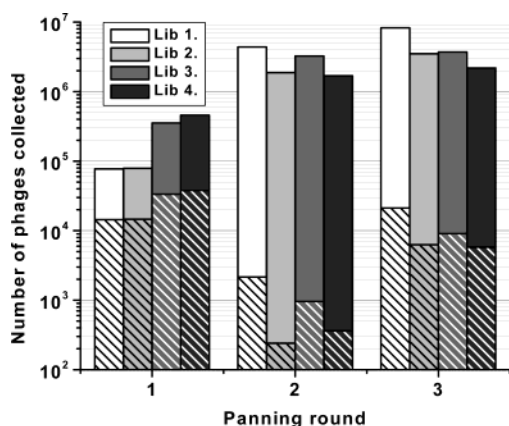
Minced beef was spiked with varying concentrations of SHZ, thoroughly mixed, and incubated for 24 h at 4 °C. To each sample of beef, 10 mL/g of assay buffer was then added and the sample was thoroughly vortexed. After the sample was centrifuged (4000g, 20 min), the supernatant was used in the assay.

Chicken serum was spiked with varying concentrations of SHZ, thoroughly mixed, and incubated for 1 h in 25 °C. The serum samples were then briefly vortexed and diluted 10-fold in assay buffer before the analysis.

Rabbit anti-mouse IgG-coated microtiter plates were coated with scFv M.3.4 as above. After they were washed, 100  $\mu$ L of assay buffer containing 12 ng/mL of Eu-SHZ was applied to each well, followed by a 100  $\mu$ L aliquot of the sample. The plates were incubated for 30 min at room temperature with gentle shaking and washed four times. A 200  $\mu$ L aliquot of enhancement solution was added to each well, and after 30 min of incubation with gentle shaking, time-resolved fluorescence was measured. Six different concentrations were measured for each sulfonamide, and sigmoidal curves were fitted to these data. The  $R^2$  values obtained from the comparison of the fitted curves to the raw data varied between 1 and 0.98.

## RESULTS

**Construction and Enrichment of the Site-Directed Mutagenesis Libraries.** On the basis of the model of antibody 27G3 (27) and the mutation data of previous studies (26, 27), a selection of amino acid residues in the CDR loops of scFv A.3.5 was targeted for site-directed mutagenesis (**Table 1**). Residues L:D28, L:K30, L:Y32, L:Q50, L:G51, L:N53, H:T35, H:N52, and H:A52a were picked for mutagenesis, because in the model the side chains of these residues were located in close proximity to the assumed antigen binding site and because several mutants with mutations in these residues were found in the previous studies (26, 27). The rest of the residues in **Table 1** were selected for mutagenesis, regardless of the fact that no mutations in these residues had been found in the previous studies (26, 27), because in the model these residues were located close by the assumed antigen binding site of the antibody. When deciding the variety of amino acid changes applied to each residue, the environment of each residue in the model was considered and only mutations that we believed each particular location could accommodate were chosen. The variety of mutations selected for each residue is shown in **Table 1**. A set of primers with degenerated bases for the introduction of the planned mutations to the scFv A.3.5



**Figure 4.** Number of phages collected during each panning round. Shown for each of the four different libraries is the number of phages collected from the input pool ( $3 \times 10^{13}$  phages for first round and  $5 \times 10^{11}$  phages for rounds 2 and 3) with the panning antigen (the bars) and by unspecific binding (the shaded area) during each round of panning.

gene were designed (Table 2). Four different mutant libraries were assembled with these primers in SOE-PCR reactions. In each of these four libraries, only two structurally adjacent CDR loops had mutations (Table 1) in them (Lib 1, CDR-H3 + CDR-L2; Lib 2, CDR-L1 + CDR-L3; Lib 3, CDR-L1 + CDR-L2; and Lib 4, CDR-H1 + CDR-H2).

All four PCR products were cloned to a pAKp3fl phagemid vector (Figure 2). The scFv mutants were produced as fusions to full-length filamentous phage coat protein III from this vector. The coat protein III had an artificial trypsin cut site engineered to the N terminus of the protein. The usage of vector pAKp3fl enabled the enrichment of the mutant libraries with hyperphage display (29). After transformation to *E. coli* XL1-Blue cells, the libraries contained approximately  $2 \times 10^7$  independent clones each.

The libraries were biopanned with three rounds of hyperphage display (29), where sulfa binders were repeatedly enriched by collecting phages with Bio-SHZ attached to streptavidin-coated microtiter wells followed by phage amplification *in vivo*. The amount of collection antigen used was reduced with each passing round (panning round 1, Bio-SHZ dilution 1:3000; 2, 1:3000; and 3, 1:20 000). Figure 4 shows how many phages were collected from each library with the SHZ antigen during each round of panning and how many of those were collected by unspecific background binding. The increasing number of collected phages as the rounds progress, even when the amount of collection antigen decreases, and the fact that the unspecific background stays in very low levels indicates that phages displaying antigen binders were indeed enriched.

**Isolation of Active Clones.** For the purpose of isolating the active clones from the libraries, phagemid DNA was purified from cultures of cells infected with each enriched library and scFv genes were transferred to the pAK100CL vector (Figure 2) with *SfiI* digestion and ligation. Expressed from this vector, scFv antibodies were produced as mouse IgG constant light domain fusions, which enabled immobilization on anti-mouse IgG-coated surfaces. Twenty individual clones from each of the four libraries were grown in minicultures and Eu-SHZ binding activity was measured. Of the selection of 20 clones picked from library 1, seven clones showed significant ( $S/N > 7$ ) SHZ binding activity, whereas five of the library 2 clones and six of the library 3 clones also did so. Not a single clone with significant Eu-SHZ binding activity was found from library 4. Several clones from all enriched libraries displayed measurable

**Table 3.** Performance of Selected Clones in a Competitive Sulfonamide Assay for 13 Different Sulfonamides

sulfa <sup>a</sup>	IC <sub>50</sub> concentration ( $\mu\text{g L}^{-1}$ )					
	27G3	A.3.5	M.1.12	M.1.17	M.3.1	M.3.4
SMT	1.2	0.24	0.11	0.029	0.019	0.05
STZ	8.7	2.5	0.12	0.33	0.085	0.15
SCP	9	1.3	0.41	0.24	0.066	0.19
SMP	19	3.1	0.63	0.38	0.12	0.25
SDO	22	0.83	0.44	0.23	0.11	0.11
SPY	35	9.6	1.3	1	0.27	0.42
SMX	69	6.8	0.94	0.58	0.28	0.34
SDZ	160	5.8	0.48	0.47	0.26	0.24
SMZ	310	11	0.33	0.83	0.26	0.68
SFX	420	19	3.8	3.4	2.21	2.75
SQX	760	230	18	31	16	14
SDM	1100	360	35	60	23	48
SHZ	4200	480	29	64	47	19

<sup>a</sup> Sulfonamides not previously defined are abbreviated as follows: sulfathiazole (STZ), sulfachloropyridazine (SCP), sulfamethoxypyridazine (SMP), and sulfamerazine (SMZ).

**Table 4.** Performance of Mutant M.3.4 in a Competitive Assay of SHZ from Different Sample Matrices

sample	IC <sub>50</sub> ( $\mu\text{g L}^{-1}$ )	$R^2$
buffer	20	0.98
1:10 semiskimmed milk	34	0.98
1:10 chicken serum	27	1.00
1:10 minced beef	40	1.00

binding activity ( $S/N > 2$ ), but these were judged to be binders of low affinity and were not chosen for further study. It is likely that site-directed mutagenesis of antibody CDR loops produces libraries of mostly inactive mutants. The high frequency presence of significantly and weakly active SHZ binding clones in the enriched libraries clearly tells us that the panning process with hyperphage display had resulted in enrichment of SHZ binders.

**Characterization of Active Clones.** Each isolated antibody mutant displaying significant Eu-SHZ binding activity ( $S/N > 7$ ) was sequenced in order to ascertain that each of the mutants had a unique sequence (results not shown). The mutants were also produced in 100 mL cultures and tested with a time-resolved competitive sulfa fluoroimmunoassay for their ability to bind SHZ. In this assay, sulfonamides compete with the Eu-SHZ tracer for binding to the selected antibody. The concentration of SHZ needed to inhibit 50% of the tracer binding (IC<sub>50</sub>) was calculated for each mutant antibody (data not shown). Four clones with the lowest IC<sub>50</sub> values for SHZ (ranging from 30 to 60  $\mu\text{g/L}$ ) were picked for further study. Two of these clones were from library 1 and two from library 3. The performance of these mutants in a competitive sulfa assay for 13 different sulfonamides is shown in Table 3. The results show that not only are nine of the sulfonamides recognized with the IC<sub>50</sub> values varying within a range of little over a decade but also the IC<sub>50</sub> values for all sulfas are well below the 100  $\mu\text{g/L}$  MRL, in the case of all four of the characterized mutants.

**Mutant Functionality in Sample Matrices.** To briefly demonstrate that the mutant with the lowest IC<sub>50</sub> for SHZ, M.3.4, also works in some real sample matrices, the measurement of SHZ from semiskimmed milk, minced beef, and chicken serum was done. Table 4 shows the IC<sub>50</sub> values of SHZ and the  $R^2$  values of the sigmoidal fits in each of these sample matrices diluted 10-fold in assay buffer before the assay. The data refer to the actual SHZ concentration in the assay reaction. The results

show that the  $IC_{50}$  value of SHZ is only slightly affected by the sample matrices. The worst effect is observed in the minced beef matrix, which gives a 2-fold increase in the  $IC_{50}$  value. Nevertheless, a MRL contamination of each sample matrix (100  $\mu\text{g/g}$  of SHZ in sample), diluted 10-fold for the assay, reduced the assay signal about 25%, demonstrating that an illegal sample contamination should be detected with an immunoassay using mutant M.3.4.

## DISCUSSION

**Libraries and Enrichment.** A lot of research (1, 2, 23–27) has revolved around the subject of developing antibodies capable of binding all different sulfonamide antibiotics with an affinity strong enough for detection of sulfas below the established MRL (100  $\mu\text{g/L}$ ). However, despite the effort, such antibodies have so far not been isolated. In our previous studies (26, 27), we set out to transform a monoclonal sulfa antibody 27G3 to the direction of generic binding. We first used random mutagenesis of the whole scFv antibody gene combined to enrichment with phage display followed by, in the later study, recombination of the enriched mutants to produce the much improved antibody A.3.5. This antibody, although significantly improved over 27G3, still failed to detect three of the 13 sulfonamides below the MRL concentration.

As a result of the previous studies, we had an abundance of mutational data concerning antibody 27G3. This enabled us to undertake the current study with the goal of improving mutant A.3.5 using site-directed saturation mutagenesis: A thorough mutagenesis was targeted to the areas of the CDR loops deemed important for sulfa binding on the basis of the mutational data and the antibody homology model constructed previously (27). The advantages of site-directed mutagenesis are tremendous, since it enables the complete screening of the whole mutational space of a few residues of a protein. Before such an approach can be used, however, enough data on the protein is needed so that correct residues can be targeted. Four libraries in total were constructed, each having mutations targeted to the residues of two adjacent CDR loops. Together, the libraries covered all of the CDR loops of antibody A.3.5.

Generic antibodies probably often have only low or medium affinities for their antigens (since specificity and affinity are somewhat dependent on each other), so for the enrichment of the libraries we decided to use the new hyperphage display (29). The essence of this technique is the use of helper phages, which do not produce any wild-type pIII, thus all  $\sim 5$  pIII proteins on the surface of each recombinant phage have a scFv fragment fused to them, the phagemid vector being the only source of pIII protein. These recombinant phages can bind multivalently to antigen-coated surfaces of sufficient antigen density, thus giving better signal-to-noise ratios in panning reactions than phages with only a lone scFv displayed on their surface. This is important when panning low or medium affinity binders so that specific binding is not lost in the background. However, it has to be remembered that since each hyperphage has the same number of scFv on their surface, high-affinity antibody mutants have a competitive edge over low-affinity mutants in enrichment, even if these low-affinity antibody mutants have better expression properties in *E. coli*. This is because the mutants with better expression yields do not end up producing phages with higher valency than the low expression yield mutants, which is the case in ordinary phage display.

We synthesized a Bio-SHZ derivative for use as the collection antigen, since SHZ was the sulfonamide that antibody A.3.5 recognized with the worst affinity and we wanted to improve

the recognition of this sulfa more than any other. By enriching the four mutant libraries with hyperphage display with Bio-SHZ as the collection antigen, we managed to get rapid enrichment of binders in only three rounds; furthermore, the unspecific background of each panning round was very small (Figure 4). The panning reactions were performed in microtiter wells coated with the collection antigen prior to addition of phage libraries so that the hyperphage could bind multivalently on the randomly distributed antigen on the well surface.

**Mutant Cross-Reaction Profiles.** There have been a few studies (3, 24), which have modeled the minimum energy conformations of different sulfonamides and then calculated the electrostatic and steric properties of these conformations. These properties seem to vary considerably between different sulfonamides in the minimum energy conformation, which seems to suggest that any generic antibody must bind and thereby stabilize some higher energy conformation of each sulfonamide, which is supported by the fact that sulfonamides have rather flat potential energy maps of rotation around the dihedral angles (3); therefore, a range of conformations only slightly more energetic than the minimum are available.

All of the new mutants isolated in this study seemed to behave in quite a similar manner. Sulfamethizole (SMT) was the sulfonamide most readily recognized by the new mutants followed by nine other sulfonamides, which had their  $IC_{50}$  values spread within a range of 2 orders of magnitude from the  $IC_{50}$  of SMT, mutant M.1.12 having the narrowest range of them all (a little over 1 order of magnitude). The case of M.1.12 was a clear improvement over A.3.5, which had a cross-reaction profile spread across 2 orders of magnitude for the same 10 sulfonamides. However, all of the new mutants had on the average a 10-fold improvement of the  $IC_{50}$  value of each tested sulfonamide over A.3.5, bringing all of the mutant  $IC_{50}$  values below the MRL of 100  $\mu\text{g/L}$ .

It seems that the sulfonamide binding of the new mutants is not affected whether the  $N^1$  ring of the sulfonamide in question is of the five or six carbon variety, since, for example, sulfamethoxazole (SMX) and sulfapyridine (SPY) are recognized with almost the same affinity by all of the new mutants. However, it seems that there are a few details in the structure of sulfonamides, which are responsible for the poorer binding of sulfaquinolaxine (SQX), sulfadimethoxine (SDM), and SHZ. First of all, it seems to be beneficial for recognition that there is a nitrogen atom (as opposed to a carbon or an oxygen for example) in the  $N^1$  ring in an ortho position of the ring as shown, for example, by the 5-fold better affinity of the new mutants for SMX over sulfisoxazole (SFX). Second, it seems to be not favorable affinitywise, if both meta positions or both ortho positions of the  $N^1$  ring have methyl or larger groups attached, as demonstrated, for example, by the 100–200-fold better affinity of the new mutants for sulfadoxine (SDO) over SDM, which seems to implicate that upon binding the  $N^1$  ring of the sulfonamides turns so that any methyl or larger groups face away from the binding site surface if possible (for example, SMT and SDO can accomplish this). Thus, the worse binding of SHZ and SDM is probably caused by the fact that both of these sulfas have both meta positions of the  $N^1$  ring occupied, whereas the worse binding of SQX is maybe caused by the  $N^1$  ring turning so upon binding that the attached secondary ring is facing away from the antibody binding site surface, which causes a lack of a nitrogen atom in the ortho position of the  $N^1$  ring facing the binding site surface.

Sometimes, the performances of antibodies that function well in simple buffer systems are adversely affected by the introduc-



tion of sample matrix environments. These matrix effects can make it impossible to utilize certain antibodies in the analysis of real samples. Luckily, our new sulfonamide binders did not seem to suffer from any major matrix effects as shown with beef, serum, and milk matrices, making it possible to utilize these binders in the analysis of real samples in the future.

## CONCLUSIONS

In our earlier studies (26, 27), we demonstrated, by improving the broad specificity binding properties of sulfonamide antibody 27G3, that protein engineering is a powerful technique when the creation of broad specificity antibodies is attempted. In this study, we took the engineering even further and managed to produce, according to our knowledge, the first published antibodies capable of binding at least 13 different sulfonamides with an affinity high enough so that the antibodies can be used for the detection of these sulfonamides with a sensitivity greater than the established MRLs in the U.S. and EU. Additionally, we demonstrated that at least one of these antibodies, M.3.4, can be used to measure sulfonamides from at least three different sample matrices without any major matrix effects on IC<sub>50</sub> values.

## LITERATURE CITED

- (1) Sheth, H.; Sporns, P. Development of a single ELISA for detection of sulfonamides. *J. Agric. Food Chem.* **1991**, *39*, 1696–1700.
- (2) Haasnoot, W.; Cazemier, G.; Du Pre, J.; Kemmers-Voncken, A.; Bienenmann-Ploum, M.; Verheijen, R. Sulphonamide antibodies: From specific polyclonals to generic monoclonals. *Food Agric. Immunol.* **2000**, *12*, 15–30.
- (3) Muldoon, M.; Holtzapple, C.; Deshpande, S.; Beier, R.; Stanker, L. Development of a monoclonal antibody-based cELISA for the analysis of sulfadimethoxine. 1. Development and characterization of monoclonal antibodies and molecular modeling studies of antibody recognition. *J. Agric. Food Chem.* **2000**, *48*, 537–544.
- (4) Koenen-Dierick, K.; Okerman, L.; De Zutter, L.; Degroot, J.; Van Hoof, J.; Srebrnik, S. A one-plate microbiological screening test for antibiotic residue testing in kidney tissue and meat: an alternative to the EEC four-plate method? *Food Addit. Contam.* **1995**, *12*, 77–82.
- (5) Korsrud, G.; Boison, J.; Nouws, J.; MacNeil, J. Bacterial inhibition tests used to screen for antimicrobial veterinary drug residues in slaughtered animals. *J. AOAC Int.* **1998**, *81*, 21–24.
- (6) Nouws, J.; Van Egmond, H.; Loeffen, G.; Schouten, J.; Keukens, H.; Smulders, I.; Stegeman, H. Suitability of the Charm HVS and a microbiological multiplate system for detection of residues in raw milk at EU maximum residue levels. *Vet. Q.* **1999**, *21*, 21–27.
- (7) Abian, J.; Churchwell, M.; Korfmacher, W. High-performance liquid chromatography thermospray mass spectrometry of 10 sulfonamide antibiotics. Analysis in milk at the ppb level. *J. Chromatogr.* **1993**, *629*, 267–276.
- (8) Cooper, A.; Creaser, C.; Farrington, W.; Tarbin, J.; Shearer, G. Development of multi-residue methodology for the HPLC determination of veterinary drugs in animal tissues. *Food Addit. Contam.* **1995**, *12*, 167–176.
- (9) Lin, C.; Hong, C.; Kondo, F. Simultaneous determination of residual sulfonamides in the presence and absence of p-aminobenzoic acid by high-performance liquid chromatography. *Microbios* **1995**, *83*, 175–183.
- (10) Volmer, D. Multiresidue determination of sulfonamide antibiotics in milk by short-column liquid chromatography coupled with electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass. Spectrom.* **1996**, *10*, 1615–1620.
- (11) Abjean, J. Planar chromatography for the multiclass, multiresidue screening of chloramphenicol, nitrofurans and sulfonamide residues in pork and beef. *J. AOAC Int.* **1997**, *80*, 737–740.
- (12) Le Boulair, S.; Bauduret, J.-C.; Andre, F. Veterinary drug residues survey in meat: an HPLC method with a matrix solid-phase dispersion extraction. *J. Agric. Food Chem.* **1997**, *45*, 2134–2142.
- (13) Dost, K.; Jones, D.; Davidson, G. Determination of sulfonamides by packed column supercritical fluid chromatography with atmospheric pressure chemical ionisation mass spectrometric detection. *Analyst* **2000**, *125*, 1243–1247.
- (14) Ackermans, M.; Beckers, J.; Everaerts, F.; Hoogland, H.; Tomassen, M. Determination of sulphonamides in pork meat extracts by capillary zone electrophoresis. *J. Chromatogr.* **1992**, *596*, 101–109.
- (15) Hows, M.; Perrett, D.; Kay, J. Optimisation of a simultaneous separation of sulphonamides, dihydrofolate reductase inhibitors and beta-lactam antibiotics by capillary electrophoresis. *J. Chromatogr. A* **1997**, *768*, 97–104.
- (16) Heering, W.; Usleber, E.; Dietrich, R.; Märtlbauer, E. Immunochemical screening for antimicrobial drug residues in commercial honey. *Analyst* **1998**, *123*, 2759–2762.
- (17) Elliott, C.; Baxter, G.; Crooks, S.; McCaughey, W. The development of a rapid immunobiosensor screening method for the detection of residues of sulphadiazine. *Food Agric. Immunol.* **1999**, *11*, 19–27.
- (18) Akkoyun, A.; Kohen, V. F.; Bilitewski, U. Detection of sulphamethazine with an optical biosensor and anti-idiotypic antibodies. *Sens. Actuators* **2000**, *B70*, 12–18.
- (19) Kohen, F.; Gayer, B.; Amir-Zaltsman, Y.; O'Keefe, M. Generation of an anti-idiotypic antibody as a surrogate ligand for sulfamethazine in immunoassay procedures. *Food Agric. Immunol.* **2000**, *12*, 193–201.
- (20) Lee, N.; Holtzapple, C.; Muldoon, M.; Deshpande, S.; Stanker, L. Immunochemical approaches to the detection of sulfathiazole in animal tissues. *Food Agric. Immunol.* **2001**, *13*, 5–17.
- (21) Spinks, C.; Schut, C.; Wyatt, G.; Morgan, M. Development of an ELISA for sulfachlorpyridazine and investigation of matrix effects from different sample extraction procedures. *Food Addit. Contam.* **2001**, *18*, 11–18.
- (22) Situ, C.; Crooks, S.; Baxter, A.; Ferguson, J.; Elliott, C. On-line detection of sulfamethazine and sulfadiazine in porcine bile using a multichannel high-throughput SPR biosensor. *Anal. Chim. Acta* **2002**, *473*, 143–149.
- (23) Muldoon, M.; Font, I.; Beier, R.; Holtzapple, C.; Young, C.; Stanker, L. Development of a cross-reactive monoclonal antibody to sulfonamide antibiotics: Evidence for structural conformation-selective hapten recognition. *Food Agric. Immunol.* **1999**, *11*, 117–134.
- (24) Spinks, C.; Wyatt, G.; Lee, H.; Morgan, M. Molecular modeling of hapten structure and relevance to broad specificity immunoassay of sulfonamide antibiotics. *Bioconjugate Chem.* **1999**, *10*, 583–588.
- (25) Spinks, C.; Wyatt, G.; Everest, S.; Jackman, R.; Morgan, M. Atypical antibody specificity: Advancing the development of a generic assay for sulphonamides using heterologous ELISA. *J. Sci. Food Agric.* **2002**, *82*, 428–434.
- (26) Korpimäki, T.; Rosenberg, J.; Virtanen, P.; Karskela, T.; Lamminmäki, U.; Tuomola, M.; Vehniäinen, M.; Saviranta, P. Improving broad specificity hapten recognition with protein engineering. *J. Agric. Food Chem.* **2002**, *50*, 4194–4201.
- (27) Korpimäki, T.; Rosenberg, J.; Virtanen, P.; Lamminmäki, U.; Tuomola, M.; Saviranta, P. Further improvement of broad specificity hapten recognition with protein engineering. *Protein Eng.* **2003**, *16*, 37–46.
- (28) Haasnoot, W.; Bienenmann-Ploum, M.; Kohen, F. Biosensor immunoassay for the detection of eight sulfonamides in chicken serum. *Anal. Chim. Acta* **2003**, *483*, 171–180.

- (29) Rondot, S.; Koch, J.; Breitling, F.; Dübel, S. A helper phage to improve single-chain antibody presentation in phage display. *Nat. Biotechnol.* **2001**, *19*, 75–78.
- (30) Krebber, A.; Bornhauser, S.; Burmester, J.; Honegger, A.; Willuda, J.; Bosshard, H.; Plückthun, A. Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. *J. Immunol. Methods* **1997**, *201*, 35–55.
- (31) Kabat, E.; Wu, T.; Perry, H.; Gottesman, K.; Foeller, C. *Sequences of Proteins of Immunological Interest*, 5th ed.; NIH Publication 91-3242; U.S. Department of Health and Human Services: Washington, DC, 1991.
- (32) Sambrook, J.; Fritsch, E.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.
- (33) Horton, R.; Cai, Z.; Ho, S.; Pease, L. Gene splicing by overlap extension: Tailor-made genes using the polymerase chain reaction. *Biotechniques* **1990**, *8*, 528–535.
- (34) Hemmilä, I.; Dakubu, S.; Mikkala, V.; Siitari, H.; Lövgren, T. Europium as a label in time-resolved immunofluorometric assays. *Anal. Biochem.* **1984**, *137*, 335–343.

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