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Development of an Enzyme-Linked Immunosorbent Assay for the Determination of the Linear Alkylbenzene Sulfonates and Long-Chain Sulfophenyl Carboxylates Using Antibodies Generated by Pseudoheterologous Immunization

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ELISA methods have been developed for screening contamination of water resources by linear alkyl benzene sulfonates (LAS) or the most immediate degradation products, the long chain sulfophenyl carboxylates, SPCs. The assay uses antibodies raised through pseudoheterologous immunization strategies using an equimolar mixture of two immunogens (SFA−KLH and $13C_{13}$-SPC−KLH) prepared by coupling *N*-(4-alkylphenyl)sulfonyl-3-aminopropanoic acid (SFA) and *p*-(1-carboxy-13-tridecyl)-phenylsulfonic acid ($13C_{13}$-SPC) to keyhole limpet hemocyanin (KLH). The immunizing haptens have been designed to address recognition versus two different epitopes of the molecule. The SFA hapten maximizes recognition of the alkyl moiety while preserving the complexity of the different alkyl chains present in the LAS technical mixture. The $13C_{13}$-SPC hapten addresses recognition of the common and highly antigenic phenylsulfonic group. The antisera raised using this strategy have been shown to be superior to those obtained through homologous immunization procedures using a single substance. By using an indirect ELISA format, LAS and long-chain SPCs can be detected down to 1.8 and 0.2 μg L^{-1}, respectively. Coefficients of variation of 6 and 12% within and between assays, respectively, demonstrate immunoassay reproducibility. The assay can be used in media with a wide range of pH and ionic strength values. Preliminary experiments performed to assess matrix effects have demonstrated the potential applicability of the method as a screening tool to assess contamination by these types of surfactants in natural water samples.

INTRODUCTION

Linear alkylbenzene sulfonates (LAS) are a mixture of closely related isomers and homologues, each containing an aromatic ring sulfonated at the para position and attached to a linear alkyl chain. LAS are one of the major anionic surfactants used on the market today in cleaning products for home, institutional, and industrial use, for example, car wash liquids, laundry detergents, liquid dish detergents, hard surface cleaners, dry cleaning products, and waterless hand and industrial cleaners. The European consumption of LAS was estimated to be ∼400 Kton in year 2000, and the global consumption is around 1.6 million tons/year.[1] As a consequence of this extensive use, residues of LAS and their degradation products are found in almost all types of environmental water and soil samples near urban and industrial areas at significant concentration levels.[2] As an example, the daily mass input of LAS and their biodegradation intermediates from the Sancti Petri Channel to Cadiz Bay was 44.6 kg.[3] In a study of the year 2002[4] in Rio Macacu (Brazil), LAS concentrations ranged between 14 and 155 μg L^{-1}, and for sulfophenyl carboxylic (SPCs) degradation products, from 1.2 to 14 μg L^{-1}. Similarly, in Europe, the levels of SPCs in the Llobregat (Spain) and Rhine (Germany) Rivers amounted to 5 and 1.8 μg L^{-1}, respectively. Treatments in the corresponding waterworks produced drinking water with SPC levels around 2 μg L^{-1} (Spain) and 0.05 μg L^{-1} (Germany).[5]

LAS are not especially toxic by themselves, but because of their amphoteric structure, they can contribute to the permeation of other pollutants (i.e., heavy metals and pesticides) through biological membranes into aquatic organisms.[6,7] Moreover, these chemicals degrade rapidly aerobically, whereas they do not degrade under anaerobic conditions. Therefore, there is a risk of bioaccumulation in aquatic plants and organisms. Their total biodegradation still requires 5−10 days under normal conditions.[1] The first degradation products are long-chain sulfophenyl carbox-

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ilic acids,[8] formed via ω-oxidation of the alkyl chains. Subsequent β-oxidation steps result in congeners with shorter alkyl chains, some of them extremely polar, that may remain in the environment, since further degradation of the aromatic ring occurs slowly.

The analytical determination of LAS in environmental samples has relied on the use of chromatographic and spectrometric techniques, such as liquid chromatography/mass spectrometry (LC/MS).[9−11] The use of gas chromatography/mass spectrometry (GC/MS) has also been reported,[12] but it involves a derivatization step. Cleanup/preconcentration steps consisting of liquid−liquid extraction with organic solvents followed by solid-phase extraction (SPE) procedures are always required. Their ability to adsorb to the solid surfaces, through their hydrophobic side, prevents representative results of real environmental concentrations.[13] Moreover, the complexity of the chromatograms obtained may also make routine screening and multiple analyses difficult. As an effective alternative, immunochemical techniques could not only afford the necessary detectability and specificity for the target analyte but also offer other advantages, such as reliability, simplicity, low cost, and high sample throughput capabilities.[14−19]

The preparation of optimum haptens as immunogens and competitors has been regarded as the most crucial step in the development of an immunochemical technique for small molecules. Many literature examples prove that an appropriate hapten design determines the features of the resulting antibodies, which mainly govern the specificity and the selectivity of an immunochemical technique.[14,20−23] Theoretical molecular models and calculations can be useful tools to assist prediction of which hapten will be the most appropriate to raise antibodies;[24−26] however, LAS exhibit the particularity of being a complex mixture of substances (different alkyl chain lengths and positional isomers), which makes antibody production even more challenging. Fujita et al. have reported the preparation of antibodies using 5-(4-sulfophenyl)-pentanoic acid, a single substance with a short alkyl chain, as an immunizing hapten.[27] This chemical structure addressed antibody recognition versus the sulfonic group, which is one of the most antigenic determinants in this molecule; however, studies performed in our group indicate that the contribution of the alkyl chain in the stabilization of the antibody−analyte should not be underestimated.[28] In the field of catalytic antibodies, several research groups have made use of heterologous immunization strategies to elicit antibody immunoresponse against two different epitopes of the molecules, simplifying hapten synthesis.[29−31] Thus, although an ideal hapten preserving both groups would have a spacer arm at the ortho or meta positions, the synthetic pathway leading to these types of chemical structures could be troublesome. The heterologous immunization strategy devised by the group of Prof. Masamune[31−33] consists of successfully immunizing the animal with two different but structurally related haptens. It has mainly been used for zwirterionic transition-state analogues with positive and negative charges, for which chemical preparation of the corresponding hapten could be problematic. Instead, the heterologous immunization using two individual haptens containing a different charge provided an opportunity to simultaneously generate an acidic and a basic catalytic residue in the antibody combining site. Thus, Ersoy et al.,[29,34] designed three haptens to produce, by heterelogous immunization, nucleophile-mediated (phenol) amide bond cleaving catalytic antibodies having a binding pocket with (i) a hydrophobic area, (ii) an acidic residue complementary to the oxyanionic transition state, and (iii) a basic residue to aid deprotonation of a phenol nucleophile and protonation of the departing amine. In all those examples, the catalytic activity of the antibodies generated through heterologous strategies is higher than those obtained by homologous immunization. Attending to these precedents, the present paper describes for the first time the use of this immunization approach to obtain antibodies against an amphoteric molecule such as LAS with two well differentiated epitopes in its chemical structure. The results show that the antibodies obtained by applying this strategy provide better immunoassays than those obtained through homologous immunization strategies.

EXPERIMENTAL SECTION

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).

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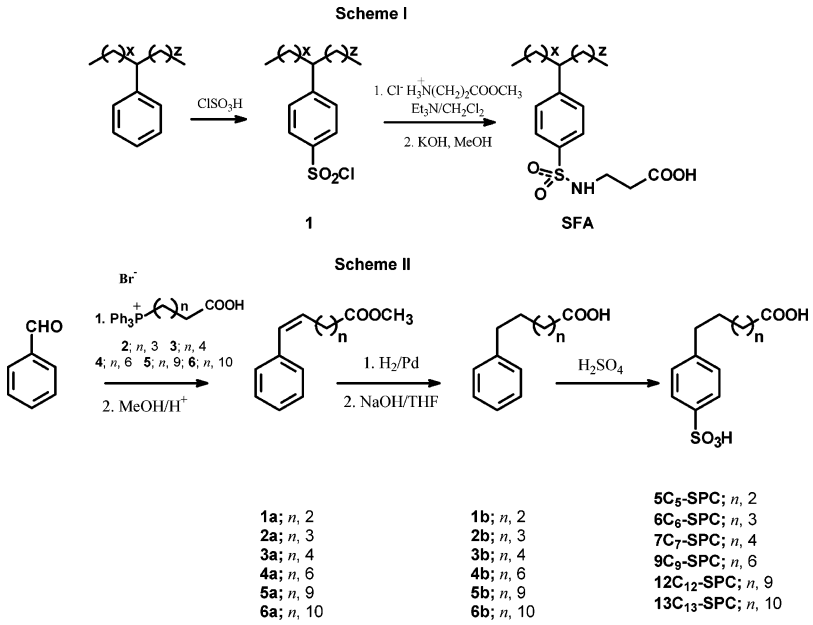

Figure 1. Schemes showing the synthetic pathways used to prepare immunizing (SFA and $13C_{13}$-SPC) and competitor haptens.

Unless otherwise indicated, purification of the reaction mixtures was accomplished by "flash" chromatography using silica gel as the stationary phase. ^1H and ^{13}C NMR spectra were obtained with a Varian Unity-300 (Varian Inc., Palo Alto, CA) spectrometer (300 MHz for ^1H and 75 MHz for ^{13}C). The chemical reagents used in this synthesis were obtained from Aldrich Chemical Co. (Milwaukee, WI). The mixture of alkylbenzene synthetic precursors of LAS was kindly provided by PETRESA S.A. (San Roque, Cádiz, Spain).

Synthesis of the SFA (Sulfonamide) Hapten. (See scheme 1 in Figure 1) Spectroscopic and spectrometric data are given as Supporting Information.

***N*-(4-Alkylphenyl)sulfonyl-3-aminopropanoic Acid (SFA).** Chlorosulfonic acid (1.7 mL, 25 mmol, 3 equiv) was placed in a two-neck round-bottom flask provided with magnetic stirring and under Ar atmosphere. One of the necks was connected to a trap with 1 M NaOH to neutralize the HCl formed. The other neck was used to add slowly the alkylbenzene technical mixture (1 g, 4.2 mmol) at room temperature. The initial pale yellow color of the reaction mixture became intense red. The reaction was finished after 1:30 h, as observed by TLC (hexane as mobile phase). The crude mixture was poured over a water−ice mixture (100 mL), and the white precipitate formed was extracted with hexane, washed with saturated $NaHCO_3$, dried with anhydrous MgSO4, filtered, and evaporated. The crude residue was then purified by silica gel flash chromatography using hexane as mobile phase to obtain the corresponding mixture of 4-alkylphenylsulfonyl chlorides as a pale yellow oil (400 mg, 30% yield). A solution of the mixture of 4-alkylphenylsulfonyl chlorides (200 mg, 0.6 mmol) in anhydrous CH_2Cl_2 (2 mL) was slowly added under Ar atmosphere to a solution of triethylamine (190 mg, 1.4 mmol, 2.3 equiv), and the chlorhydrate of methyl 3-aminoproanoate (107 mg, 0.77 mmol, 1.3 equiv) in the same solvent (3 mL) was placed in a three-neck round-bottom flask with magnetic stirring. The mixture was left to react at RT until the starting material disappeared by TLC (hexane/ethyl acetate, 1:1). The solvent was evaporated, suspended in saturated $NaHCO_3$, and extracted with EtAcO (ethyl acetate). The organic layer was dried with $MgSO_4$, filtered, and evaporated to dryness to obtain a mixture of methyl *N*-(4-alkylphenyl)sulfonyl-3-aminopropanoates (140 mg, 60% yield) as a yellow oil. Finally, the esters were hydrolyzed in MeOH (1.3 mL) with 1 M KOH (1 mL, 1 mmol, 3 equiv) for 2 h. The solvent was then evaporated, and the residue was dissolved with 1 N HCl and extracted with EtAcO to obtain SFA (90 mg, 67% yield) as a yellow oil.

Synthesis of SPC Haptens. Different SPCs ($5C_5$, $6C_6$, $7C_7$, $9C_9$, $12C_{12}$ and $13C_{13}$) were synthesized from the corresponding ω-phenylalkylcarboxylic acids **1b**−**6b** by sulfonation of the aromatic ring. The 5-phenylpentanoic acid **1b** and the (4-carboxybutyl)triphenylphosphonium bromide used to prepare **2a** were obtained from commercial sources. The phenylcarboxylic acids **2b**−**6b** were prepared through a Wittig reaction using benzaldehyde and the respective phosphonium bromide salts **2**−**6**, followed by reduction of the double bond formed with H_2 using Pd/C as catalyst (see scheme 2 in Figure 1). A general procedure is described below. Synthetic details and spectroscopic and spectrometric data for each particular compound are provided as Supporting Information.

Preparation of (ω-Carboxyalkyl)triphenylphosphonium Bromides 2−6. *General Protocol.* The corresponding ω-bromoalkanoic acid (9 mmol, 1 equiv) and the triphenylphosphine (9 mmol, 1 equiv) were placed in a round-bottom flask equipped with a cooling jacket and a magnetic stir bar. The mixture was heated to 100 °C under Ar atmosphere. After 4 h, a total conversion of the acid was observed by ^1H NMR. The reaction was left overnight under vacuum at 100 °C. The yield was quantitative.

Preparation of ω-Phenylalkylcarboxylic Acids 1b−6b. *General Protocol. Step 1: Synthesis of the Methyl ω-Phenylalkenoates 1a−6a.* Anhydrous DMSO (6.1 mL, 0.1 mol, 3.5 equiv) was added to a suspension of 60% NaH (1.24 g, 0.3 mol, 2.1 equiv; previously washed with anhydrous pentane (3 × 15 mL) and dried), placed in a round-bottom flask equipped with a magnetic stir bar and a cooling jacket, under Ar atmosphere. The mixture was heated at 65 °C for 30 min until no more formation of H_2 was observed and

the solution became pale green-yellow. At that moment, the solution was allowed to reach room temperature, and the corresponding phosphonium bromide salt **2**−**6** (6 mmol, 1 equiv) dissolved in anhydrous DMSO (5 mL) was added dropwise. A characteristic color shift to an intense red was produced, indicating the formation of the corresponding ylide. After 15 min, freshly distilled benzaldehyde (0.65 mL, 6 mmol, 1 equiv) was added and allowed to react for 2 h at RT under stirring until the starting material had disappeared according to TLC (hexane/ethyl acetate/AcOH, 1:1:0.1). The crude of the reaction was acidified with 1 N HCl and extracted with ethyl ether. The organic phase was washed with H_2O, dried with $MgSO_4$, and filtered. The solvent was evaporated to dryness to obtain mixtures of the *Z*/*E* ω-phenylalkenoic acids that were subsequently esterified in MeOH with a few drops of concentrated H_2SO_4 at RT until the total disappearance of the starting material by TLC. The solvent was evaporated, and the residue was dissolved in ethyl ether and washed with saturated $NaHCO_3$. The organic phase was dried with $MgSO_4$, filtered, and evaporated. The crude mixture was purified by silica gel flash chromatography using hexane/ethyl ether as mobile phase to obtain the corresponding methyl esters as a *Z*/*E* mixture.

Step 2: Synthesis of the ω-Phenylalkanoic Acids 1b−*6b.* The double bond of the ester (3.92 mmol) was reduced with H_2 using Pd/C (10% Pd, 208.4 mg, 0.19 mmol Pd) as catalyst in MeOH (10 mL). The suspension was purged several times with vacuum/H_2 cycles to remove the O_2 present in the media and finally was kept under H_2 at atmospheric pressure. The reaction mixture was stirred for ∼2 h at RT until the disappearance of the starting material was observed by TLC (hexane/ethyl ether, 1:1). The suspension was then purged again with vacuum/N_2 cycles to eliminate the H_2. The catalyst was removed by filtration, and the MeOH was evaporated to dryness to obtain the corresponding methyl ω-phenylalkanoates. The esters were hydrolyzed in THF and with 0.5 N NaOH at RT until the disappearance of the ester by TLC. The THF was removed under vacuum, and the aqueous solution was acidified with concentrated HCl and extracted with Et_2O. The organic phase was dried with MgSO4, filtered, and evaporated to dryness to obtain the desired ω-phenylalkanoic acids **1b**−**6b**.

Preparation of the Sulfophenyl Carboxylates $5C_5$-, $6C_6$-, $7C_7$-, $9C_9$-, $12C_{12}$-, and $13C_{13}$-SPCs. Sulfonation of the phenyl carboxylic acids **1b**−**6b** was performed following a similar procedure as described.[35,36] The corresponding phenylcarboxylic acids **1b**−**6b** (2.8 mmol, 1 equiv) were added to a round-bottom flask containing concentrated H_2SO_4 (1 mL, 18.22 mmol, 6.5 equiv) and equipped with a cooling jacket heated at 100 °C. The reaction mixture was stirred for 2 h and then slowly poured into H_2O (80 mL). $5C_5$-, $6C_6$-, and $7C_7$-SPCs were isolated as calcium salts by washing the aqueous solution with Et_2O (3 × 25 mL) and then neutralizing it with $CaCO_3$ (6 g, 75 mmol). The solid formed was removed by filtration, and the aqueous solution was evaporated under reduced pressure to dryness to finally obtain the desired SPCs as calcium salts. $9C_9$-, $12C_{12}$-, and $13C_{13}$-SPCs were purified from the reaction mixture as sodium salts by extracting the aqueous layer with ethyl acetate. The organic phase was dried with $MgSO_4$, filtered, and evaporated to dryness to finally obtain a dark oil that was subsequently washed with a saturated solution of $NaHCO_3$. The yellow precipitate obtained was filtered and washed with ethyl acetate to yield a white solid corresponding to the desired compound.

Immunochemistry. *General Methods and Instruments.* The MALDI-TOF-MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometer) used for analyzing the protein conjugates was a Perspective BioSpectrometry Workstation equipped 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 Corporation (Salem, NH). The pH and the 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, DK). Washing steps were performed on a SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). 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 v2.6 (Molecular Devices) and GraphPad Prism (GraphPad Sofware Inc., San Diego, CA). Unless otherwise indicated, data presented correspond to the average of at least two well replicates.

Chemicals and Immunochemicals. Immunochemicals and the seasalts used to prepare artificial seawater were obtained from Sigma Chemical Co. (St. Louis, MO). The preparation of the protein conjugates and the antisera is described below. Most of the chemicals used for crossreactivity studies were acquired from Aldrich Chemical Co. (Milwaukee, WI). The technical mixture of LAS was kindly provided by PETRESA S.A. (San Roque, Cádiz, Spain). The exact percentage weight of each LAS homologue was <C10, 0.4%; C10, 12%; C11, 34%; C12, 30%; C13, 22%; C14, 0.4%, and 0.1% of parafines. The standards used for the calibration of the ELISA experiments were prepared by serial dilutions in DMSO (32 nM − 5000 μM) and diluted 200 times with PBST-I prior to when the assay was run. The branched short chain SPCs were synthesized in our laboratory as described.[36]

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

Preparation of the Immunogens and other Secondary Immunoreagents. The immunoreagents have been named combining the abbreviations of the haptens (SFA or XC_x-SPC) with that of the proteins or enzymes.

Mixed Anhydride (MA) Method. Following described procedures,[37,38] haptens SFA and $13C_{13}$-SPC (15 μmol) were reacted with tributylamine (4 μL, 16.5 μmol) and isobutylchloroformate

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Table 1. Hapten Densities of the BSA Conjugates[a]

immunoreagent	δ-hapten[b]
SFA-BSA	18.8
$5C_5$-SPC−BSA	3.2
$6C_6$-SPC−BSA	13.4
$7C_7$-SPC−BSA	17.9
$9C_9$-SPC−BSA	10.7
$12C_{12}$-SPC−BSA[c]	20
$13C_{13}$-SPC−BSA[c]	13

[a] Hapten densities were calculated by MALDI-TOF-MS. [b] Moles of hapten per mole of protein.

(3 μL, 18 μmol) in DMF (160 μL). The solution containing the activated hapten was then divided into two equivalent fractions and added to the KLH and BSA solutions (30 mg/each) in 0.2 M borate buffer (1.8 mL).

Active Ester (AE) Method. Following described procedures,[39] the haptens SFA, $5C_5$-, $6C_6$-, $7C_7$-, $9C_9$-, $12C_{12}$-, and $13C_{13}$-SPCs (60 μmol) were reacted with freshly prepared solutions of *N*-hydroxysuccinimide (NHS, 8.62 mg, 75 μmol) and dicyclohexylcarbodiimide (DCC, 30.90 mg, 150 μmol) in anhydrous DMF (200 μL) for ~2 h at room temperature until the appearance of the urea precipitate that was removed by centrifugation. The supernatant of each solution was split into four portions and then added dropwise to the BSA, CONA, OVA (10 mg/each), and HRP (2 mg) solutions 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 0.01 M PBS at 1 mg mL^{-1}.

Hapten Density Analysis. Hapten densities of the BSA conjugates were calculated by MALDI-TOF-MS by comparing the molecular weight of the native proteins to that of the conjugates (see Table 1). 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 (v/v) with a 0.1% TFA) with 2 μL of a solution of the conjugates or proteins (10 mg mL^{-1} in CH_3CN/H_2O 70:30 (v/v) with 0.1% TFA), except for $12C_{12}$- and $13C_{13}$-SPC, which were prepared in Milli-Q water (10 mg mL^{-1}). The hapten density (δ hapten) was calculated according to the following equation:

$$\{\text{MW (conjugate)} - \text{MW (protein)}\}/\text{MW (hapten)}\}$$

Polyclonal Antisera. The immunization protocol was performed on female New Zealand white rabbits weighing 1−2 kg, as previously described.[39] Rabbits 93 and 94 were immunized with SFA−KLH, rabbits 95 and 96 were immunized with $13C_{13}$-SPC−KLH, and rabbits 97 and 98 were immunized with a mixture (1:1) of SFA−KLH and $13C_{13}$-SPC−KLH. We have named this immunization procedure "pseudoheterologous immunization strategy" to show that it is based on the same concept as the heterologous immunization procedure proposed by Tsumuraya et al.[31] In that case, however, the authors alternate the immunogens on each boosting injection, whereas in this case, the immunogens were used as a mixture. The corresponding antisera (As) obtained were named with the rabbit numbers. Evolution of the antibody titer was assessed by measuring the binding of serial dilutions of the different antisera to microtiter plates coated with SFA-BSA (AE) for As93−94, with $13C_{13}$-SPC-BSA (AE) for As95−96, and with SFA-BSA and $13C_{13}$-SPC−BSA (AE) for As97−98. After an acceptable antibody titer was observed, the animals were exsanguinated, and the blood was collected in vacutainer tubes equipped with a serum separation gel. Antisera were obtained by centrifugation and stored at −40 °C in the presence of 0.02% NaN_3.

ELISA Methods. The general ELISA protocols used to select the most appropriate combination of immunoreagents are given as Supporting Information. Here we only describe the optimized methods.

Optimized Direct ELISA (As98/$7C_7$-HRP). Microtiter plates were coated with As98 (1/8000 in coating buffer, 100 μL/well) overnight at 4 °C and covered with adhesive plate sealers. The following day, the plates were washed with PBST (four times, 300 μL/well) and the LAS standards (0.16−25 000 nM in PBST-I, 0.5% DMSO) were added to the microtiter plates (50 μL/well). After 15 min at RT, a solution of $7C_7$-HRP (2 ng L^{-1} in PBST-I) was added (50 μL/well), and the plates were incubated for an additional 30 min at RT. The plates were washed again as before, and the substrate solution was added (100 μL/well). Color development was stopped after 30 min at RT with 4 N H_2SO_4 (50 μL/well), and the absorbances were read at 450 nm.

Optimized Indirect ELISA (As98/$7C_7$-CONA). Microtiter plates were coated with $7C_7$-CONA (0.08 μg mL^{-1} in coating buffer, 100 μL/well) overnight at 4 °C and covered with adhesive plate sealers. The following day, the plates were washed with PBST (four times, 300 μL/well), and LAS standards (0.16−25 000 nM, in PBST-I, 0.5% DMSO) were added to the microtiter plates (50 μL/well), followed by the antisera As98 (1/16 000 in PBST-I, 50 μL/well). After 30 min of incubation time at RT, the plates were washed again as before, and a solution of anti IgG-HRP (1/6000 in PBST) was added (100 μL/well) and incubated for an additional 30 min at RT. The plates were washed again, and the substrate solution was added (100 μL/well). Color development was stopped after 30 min at RT 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.

Cross-Reactivity Determinations. Stock solutions were prepared in DMSO at a concentration of 1 mM for different long-chain SPCs, phenylsulfonic acids, naphthalenesulfonates, the commercial mixture of LAS, and the individual congeners. Standard solutions were prepared for each analyte in PBST-I (0.16−25 000 nM) and measured with the ELISA. For some analytes, it was possible to build a standard curve that fitted to the four-parameter equation mentioned above. The cross-reactivity

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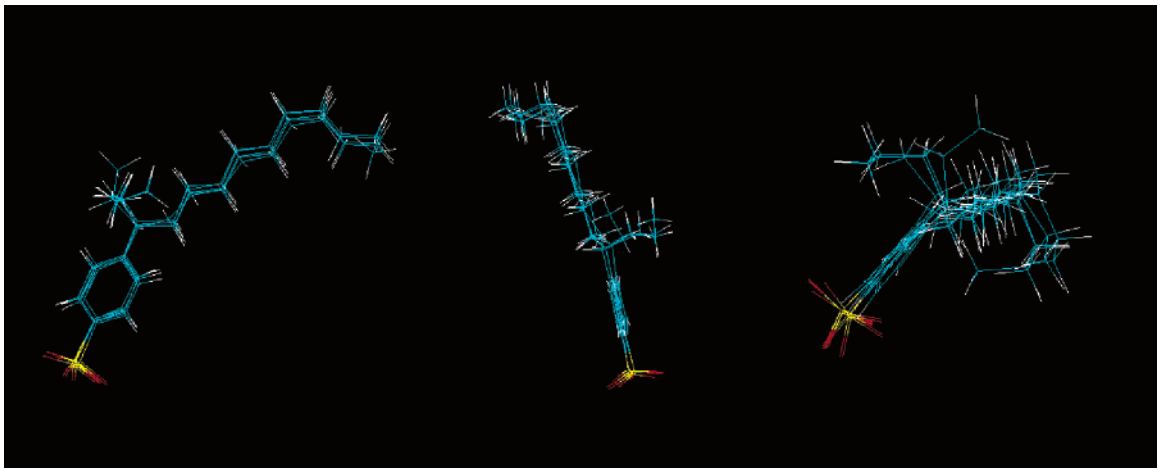

Figure 2. Stick and wedges display of the optimized geometries according to PM3 models of selected LAS congeners and isomers shown from different angles. The optimized geometries have been overlapped to show the complexity of the target analyte. The elements are presented in the following manner: light blue, carbon; dark blue, nitrogen; black, hydrogen; red, oxygen; green, chlorine.

values were calculated according to the equation

$$(IC_{50}\ \text{LAS}/IC_{50}\ \text{tested compounds}) \times 100$$

Accuracy. This parameter was assessed by preparing nine different blind spiked samples in Milli-Q water. Prior to measuring them with the assay, the samples were buffered with PBST-I to adjust the ionic strength and the pH. Analyses were performed in triplicate.

Matrix Effect Studies. Artificial seawater (35 ‰ of sea salts obtained from Sigma) and different natural water samples (tap water (Barcelona city), river water (sample from Aigüestortes Natural Park, Pyrenees, Spain), well water (Barcelona city), and seawater (Mediterranean coast)) were collected and used to assess performance of the indirect ELISA method. Samples were buffered and used to prepare standard curves of LAS. The sigmoid curves obtained were compared with the curve prepared in the assay buffer.

RESULTS AND DISCUSSION

Hapten Design. To produce antibodies, we must consider that LAS are anionic surfactants consisting of a complex mixture of congeners (alkyl chain lengths ranges typically between 10 and 14 carbon units) and isomers (the benzene ring is attached to a linear alkyl chain at any position except the terminal carbons[40]) of alkylphenylsulfonic acids. Moreover, LAS have two very well differentiated areas in their chemical structure inherent to their surfactant properties, a very polar one determined by the phenylsulfonic group and another much more hydrophobic determined by the alkyl moiety, which determine their amphoteric behavior in water. Although the first area is equal in all congeners, the second defines a quite complex hydrophobic zone considering the mixture of compounds (see Figure 2). The heterogeneity of LAS and the potential synthetic difficulties envisaged for the introduction of the spacer arm at the ortho or meta position prompted us to use heterologous immunization strategies using two types of haptens. The approach allows generation of multiple charged residues in the antibody binding sites while avoiding complicated hapten syntheses by immunizing alternatively with different functionalized haptens. Applying this strategy, hapten SFA would maximize the recognition of the complex alkyl moieties of LAS, maintaining the same mixture of substances as in the technical LAS, whereas hapten $13C_{13}$-SPC would address recognition of the phenylsulfonic group.

Synthesis of the Immunizing Haptens. For the preparation of the SFA hapten, the alkylbenzene technical mixture was used to prepare LAS in the industrial process as starting material. The first step consisted of introducing a chlorosulfonic group in the para position of the aromatic ring to obtain **1** through an S_EAr reaction using chlorosulfonic acid.[41] Second, the spacer arm was introduced with an S_N2 reaction by reacting **1** with the methyl ester of the 3-aminopropanoic acid. Finally, the hydrolysis of the ester gave the desired sulfonamide hapten SFA (see scheme 1 in Figure 1). The overall yield of this synthetic pathway was 12%. Previous attempts to introduce a longer spacer arm (i.e., aminobutyric acid) led to the formation of the corresponding cyclic lactame as a result of the second nucleophilic attack of the amino group of the sulfonamide formed over the carboxylic group. The synthesis of the immunizing hapten $13C_{13}$-SPC was accomplished through a Wittig reaction with benzaldehyde and 12-(carboxydodecyl)-triphenylphosphonium bromide using the methylsulfinyl carbanion ($CH_3-SO-CH_2^-\ Na^+$) as base.[42] This base was generated in situ with NaH. After esterfication of the reaction crude and purification by silica gel, a mixture of 60:40 *Z*/*E* isomers of **6a** could be isolated and identified according to ^1H and ^{13}C RMN. Thus, in the ^1H RMN spectra, the appearance of multiple signals corresponding to conjugated double bonds indicated the presence of both isomers (double triplet at δ 5.65 ppm and a doublet at δ 6.40 ppm were assigned to the *Z* isomer, whereas a double triplet at δ 6.22 ppm and the doublet a δ 6.37 ppm were attributed to the *E* isomer). Similarly, the ^{13}C RMN spectra showed characteristic chemical shifts for the double bonds of both isomers at δ

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(41) Ishida, Y.; Ohta, K.; Itoh, S.; Nakahama, T.; Miki, H.; Yoshikawa, H. *J. Pestic. Sci.* **1993**, *18*, 175−182.

(42) Corey, E. J.; Chaykovsky, M. *J. Am. Chem. Soc.* **1965**, *87*, 1345−1353.

126 and 131 for the *Z* isomer and at δ 129 and 133 for the *E* isomer. Reduction of the double bond using H_2/Pd(C), followed by the hydrolysis of the ester lead to the desired phenylalkanoic acid. The disappearance of the characteristic signals of the double bonds in the ^{1}H RMN spectra and the appearance of a triplet at δ 2.65 ppm, corresponding to the hydrogens of the benzylic position, indicated that reduction had occurred successfully. Finally, sulfonation at the para position with H_2SO_4 at 100 °C led to the formation of the desired $13C_{13}$-SPC, as evidenced by the appearance of the characteristic signals of a para substitution in the aromatic region of the NMR spectra at a δ 7.10 and 7.48 ppm. The compound was extracted from the reaction mixture as a sodium salt (see scheme 2 in Figure 1). In this reaction, it was extremely important to add the phenylalkanoic acid with the temperature of the sulfuric acid at 100 °C to avoid formation of the corresponding sulfonic derivatives at the ortho position.

Antibodies. The immunogens were prepared by covalently coupling the haptens, through their carboxylic groups, to the lysine amino acid residues of the KLH using the mixed anhydride method. Two rabbits were used for each immunogen, and a third group of two rabbits was used to immunize them with the mixture of both immunogens. Although in most of the examples reported heterologous immunization protocols are performed alternating two immunogens on each boosting injection (i.e., A, B, A, B, A, B), in our case, we used an equimolar mixture of both immunogens on each boosting injection (pseudoheterologous, AB, AB, AB, ...). After several boosting injections, three types of antisera (As) were collected: As93 and As94 from rabbits immunized with SFA−KLH, As95 and As96 from rabbits immunized with $13C_{13}$-SPC−KLH, and As97 and As98 from rabbits immunized with the mixture of both immunogens.

Secondary Immunoreagents. In addition to the SFA and $13C_{13}$-SPC haptens, a battery of competitor SPC-type haptens differing on the length of the spacer arm were prepared to assess the effect of the hapten heterology on immunoassay performance. These haptens were synthesized following procedures similar to those described for hapten $13C_{13}$-SPC (see scheme 2 in Figure 1) and purified as sodium ($9C_9$- and $12C_{12}$-SPCs) or as calcium ($5C_5$-, $6C_6$-, and $7C_7$-SPCs) salts. Immunochemical reagents were prepared for developing direct and indirect ELISAs by covalently coupling their carboxylic groups to HRP (ET, enzyme tracers) and BSA, CONA, or OVA (CA, coating antigens) using the active ester method. The hapten densities evaluated by MALDI-TOF-MS are shown in Table 1 for the corresponding haptenized BSA immunoreagents.

Screening of the Avidity of the As. The avidity was tested using noncompetitive ELISA formats. The results of these experiments are shown in Figure 3. It can be observed that in both ELISA formats, the antisera obtained by pseudoheterologous immunization (As97−98) had the broadest recognition and that the As raised against the phenylsulfonic epitope (As95−96) show better antibody titers. Thus, no recognition of any of the HRP tracers and only a slight recognition of the SFA conjugates (BSA, CONA, and OVA) were observed for the As93−94 (homologous immunization with SFA−KLH) in the direct and indirect ELISA formats, respectively. In contrast, As95−96 (homologous immunization with $13C_{13}$−KLH) showed very good antibody titers for the different SPC haptens in the indirect ELISA format,

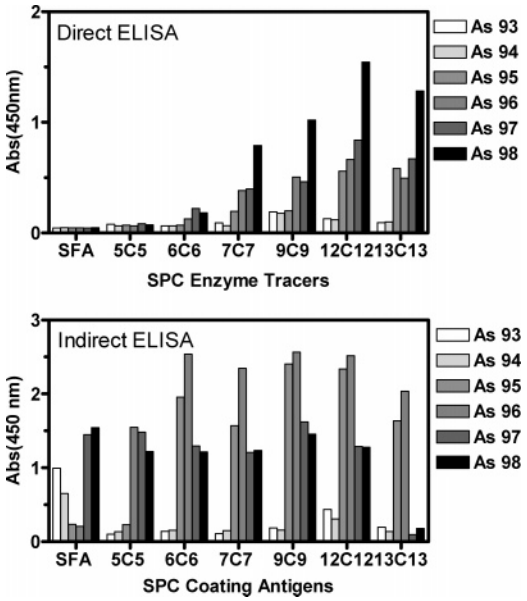

Figure 3. Results from the antibody titration experiments performed using antisera generated against SFA−KLH (As93−94, homologous immunization), $13C_{13}$-SPC−KLH (As95−96, homologous immunization), and a 1:1 mixture of both immunogens (As97−98, pseudo-heterologous immunization) versus different haptens (SFA, $5C_5$-, $6C_6$-, $7C_7$-, $9C_9$-, $12C_{12}$-, and $13C_{13}$-SPCs) coupled to HRP (direct ELISA, graph A) or to CONA (indirect ELISA, graph B). Similar results were obtained with BSA or OVA. Direct ELISA: As, 1/1000; ET, 1 μg mL^{-1}. Indirect ELISA: As, 1/1000; antigens, 1 μg mL^{-1}.

although in the direct ELISA, only those HRP tracers of SPCs with long spacer arms were recognized ($9C_9$-, $12C_{12}$-, and $13C_{13}$-SPCs) at concentrations of 0.5 μg L^{-1} or higher. Finally, As97−98 recognized all of both the SFA and SPC antigens and most of the SPC tracers in the indirect and direct ELISA formats, respectively. A clear correlation between the length of the spacer arms of the SPC tracers and the antibody recognition was observed for As95−96 (homologous immunization) and As97−98 (pseudoheterologous immunization) in the direct ELISA format, but this pattern was only observed for the As95−96 in the indirect ELISA format. The same behavior was observed for all three types, BSA, CONA, and OVA, protein conjugates. Regarding competitor haptens, the direct ELISA format shows that $7C_7$-SPC seems to have the critical spacer arm length. Efficient recognition starts with this hapten, whereas haptens with spacer lengths superior to 12 or 13 carbon atoms could be detrimental for the recognition. Thus, in both ELISA formats, a drop in the recognition for $13C_{13}$-SPC antigens and tracers was observed.

Development of Competitive ELISAs. Those As/ET (direct ELISA) and As/CA (indirect ELISA) combinations showing acceptable titers (A_{max} > 0.5 units; As dilution > 1/1000; [ET] < 0.5 μg mL^{-1}; [Ag] < 0.5 μg mL^{-1}) were further investigated to test recognition of the technical mixture of LAS in solution. As a result of those experiments, several competitive assays were obtained in both formats. Table 2 shows those assays with IC_{50} values lower than 300 μg L^{-1}. In contrast to the As raised against $13C_{13}$-SPC−KLH (As95−96) or to the mixture of immunogens (As97−98), no usable assays were obtained with the As raised against SFA−KLH (As93−94). On a direct ELISA format, only one As/ET combination (As98/$7C_7$-SPC−HRP), using As generated by pseudoheterologous immunization procedures, provide

Table 2. Immunoassay Features of the Different Competitive ELISAs[a]

immunogen	assay	A_{max}	A_{min}	IC_{50}, $\mu g\ L^{-1}$	slope	R^2
$13C_{13}$-SPC−KLH	As96/$6C_6$-SPC−OVA	1.60	0.10	180.5	−0.8	0.97
	As96/$7C_7$-SPC−CONA	1.60	0.06	119.8	−0.7	0.99
	As96/$7C_7$-SPC−OVA	0.68	0.09	51.4 ± 18.4[d]	−0.4	0.97
	As96/$9C_9$-SPC−BSA	1.75	0.13	153.9	−0.9	0.81
	As96/$9C_9$-SPC−CONA	0.88	0.11	73.0 ± 29.2[c]	−0.5	0.99
	As96/$12C_{12}$-SPC−BSA	1.66	0.10	84.8 ± 28.4[c]	−0.5	0.92
	As96/$12C_{12}$-SPC−CONA	0.99	0.12	149.9	−0.8	0.96
	As96/$13C_{13}$-OVA	1.02	0.03	163.9	−0.9	0.97
SFA−KLH	As 98/$7C_7$-SPC−HRP[b]	0.41	0.04	132.6	−0.57	0.64
$13C_{13}$-SPC−KLH	As97/$12C_{12}$-SPC−BSA	0.57	0.02	140.0	−1.3	0.98
	As98/$5C_5$-SPC−OVA	1.28	0.10	152.3	−1.1	0.88
	As98/$6C_6$-SPC−OVA	1.33	0.01	153.9	−0.9	0.97
	As98/$7C_7$-SPC−CONA	1.00	0.07	37.1 ± 3.2[c]	−0.7	0.92
	As98/$9C_9$-SPC−CONA	1.61	0.22	57.2 ± 9.0[c]	−0.5	0.99
	As98/$9C_9$-SPC−BSA	1.11	0.13	120.8	−0.8	0.97

[a] Only those assays showing reasonable parameters and IC_{50} values below 300 $\mu g\ L^{-1}$ are shown. The values are extracted from the four-parameter equation used to fit the standard curves. [b] Direct assay. All the other combinations shown are indirect assays. [c] The data presented correspond to the average of 5 calibration curves run in 5 different days.

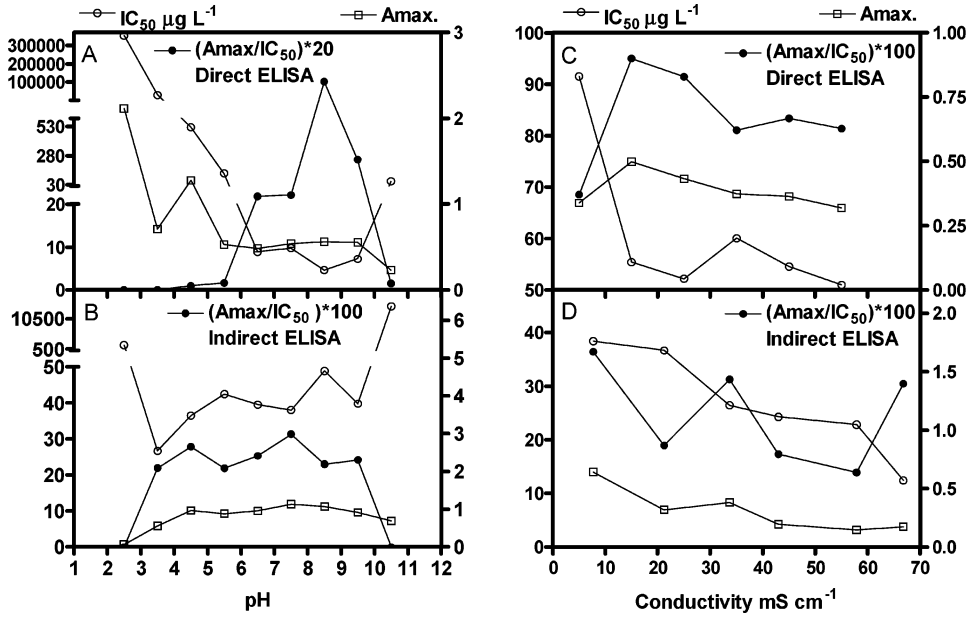

Figure 4. Effect of the pH and the ionic strength on the A_{max} and IC_{50} of the direct (graphs A and B) and indirect (graphs C and D) ELISA assays developed for LAS analysis. The data are extracted from the four-parameter equation used to fit the standard curves.

acceptable features to continue with the evaluation. In contrast, several assays were obtained under an indirect ELISA configuration, using As obtained by both homologous and pseudo-heterologous immunization strategies. However, repetitive experiments using the best assays obtained with each set of As demonstrated that As98/$7C_7$-SPC−CONA was the most reproducible assay (see standard deviations for the IC_{50} values in Table 2). In both ELISA formats, the As giving the best assays were obtained through pseudoheterologous immunization methods. Moreover, hapten $7C_7$-SPC has always provided the assays with the best features, supporting that seven carbon units is a critical spacer length also under competitive configurations.

Evaluation and Characterization of the ELISA Methods. Both direct (As98/$7C_7$-SPC−HRP) and indirect (As98/$7C_7$-SPC−CONA) ELISAs were further investigated with the aim to improve performance and to characterize their behavior in media with different physicochemical parameters (pH, ionic strength, etc.). Preincubating the analyte with the As, previous to the competitive step, for 15 and 60 min in direct and indirect assays, respectively, improved the detectability. Similarly, both direct and indirect formats appeared to perform much better in the absence or very low concentration of Tween 20 (<0.01%). The assays perform well on media with a wide range of pH values without affecting the immunoassay features, although the detectability of the direct assay decreases drastically in media with pH values below 6 (see Figure 4, graph A), and the indirect assay is more robust (see Figure 4, graph B). Regarding ionic strength, both assays perform well on a wide interval, although the maximum absorbance of both assays tends to decrease when ionic strength increases. The detectability of both assays remains almost constant for the direct assay after 15 mS cm^{-1} or with a slight tendency to improve in media with high ionic strength (see Figure 4, graphs C and D). According to these data, a working protocol for both formats was established as it is described in the Experimental Section.

Table 3. Interference Caused by Structurally Related Chemicals on the Immunoassays, Expressed as the Percentage of Cross-Reactivity (%CR)[a]

group	analyte	direct ELISA % CR	indirect ELISA % CR
LAS	LAS	100	100
LAS congeners	2phenylC$_8$	127	56
	2phenylC$_{10}$	174	155
	2phenylC$_{12}$	14	95
	2phenylC$_{14}$	2	13
	2phenylC$_{16}$	<1	4
	2phenylC$_{18}$	<1	4
	2phenylC$_{20}$	<1	3
SPCs	5C$_5$	<1	<1
	6C$_6$	<1	2
	7C$_7$	21	5
	9C$_9$	2875	379
	12C$_{12}$	76 666	6600
	13C$_{13}$	23 000	6600
	2C$_3$	9	1
	2C$_4$	12	<1
	2C$_5$	9	3
	3C$_4$	2	<1
	3C$_5$	<1	<1
	3C$_6$	<1	<1
phenylsulfonic acids	*p*-Ts	<1	<1
	p-Xs	<1	<1
	BDS	<1	<1
	EBS	<1	<1
sulfonamide	SFA	5	3
naphthalene sulfonates	1-NS	<1	<1
	1,5-NDS	<1	<1
	1,3,5-NTS	<1	<1

[a] Cross-reactivity is expressed as a percent of the IC_{50} of the LAS divided by the IC_{50} of the assayed compounds. NS, naphthalene sulfonate; NDS, naphthalene disulfonate; NTS, naphthalene trisulfonate; *p*-TS, *p*-toluensulfonic acid; *p*-XS, *p*-xylene-2-sulfonic acid; BDS, benzene-1,3-disulfonic acid; EBS, ethylbenzenesulfonic acid.

Specificity of these assays was assessed by measuring standard curves prepared with a battery of structurally related substances such as sulfonamides, phenylsulfonic acids, SPC metabolites, and naphthalenesulfonates. Moreover, the most abundant groups of isomers of alkylphenylsulfonic congeners, with a defined alkyl chain length, present in the commercial mixture were also analyzed in order to know the recognition pattern of the different components of the technical LAS. Table 3 shows the results of these studies expressing the recognition of each of those compounds by their percentage of cross-reactivity with respect to the IC_{50} of LAS. As can be observed, the 2-phenylC$_{10}$ and C$_{12}$ congeners are the best recognized in the indirect ELISA format. A limit of detection of 1.2 μg L^{-1} (IC_{50} is 56.7 μg L^{-1}) was estimated for the 2-phenylC$_{12}$ congener, one of the major components of the technical mixture. The 2-phenylC$_8$ is also very well recognized in the direct format. Congeners with alkyl chains higher than 14 carbon atoms, which are minor components of the technical mixture, are not recognized significantly in the assays. In contrast, the use of 13C$_{13}$-SPC−KLH as immunogen has determined a high recognition of long chain SPCs. According to the reported degradation kinetics,[43] formation of the 13C$_{13}$-, 12C$_{12}$-, and 9C$_9$-SPCs occurs quite fast. Therefore, the presence of those substances should be expected together with LAS in all the samples.

(43) Leon, V. M.; Gomez-Parra, A.; Gonzalez-Mazo, E. *Environ. Sci. Technol.* **2004**, *38*, 2359−2367.

The IC_{50} values accomplished for the long chain SPCs are in the nanomolar range (i.e., 12C$_{12}$-SPC, 2−3 nM, depending on the assay format). In contrast, short-chain SPCs or other substances possessing sulfonic or phenylsulfonic acids, such as *p*-toluene sulfonic, *p*-xylene sulfonic, or naphthalene sulfonates, are not significantly recognized by these assays.

Features of the ELISAs. The features of the assays carried out under the conditions established are shown in Table 4, and Figure 5 shows the standard curves obtained for both indirect and direct ELISA assays. Because of the excellent recognition of LAS and long-chain SPCs, both assays can be used for screening purposes to detect contamination by these anionic surfactants of environmental and potable water samples. The assay reported by Fujita [27] did recognize LAS with an IC_{50} of 40 μg L^{-1}, but no data is reported regarding recognition of long- or short-alkyl-chain SPCs. The limit of detection (concentration producing 90% of the A_{max}) accomplished by the indirect ELISA was 1.7 ± 0.6 μg L^{-1} (IC_{50} is 28.1 ± 3.2 μg L^{-1}) using the technical mixture of LAS as standard and 0.17 ± 0.04 μg L^{-1} (IC_{50} is 0.83 ± 0.06 μg L^{-1}) for the long-chain SPCs, taking 12C$_{12}$-SPCs as the representative congener. The direct ELISA format reported here also shows excellent detectability values for LAS and 12C$_{12}$-SPCs (see Table 4), although variability of this assay was higher (CV within assay, 14.3% and between assays performed on different days, 21.2%) than in the indirect ELISA format (CV within assay, 5.6% and between assays performed on different days, 11.8%). This lack of reproducibility of the direct ELISA was mainly attributed to the poor stability of the enzyme tracer. Several new stock solutions and new batches of 7C$_7$-SPC−HRP tracer had been prepared during the optimization and evaluation experiments to try to find out the reason for this variability and the optimum storage conditions. At present, the stabilization of the tracer has still not been solved.

Assay As98/7C$_7$-SPC−CONA was used to measure a set of blind samples with the aim to evaluate immunoassay accuracy. Figure 6 shows the correlation found between the measured and spiked values using the indirect ELISA. The coefficient of correlation was very good (r^2 = 0.99), and the slope was very close to 1, indicating that the results obtained match very well the spiked value. Similarly, the matrix effects caused by different water samples were evaluated. Although the pH was in all cases very close to 7.5, the ionic strength was very low for the river, tap, and well water samples (conductivity values measured were 0.9, 0.6, and 2.0 mS cm^{-1}, respectively) and quite high for the seawater samples (50 and 46.1 mS cm^{-1} for natural and artificial samples, respectively); therefore, prior assay measurements of the physicochemical parameters were adjusted by buffering the samples. Although tap and river water samples did not produce significant interferences in the assay, nonspecific signals were observed when well and seawater samples were measured. In all cases, these undesired matrix effects observed could be avoided by just diluting the sample in the assay buffer; however, this shifted the actual detectability values. Thus, the standard curves shown in Figure 7 for the different matrixes assessed demonstrate the parallelism with the curve prepared in the assay buffer after application of the corresponding dilution factor with the assay buffer. As a result, the LODs for well water and natural seawater samples shifted to 13.2 and 1.3 μg L^{-1}, respectively, for LAS and 12C$_{12}$-SPCs, because a minimum dilution factor of 8 times had to

Table 4. Features of the Optimized Immunoassays[a]

	direct ELISA As98/7C_7-SPC−HRP[b]		indirect ELISA As98/7C_7-SPC−CONA[c]	
	LAS[d]	12C_{12}-SPC[e]	LAS[d]	12C_{12}-SPC[e]
A_{min}	0.06 ± 0.03	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.05
A_{max}	0.70 ± 0.03	0.63 ± 0.03	0.75 ± 0.01	0.91 ± 0.01
slope	−0.56 ± 0.09	−0.69 ± 0.08	−0.84 ± 0.04	−1.32 ± 0.06
IC_{50}, $\mu g\ L^{-1}$	43.47 ± 22.30	1.12 ± 0.08	28.1 ± 3.2	0.83 ± 0.06
LOD, $\mu g\ L^{-1}$	2.6 ± 4.9	0.12 ± 0.08	1.7 ± 0.6	0.17 ± 0.04
dynamic range, $\mu g\ L^{-1}$	(6.6 ± 16.3)−(381.7 ± 555)	9.65 to 0.26	(5.1 ± 1.1)−(137.2 ± 35.0)	0.35−2.45
R^2	0.98	0.99	0.99	0.99

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve. [b] The data presented correspond to the average of 6 calibration curves run on three different days. [c] The data presented correspond to 11 calibration curves run on three different days. [d] Values obtained when using the LAS mixture as standard. [e] Values obtained when using the main representative SPC congener 2phenylC_{12} as standard. Each curve was built using two-well replicates.

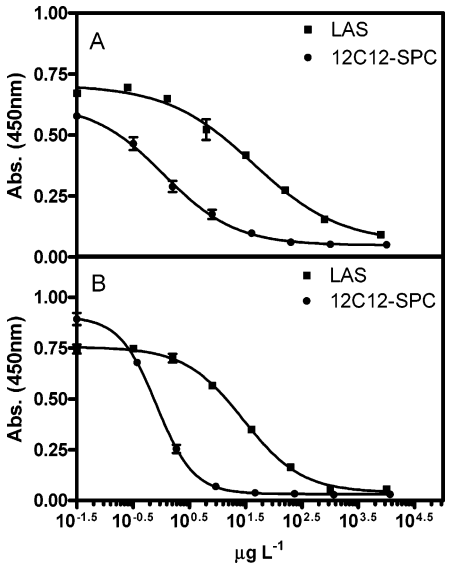

Figure 5. Standard curves obtained for LAS and the long-alkyl-chain SPCs (12C_{12}-SPC as representative congener) with the direct (A, As98/7C_7-SPC−HRP) and indirect (B, As98/7C_7-SPC−CONA) ELISAs. The data presented correspond to the average and standard deviation of six assays run on different days. Standard curves were built using three-well replicates. The features of these assays are summarized in Table 4.

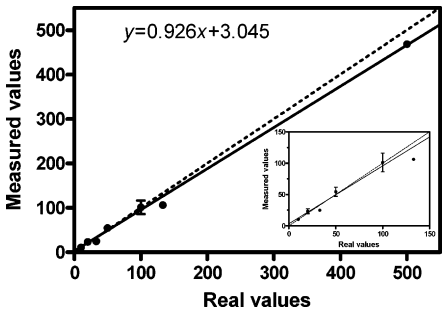

Figure 6. Correlation between the spiked concentrations and the corresponding values measured by ELISA. Blind samples were prepared in Milli-Q water. Samples were buffered with PBST-I to adjust pH and ionic strength before ELISA measurements. The data correspond to the average of three-well replicates. The dotted line corresponds to a perfect correlation (slope = 1). The coefficient of correlation is 0.99. The inserted graph is an amplification of the correlation found at lower concentration values. Each point is the average and standard deviation of analyses made on three different days.

be applied. However, at present, we cannot ensure that these samples, especially the natural seawater sample taken near of the city of Barcelona, do not contain LAS and SPCs as result of the domestic discharges and river effluents. Blank samples obtained far away from the cost would help to determine more accurately the extent of the matrix effect. Supporting this hypothesis are the results obtained with the artificial seawater sample. A 2-fold dilution factor was sufficient to avoid the undesired nonspecific signal. Finally, preliminary accuracy studies were performed with these samples by preparing blind spiked samples at three different levels and measuring them with the ELISA. As can be observed in Table 5, results obtained match very well the spiked values, and the recovery factors are close to 100% in most of the cases.

CONCLUSIONS

The work presented here shows the potential of using heterologous (or pseudoheterologous) immunization strategies to produce antibodies against small molecules. Immunochemical methods rely on the availability of the necessary immunoreagents, particularly the antibodies. Raising antibodies against small nonimmunogenic organic molecules is sometimes difficult and requires participation of truthful synthetic organic chemists to prepare the most appropriate immunizing haptens. This is often the key step of the whole process. This work is even more challenging when, as in this case, the target analyte is not a single substance but a mixture of compounds. In this paper, antibodies have been raised against LAS and the long chain SPCs using two types of immunogens and two different immunization strategies. The immunogens have been designed to maximize recognition of the alkyl moiety (SFA−KLH) or the phenylsulfonate group (13C_{13}-SPC−KLH). By using homologous immunization strategies, the last one has provided antisera with higher antibody titers, indicating the superior antigenic properties of a group containing heteroatoms and net charge in front of the hydrophobic alkyl chain. However, as demonstrated also before,[28] the size and complexity of the alkyl chain in the LAS technical mixture also participates in the stabilization of the antigen−antibody complex. Thus, the antisera raised by means of pseudoheterologous immunization procedures have provided the best assays in both direct and direct competitive ELISA formats. Moreover, the results from the noncompetitive experiments in the direct format clearly demonstrate a superior performance of these antisera. In the indirect format, these antisera showed the widest recognition. The work presented supports the idea that heterologous immunization

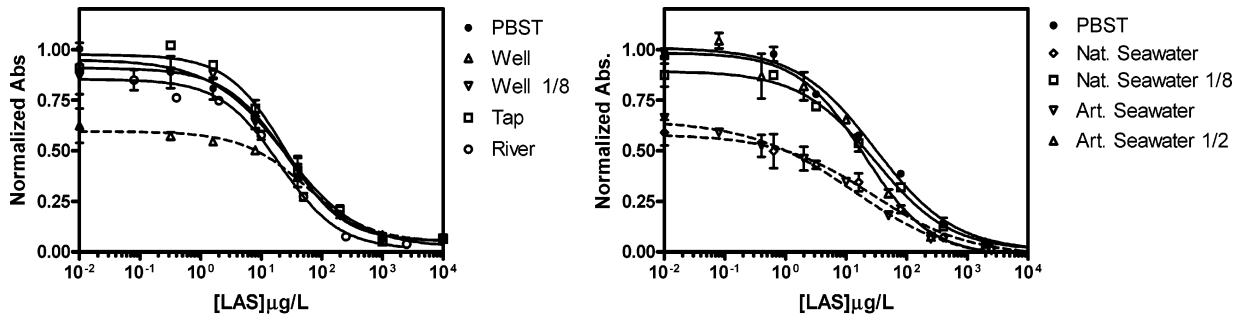

Figure 7. Graphs showing the parallelism of the standard curves prepared in different types of water samples and in the assay buffer. Although tap and river waters can be measured directly (left graph), an undesired effect is observed when trying to measure well (left graph) and seawater (right graph) samples. Dilution of the samples with the assay buffer was sufficient to avoid these effects.

Table 5. Results from the Accuracy Studies Performed with Natural Water Samples[a]

spiked value	measured measured	% recovery
river water		
100	103.5 ± 14.6	104
20	15.7 ± 1.9	79
4	4.1 ± 0.4	103
seawater[b]		
200	235.8 ± 31.0	118
40	38.8 ± 14.3	97
8	9.3 ± 1.9	116
tap water		
200	182.4 ± 41.6	91
40	43.1 ± 0.5	108
8	5.6 ± 0.4	70
well water		
400	401.7 ± 33.5	100
80	61.5 ± 2.8	77
16	16.4 ± 4.1	103

[a] Natural water samples were spiked and measured using the As 98/7C_7-SPC−CONA indirect ELISA. [b] Samples prepared with artificial seawater samples were diluted two times before ELISA measurements to avoid matrix effects. Each value corresponds to the mean of two replicates. All concentration values are expressed in μg L^{-1}.

strategies may allow raising antibodies against two different epitopes by combining two immunogens addressing recognition against two different epitopes. This immunization schemes may overcome, in some cases, the need to invest time and effort in complex synthetic methods to prepare suitable haptens preserving the most important antigenic determinants. As result of this work, highly sensitive and robust ELISAs have been developed for determination of the widely used domestic and industrial anionic surfactant LAS and the first degradation products, the long alkyl chain SPCs. The indirect format has been found to be more robust, since it avoids all the problems encountered with the stabilization of the enzyme tracer. The limit of detection reached by this assay is almost at the same level as the chromatographic analytical techniques used nowadays to analyze these substances, with the advantage that simultaneous analyses could be performed directly in the aqueous media without the need to go through tedious extraction and preconcentration steps. The immunochemical method reported here should be considered as an analytical screening method, since it does not discriminate between LAS and long chain SPCs, when both types of compounds are expected to be found together in environmental and biological samples. In fact, the sensitivity versus these long chain SPCs, formed during the first steps of the degradation process, is much higher. Thus $12C_{12}$-SPC, which is expected to be the most abundant SPC once degradation has started, is recognized with a LOD around 0.2 μg L^{-1}. Short-chain SPCs and other substances possessing aromatic and sulfonic groups in their structure are not recognized. Positive results obtained with this assay can only be attributed to the presence of LAS or compounds derived from the initial degradation process. Regular screening of LAS and long-chain SPCs by chromatographic methods can be intricate because of the complex composition of the technical mixture plus their high solubility in aqueous media, which reduces the throughput capabilities of the actual analytical methods. The immunoassay presented here can be an excellent tool to improve monitoring efficiency of LAS and SPCs in environmental and potable water samples.

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SUPPORTING INFORMATION AVAILABLE

Additional experimental procedure details and data. This material is available free of charge via the Internet at http://pubs.acs.org.

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