

Analysis of Nonylphenol: Advances and Improvements in the Immunochemical Determination Using Antibodies Raised against the Technical Mixture and Hydrophilic Immunoreagents

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The development of an enzyme-linked immunosorbent assay (ELISA) for the detection of technical nonylphenol (NP) is reported. The preparation of specific antibodies has been addressed using an immunizing hapten with a four-carbon atom spacer arm placed at the ortho position that preserves both the hydroxyl group and the complexity of the branched nonyl chain mixture of the technical NP. The synthesis of the immunizing hapten 5-(2-hydroxy-5-nonylphenyl)-pentanoic acid has been accomplished through a four-step synthetic pathway using the NP commercial technical mixture as the starting material. Three types of competitor haptens have also been prepared depending on the location of the spacer arm: in ortho position to the phenol group (type A), attached to the oxygen atom (type B), and in para position, substituting the nonyl chain (type C). Drawbacks produced by the hydrophobicity of the NP or of the hapten derivatives have been circumvented by using a highly hydrophilic carrier molecule such as a high-molecular-weight aminodextran as a coating support for antigen in an indirect ELISA format. A reproducible and sensitive indirect competitive ELISA has been finally obtained, reaching a limit of detection of $2.3 \pm 0.9 \mu\text{g L}^{-1}$ and an IC_{50} value of $29 \pm 5 \mu\text{g L}^{-1}$ (both $N = 16$). A coefficient of variation of 11% for assays performed on different days ($N = 5$; $\text{IC}_{50} = 30 \pm 3 \mu\text{g L}^{-1}$) demonstrates the assay reproducibility. The assay also recognizes the nonylphenol polyethoxylates to a different degree depending on the length of the ethoxylate chain. Recovery values in the range between 96 and 100% have been obtained using spiked blind aqueous samples although the sample preparation procedure used has been shown to have a great influence on the method accuracy. A preliminary evaluation of the analytical protocol established has been performed using real water samples.

Introduction

Alkylphenol polyethoxylates (APEOs) have, for many years, been the most commonly used nonionic surfactants through-

out the world in many fields, mainly in the formulation of detergents both for industrial and domestic applications, but also in the preparation of pharmaceutical and personal care products and in other areas such as the textile, paper, and leather industries as emulsifiers and as solubilizing, wetting, or dispersing agents. This wide range of uses has led to a worldwide production of 5×10^5 tons of total APEOs, the nonylphenol and octylphenol polyethoxylates (NPEOs and OPEOs) constituting 80% and 20% of this volume, respectively (1, 2). Therefore, large amounts of NPEO (and OPEO) are continuously discharged into the environment. Most wastewater treatment plants (WWTPs) can remove them efficiently (near 99% in some cases, depending also on the amount discharged, refs 2, 3). Their biodegradation is very fast, and it can occur in both aerobic and anaerobic conditions. The pathway involves the consecutive loss of the ethoxylate units (EO) under anoxic conditions, whereas in the presence of oxygen, the oxidation of the terminal alcohol to carboxylic acid leading to the alkylphenol ether carboxylates has also been reported. Successive losses of all the EO led to the free alkylphenols (APs), whose subsequent degradation is much slower (2). The APs have lost the surfactant properties of their parent compounds, are more hydrophobic, and are insoluble in aqueous media. Therefore, they are more persistent and tend to be adsorbed onto sediments, sludge, or particulate matter (i.e., 4).

The APEOs present a low degree of toxicity to organisms, and this increases when shortening the ethoxylate chain, with one or two EO units and the APs are the most toxic. The public and scientific concern about APs has increased considerably during recent years due to their clearly proven estrogenic effects (5) in some living organisms, including fish and mammals (6, 7), although there has not been any evidence that NP could accumulate or produce any endocrine-disrupting activity in humans (8, 9). Although the octylphenol (OP) estrogenic activity seems to be higher (10), the environmental occurrence and concentration levels of NP are greater due to the higher production volume of the NPEO. Concentrations of NP in the $\mu\text{g L}^{-1}$ range have been found in surface waters as well as in sewage effluents, sludge, and sediments (i.e., 4). Data on the real human exposure is scarce, due in part to some analytical limitations. Nevertheless, a statistical analysis of the human urinary excretion of both 4,4'-isopropylidenediphenol and nonylphenol by 395 individuals from a reference population in the US has been recently reported (11). This study showed that around 50% of the population was found to be positive ($\geq 0.1 \mu\text{g L}^{-1}$) for 4-*n*-nonylphenol, a minor isomer of the technical mixture. Gas and high-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) or tandem MS (MS/MS) can reach detection limits below the $\mu\text{g L}^{-1}$ or even in the ng L^{-1} level (i.e., 12), although a chemical derivatization of the analyte is often necessary prior to the analysis (13) as well as an additional purification and concentration step. Moreover, unlike the commercial OP, which is a single compound with a branched alkyl chain with eight atoms of carbon, the technical NP found in the environment or used in the production of the NPEO is a complex mixture of isomers with different branched patterns of the nonyl group. Thus, the chromatograms usually show a broad number of peaks whose separate identification is difficult and hinders accurate quantification. All these facts can prolong the analysis, culminating in a tedious, and often expensive, process.

As an alternative, immunochemical methods offer the possibility to simultaneously analyze many samples in a reasonable time frame. Additionally they are well-known for

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their selectivity, detectability, and reliability. Usually minimal sample pretreatment is required, which also contributes to their low cost and analysis speed. Thus, in the literature, some attempts have been reported to produce both polyclonal (14, 15) and monoclonal antibodies against NP (15, 16). However, immunochemical determination of NP has been invariably plagued by the unfavorable characteristics of the analyte. On one hand, the heterogeneity of the chain isomers of the technical mixture may limit the possibilities to raise high-quality NP antibodies. On the other hand, a second source of difficulties come from the particular physicochemical properties of NP and the fact that immunochemical analyses at the trace level have to be made using aqueous diluents. This fact strongly compromises the accuracy and precision of the analytical method. The hydrophobicity of the molecule ($\log P_{ow}$ 4.48, ref 17) determines its low water solubility (5 mg L^{-1}) and the tendency of this substance to adsorb to the solid surfaces (i.e., plasticware). Despite the immunochemical techniques developed for NP (ELISAs (14–16, 18), dipstick assays (19), polarization fluoroimmunoassays (20, 21), flow injection immunoassays (22), and capillary immunoassays (23)), this issue has never been seriously addressed.

With the goal of overcoming these aforementioned problems, we present here a rational approach based on the preparation of antibodies using an immunizing hapten with a four-carbon atom spacer arm placed at the ortho position that preserves both the hydroxyl group and the complexity of the branched nonyl chain mixture of the technical NP. Drawbacks produced by the hydrophobicity of the NP or the hapten derivatives have been resolved by using a highly hydrophilic immunoreagent, such as a high-molecular-weight aminodextran as a coating antigen in an indirect ELISA format. Accuracy and precision of the immunochemical determination of NP in aqueous buffer has been evaluated. To the best of our knowledge, there are no precedents for the use of these rational approaches to produce high-quality antibodies and to develop a reliable immunochemical assay to determine NP in aqueous media.

Experimental Section

Chemicals and Immunochemicals. Immunochemicals such as goat anti-rabbit IgG coupled to horseradish peroxidase (anti-IgG–HRP), poly-L-lysine hydrobromide (PLL) (molecular weight, MW \approx 39 000–48 100), and proteins such as horseshoe crab hemocyanin (HCH), bovine serum albumin (BSA), ovalbumin (OVA), conalbumin (CONA), and HRP were obtained from Sigma Chemical Co. (St. Louis, MO). Bradford dye reagent for protein assay was from BioRad (Hercules, CA). Aminodextran (AD) (MW 70 000) with an amino functionalization ratio of 21.5 amino group/mol_{dextran} was purchased from Molecular Probes (Leiden, Netherlands). HiTrap desalting columns prepacked with Sephadex G-25 were from Amersham Pharmacia (Uppsala, Sweden). The milk used in the blocking step of the immunoassay was commercial skimmed powdered milk for babies. The NPEO used for cross-reactivity studies were acquired from Sigma (NP10EO and NP7EO, Tergitol series). NP(1–2)EO and NP1EC standards were supplied by Dr. Damià Barceló from the department of Environmental Chemistry of IIQAB–CSIC (Barcelona, Spain). Linear alkylbenzenesulfonates (LAS) standards were a gift from PETRESA (Cádiz, Spain). The preparation of the protein conjugates and the antisera (As) is described below. Unless otherwise indicated, the immunochemicals prepared were stored dry and frozen at -40°C under argon atmosphere. Stock solutions (1 mg mL^{-1}) were prepared in phosphate-buffered saline (PBS) and stored at -40°C . The working aliquots were stored at 4°C .

Buffers. Unless otherwise indicated, PBS is 0.01 M phosphate buffer and 0.8% saline solution, and the pH is 7.5.

PBST is PBS with 0.05% Tween 20. PBST–EtOH is PBS with 0.001% Tween 20 and 10% EtOH. 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.

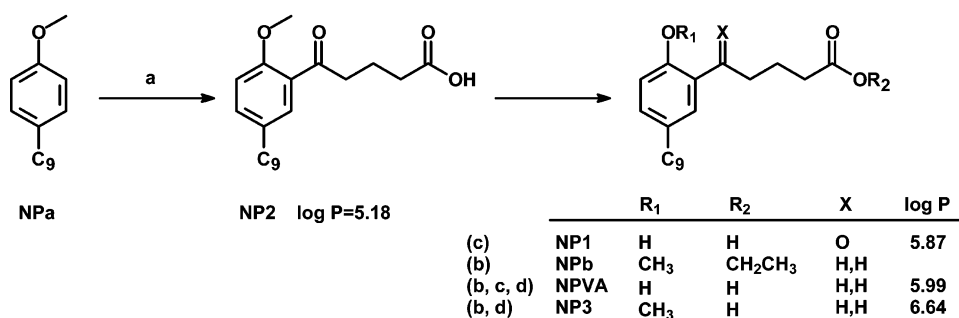
General Methods and Instruments. The matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) used for analyzing the protein conjugates was a Perspective BioSpectrometry Workstation provided with the software Voyager-DE-RP (version 4.03) developed by Perspective Biosystems Inc. (Framingham, MA) and Grams/386 (for Microsoft Windows, version 3.04, level III) developed by Galactic Industries Corp. (Salem, NH). The pH and 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, Denmark). Washing steps were performed on an SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). Absorbances were read on a Spectra-maxPlus (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 Software Inc., San Diego, CA). All washing steps used on the ELISA protocols consisted of four cycles of PBST ($300 \mu\text{L}/\text{well}$). Unless otherwise indicated, the data presented correspond to the average and standard deviation of at least two well replicates or three different experiments. Methods and instruments used for organic synthesis are available as Supporting Information.

Molecular Modeling and Theoretical Calculations. Molecular modeling was performed using the Hyperchem 6.0 software package (Hypercube Inc, Gainesville, FL). Theoretical geometries and electronic distributions were evaluated for NP and NPVA hapten (as amide derivatives) using semiempirical quantum mechanics MNDO and PM3 models (ref 24 and references therein). All the calculations were performed using standard computational chemistry criteria.

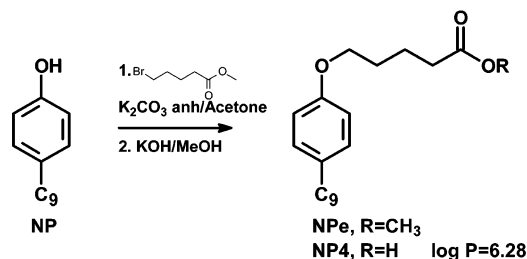
Synthesis of the Nonylphenol Haptens. Three types of haptens were synthesized depending on the site where the spacer arm was located (see Figure 1): type A, with the spacer arm in the ortho position of the phenol group (5-(2-hydroxy-5-nonylphenyl)-pentanoic acid, NPVA; 5-(2-hydroxy-5-nonylphenyl)-5-oxo-pentanoic acid, NP1; 5-(2-methoxy-5-nonylphenyl)-5-oxo-pentanoic acid, NP2; and 5-(2-methoxy-5-nonylphenyl)-pentanoic acid, NP3), type B, with the spacer arm introduced through the phenol group (5-(4-nonylphenoxy)-pentanoic acid, NP4), and type C, with the spacer arm located in the para position of the phenol group, substituting the nonyl chain (5-(4-hydroxyphenyl)-pentanoic acid, NP5; 5-(4-methoxyphenyl)-pentanoic acid, NP6; and 9-(4-hydroxyphenyl)-nonanoic acid, NP9). The synthesis of the haptens was performed as shown in Figure 1. An accurate description of the synthetic methods used together with the spectroscopic and spectrometric data used to characterize the haptens can be found in the Supporting Information.

Preparation of the Immunogen and the BSA Homologous Antigen. The conjugation of 5-(2-hydroxy-5-nonylphenyl)-pentanoic acid (NPVA) hapten through its carboxylic group to the lysine residues of HCH and BSA by the mixed anhydride (MA) method was carried out as described (25) by activating NPVA (10.2 mg , $32 \mu\text{mol}$) with isobutyl chloroformate ($5.5 \mu\text{L}$, $44 \mu\text{mol}$) and tri-*n*-butylamine ($11.3 \mu\text{L}$, $66 \mu\text{mol}$) in anhydrous DMF ($150 \mu\text{L}$) followed by the addition to the HCH solution (5 mg) in borate buffer (1.35 mL). The conjugate was purified using a Sephadex G-25 desalting column. Simultaneously, the BSA conjugate was prepared

Scheme I. Synthesis of haptens Type A



Scheme II. Synthesis of haptens Type B



Scheme III. Synthesis of haptens Type C

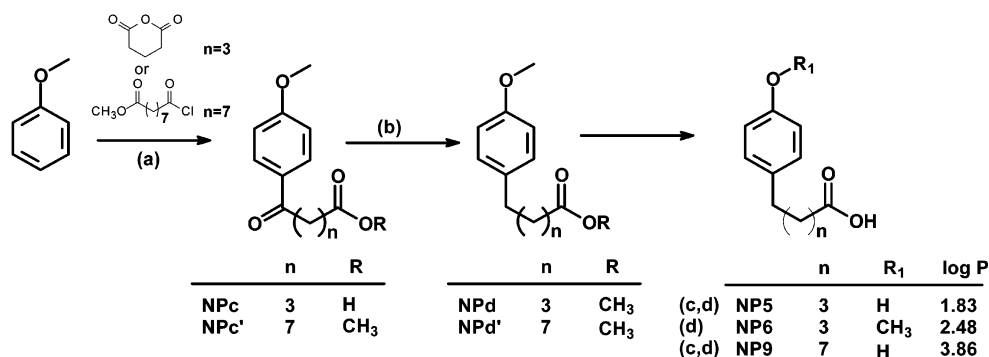


FIGURE 1. Synthetic pathways followed for the synthesis of haptens type A (Scheme I), type B (Scheme II), and type C (Scheme III). The lower letters indicate reaction conditions: a, glutaric anhydride or acid chloride, AlCl_3 , CH_3NO_2 , -20°C ; b, $\text{H}_2/\text{Pd}(\text{C})$, HClO_4 , EtOH , or MeOH ; c, $\text{BBR}_3/\text{CH}_2\text{Cl}_2$ anh. -78°C ; d, KOH/MeOH . The synthesis of hapten NP9 has been described elsewhere (54). Theoretical Log *P* values of the amide forms of the corresponding haptens have been calculated using the software package described in the Experimental Section.

following the same procedure but reacting 16 μmol of NPVA with 5 mg of the protein.

Preparation of the Coating Antigens (CA). Haptens NPVA, NP1–NP6, and NP9 were covalently conjugated to BSA, CONA (10 mg each), PLL (10.4 mg, 0.5 μmol of free lysine), and AD (16.3 mg, 5 μmol of amino groups) in borate buffer pH = 8.7 as reported (26) by first activating the haptens (5 μmol) with NHS (25 μmol) and DCC (50 μmol) in DMF (200 μL). The conjugates were extensively dialyzed against PBS (0.5 mM, 4 \times 5 L) and MilliQ water (1 \times 5 L) and lyophilized. Certain protein conjugates, after reconstitution, were not completely soluble. In those cases, the concentration of soluble fraction was determined using the Bradford protein assay (27).

Hapten Density Analysis. Hapten densities of the BSA conjugates were calculated using MALDI-TOF-MS by determining the MW of the native proteins and comparing it with that of the conjugate. Spectra were obtained by mixing 2 μL of the matrix solution, *trans*-3,5-dimethoxy-4-hydroxycinnamic acid (10 mg mL^{-1} in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 70:30, 0.1% TFA), with 2 μL of a solution of the conjugates or proteins (5 mg mL^{-1} in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 70:30, 0.1% TFA). The hapten density (δ hapten) was calculated according to the following equation: $\{\text{MW}(\text{conjugate}) - \text{MW}(\text{protein})\} / \text{MW}(\text{hapten})$. The

hapten density of the NP1–AD conjugate was determined by UV spectrophotometry in 1:1 $\text{MeOH}:\text{H}_2\text{O}$ by measuring the absorbance of the conjugate and the intact AD at $\lambda = 340$ nm. The extinction coefficient of the hapten was calculated by performing a linear regression of the absorbance at $\lambda = 340$ nm of different concentrations of the pure hapten (from 200 to 6.25 $\mu\text{g mL}^{-1}$) in the same solvent.

Polyclonal Antisera. Five female New Zealand white rabbits (105, 106, 107, 110, and 111), weighing 1–2 kg, were immunized with the immunogen NPVA–HCH, following the immunizing protocol described by Ballesteros et al. (25). The evolution of the antibody titer was assessed by measuring the binding of serial dilutions of the antisera (As, from 1/1000 to 1/32 000 in PBST) to microtiter plates coated with NPVA–BSA (MA method, 1 $\mu\text{g mL}^{-1}$). After an acceptable antibody titer was observed, the animals were sacrificed and the blood was collected on vacutainer tubes provided with a serum separation gel. Antisera were obtained by centrifugation and stored at -40°C in the presence of 0.02% NaN_3 .

Competitive ELISAs. Optimal concentrations for the CA and As dilutions were chosen through checkerboard titration experiments (see Supporting Information) to produce absorbances of around 0.7–1 units of absorbance, incubating

for 30 min at room temperature (rt), corresponding to the 70% of the maximum absorbance under nonsaturating conditions.

Optimized Indirect ELISA. Microtiter plates were coated with NP1-AD in coating buffer ($0.125 \mu\text{g mL}^{-1}$, $100 \mu\text{L/well}$) covered with adhesive plate sealers for 3 h at rt or overnight at 4°C . Following this step, the plates were washed (four times with PBST, $300 \mu\text{L/well}$), a solution of skimmed milk (1% w/v in PBS) was added ($100 \mu\text{L/well}$), and the plates were incubated for 30 min at rt. The plates were washed again, and the NP standards ($8000 \mu\text{M}$ to $0.512 \mu\text{M}$ (1760 mg L^{-1} to 0.11 mg L^{-1}) prepared in DMSO) were diluted 200 times with PBST-EtOH and added to the plate ($50 \mu\text{L/well}$) followed by the As 110 (1/1000 in PBST-EtOH, $50 \mu\text{L/well}$). The plates were washed and a solution of anti-IgG-HRP (1/6000 in PBST) was added to the wells ($100 \mu\text{L/well}$) and incubated for 30 min more at rt. Finally, the plates were washed again, and the substrate solution was added ($100 \mu\text{L/well}$). The reaction was incubated for 30 min, protected from light at rt, and then the reaction was stopped by adding $4 \text{ N H}_2\text{SO}_4$ ($50 \mu\text{L/well}$).

Cross-Reactivity Determinations. Stock solutions of several structurally related compounds were prepared fresh in DMSO (40 mM). Standard curves for each of these compounds were constructed by serial dilution ($8000 \mu\text{M}$ to $0.512 \mu\text{M}$) in DMSO and dilution 200 times in PBST-EtOH before measuring them by the optimized ELISA. The IC_{50} values were determined in the competitive experiments for each analyte and were used to determine the cross-reactivity (CR) values according to the following equation: $\{\text{IC}_{50}(\text{NP})/\text{IC}_{50}(\text{cross-reactant})\} \times 100$.

Accuracy studies were performed by measuring spiked blind samples and real sample extracts. Spiked blind samples were prepared in DMSO and then diluted 200 times in PBST-EtOH to measure them with the ELISA, either directly or after preparing further dilutions with the same buffer. Analyses were done in triplicate. The correlation was evaluated by establishing a linear regression between the spiked and the measured values. Real sample extracts from effluents of WWTPs were kindly given by Prof. Damià Barceló (Department of Environmental Chemistry, IIQAB-CSIC). Sample preparation was performed as previously described (28). The resulting methanol extracts were analyzed with the optimized ELISA by diluting them in PBST-EtOH considering a one-step dilution factor, and the data were compared with those obtained from the LC-MS analysis under the conditions previously described (29).

Results and Discussion

Hapten Design and Synthesis. Often an intuitive approach based on the chemical knowledge of the analyte is used to design the most appropriate immunizing hapten chemical structure. Using these criteria, electrostatic and hydrogen-bonding are frequently considered the most important interactions in the antigen-antibody binding. However, the actual model is much more complex and takes into account other interactions. In the early 1990s, a very sound model could explain qualitatively and almost quantitatively these interactions (i.e., refs 30, 31, and references therein). According to this model, the electrostatic and hydrogen bonding forces contribute only a small fraction (ca. 20%) of the free energy of the interaction (for a K_a around 10^{10} M^{-1} , ΔG is in the order of 68 kJ/mol). The rest comes from the so-called hydrophobic interactions, which are about 100 J/\AA^2 of the contact zone (32). The main role of electrostatic and hydrogen-bonding interactions would be to keep the two molecules in the correct orientation to allow the short range hydrophobic interactions to be established. Attending to these arguments, we thought that the characteristic complex

mixture of alkyl chains of the nonylphenol could significantly contribute to stabilize the antigen-antibody complex. Therefore, keeping the complexity of this part of the molecule was, in our opinion, essential.

The strategies used up to now have been based on the use of immunizing haptens that sacrifice the important epitopes of the molecule, both the phenol group (i.e., NP5EO, with a terminal carboxylic acid on the ethoxylate chain, ref 16) and the characteristic complex mixture of branched alkyl chains (i.e., *p*-hydroxyphenylalkylcarboxylic acids with a defined linear alkyl chain, refs 14, 15). Thus, in an attempt to produce monoclonal antibodies, Zeravik et al. (15) ended up with an assay recognizing only the linear isomer (4-*n*-nonylphenol) while two technical nonylphenol mixtures tested only cross-reacted 0.9 and 0.4%. It is worth noticing that the linear nonylphenol isomer is only a minor fraction (<1%) of the technical mixture, which makes its detection irrelevant. This result is not surprising considering that the immunizing hapten used was 9-(*p*-hydroxyphenyl)nonanoic acid. The authors only succeeded in developing an assay for the technical mixture using a polyclonal antibody raised before (14) against the same immunizing hapten, taking advantage of the almost unlimited range of specificities theoretically available on the immune system of the mammals. However, from the 12 antisera then obtained, only one showed enough affinity to sufficiently recognize the technical mixture with cross-reactivity values around 50–60% in comparison with the linear nonylphenol (100%) (15).

Alternatively, derivatization through the phenolic group, converting this to an ether, has also been reported (16). However, molecular modeling studies performed previously by our group demonstrated that inhibiting the acid-base equilibria of phenolic compounds, by converting the phenol group to an ether, produced great changes on the punctual charges of the molecule (33, 34). Moreover, placing the spacer arm through the OH of the phenolic group would lead to a much better recognition of the NPEO, as has recently been demonstrated by Goda et al. (35). Thus, both the ortho and the meta positions seemed the most appropriate positions for linking the hapten to the protein, although from the chemical point of view, the ortho position was more suitable. Chemistry at the meta position would have required nonstandard and complex synthetic procedures. Additionally, steric hindrance caused by the nearby and voluminous branched alkyl chain of the NP had to be considered. In parallel to the work presented here, Mart'ianov et al. (18, 36) have reported the direct coupling of the protein, without a spacer arm, to the ortho position of the NP via a Mannich reaction, following a strategy that respects both antigenic sites. The assay obtained has a good limit of detection (near $10 \mu\text{g L}^{-1}$), but an important drawback of their system is the low slope value (around 0.5), which places the IC_{50} around $250\text{--}300 \mu\text{g L}^{-1}$. Assays with very low slope are usually not accurate since small variations in the absorbance due to systematic errors are recorded as a significant shift of the concentration measured. On the other hand, this low slope could be attributed to a moderate affinity of the antibodies caused by the steric hindrance of the protein. Moreover, on the basis of our knowledge, we interpret that the high recognition degree of all the NPEOs assayed, some of them even more than the NP, could be due to the use of an immunizing hapten with a short spacer arm so that the hydroxyl group could be hidden by the protein (short ethoxylate chain NPEO cross-react 180–300%).

Hapten NPVA incorporating a four-carbon atom alkyl chain was thus proposed as an immunizing hapten (see Figure 2). Molecular modeling and theoretical calculations were performed to ensure that no significant changes in the geometry and electronic properties of the molecule, compared to the analyte, would take place by introducing this

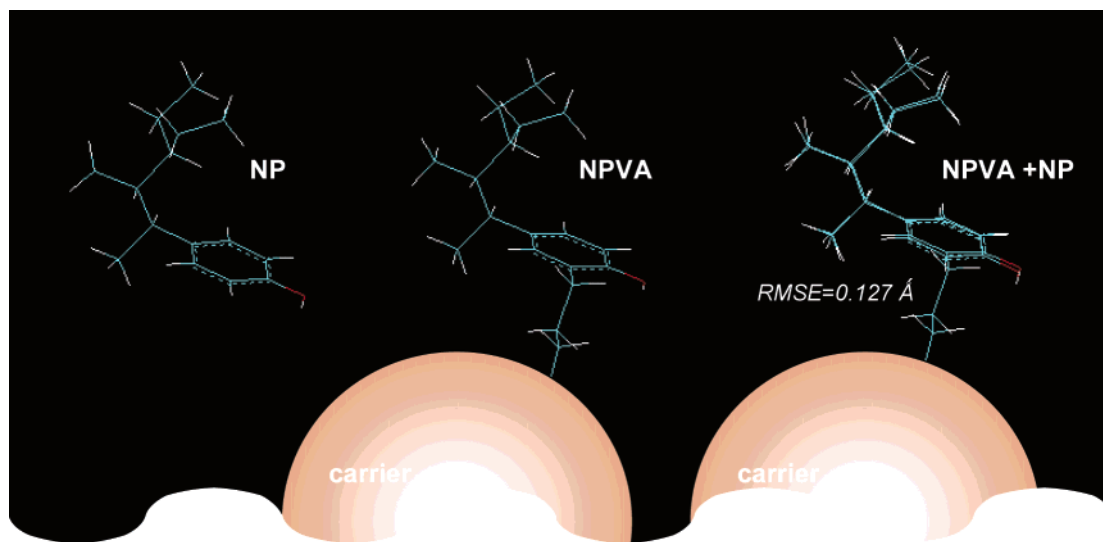


FIGURE 2. Stick and wedges display of the optimized geometries of NP (left) and hapten NPVA (middle) according to PM3 models. Model on the right shows both compounds overlapped to calculate the differences in the geometries (RMSE = 0.127 Å). Calculations have been made using the corresponding amide derivatives to mimic the conjugated haptens. The elements are presented in the following manner. Light blue, carbon; dark blue, nitrogen; white, hydrogen; red, oxygen.

spacer arm at the ortho position and that the availability of the phenol group to interact with the antibody is fully preserved (not only hydrogen donor and acceptor capability but also π - π aromatic stacking). Molecular modeling studies were made with one of the isomers of the mixture, with a trisubstituted benzylic carbon, which was considered representative of the most abundant constitutional isomers found in the technical NP. The results obtained showed that NP and NPVA had identical geometries at their minimum energy level (see Figure 2). The root-mean-square error (RMSE) value, calculated by overlapping both chemical structures (defined by the aromatic, the phenol group, and the nonyl chain), was only 0.127 Å (see Figure 2, right side), which proves that the geometry was not significantly affected by introducing the spacer at the ortho position. Similarly, the punctual charge distribution was almost the same in both molecules (data not shown). As it is shown in Figure 2, the alkyl chain is oriented above from the plane defined by the aromatic ring and in the opposite direction to that of the spacer arm (linking point to the carrier biomolecules). This conformation made us think of a potential better recognition of the nonyl chain due to a favored exposure to the immune system, compared to the hydroxyl group (closer to the protein). However, we must be aware that the orientation of the hydrophobic alkyl chain could also be affected by the tertiary structure of the protein (i.e. hydrophobic pockets) favoring other orientations once the hapten is coupled to the immunizing carrier.

Additionally, three different types of haptens were also considered as potential competitors: type A (NP1, NP2, and NP3) with the same substitution pattern as the immunizing hapten, type B (NP4) where the spacer arm is placed through the oxygen atom, and type C (NP5, NP6, and NP9) which introduces the spacer arm in the para position substituting, therefore, the nonyl chain (see Figure 1 for chemical structures). Type A haptens were obtained using the synthetic route of the immunizing hapten NPVA (see Scheme I in Figure 1). Under standard Friedel-Crafts acylation conditions (37), the reaction of the NP with glutaric anhydride and AlCl_3 as Lewis acid, in tetrachloroethane at 130 °C, led exclusively to the formation of the 4-nonylphenyl monoester of the pentanedioic acid (not shown). By protecting the phenol group with CH_3I to obtain the corresponding methyl ether, NP_a, the desired product NP2 could be obtained, although with very low yield in a complex mixture of degradation

products. Lowering the reaction temperature to 45–50 °C did not improve the reaction yield, but the use of nitromethane instead of tetrachloroethane as reaction solvent led to a cleaner reaction mixture. This solvent increased the solubility of the Lewis acid allowing the acylation reaction to take place under much milder conditions (low temperature of –20 °C). Purification of the reaction mixture by “flash” chromatography allowed isolation of the desired compound NP2 accompanied by NP_c, as a result of the dealkylation of NP_a under the reaction conditions and subsequent acylation with glutaric anhydride (see chemical structures in Figure 1, Scheme I and Scheme III). The formation of the dimers 1,5-bis-(2-methoxy-5-nonylphenyl)-pentane-1,5-dione and 1-(2-methoxy-5-nonylphenyl)-5-(4-methoxy-phenyl)-pentane-1,5-dione as byproducts (chemical structures not shown) was also observed, but in this case their formation could finally be avoided by carefully keeping the reaction temperature between –20 and –15 °C. Subsequent reduction of the ketone group of NP2 occurred with a concomitant esterification of the carboxylic acid, leading to NP_b with 94% yield. Cleavage of the methyl ether with BBr_3 , followed by the hydrolysis of the ethyl ester, finally gave the desired hapten NPVA with an overall yield of 25% from the technical NP used as the starting material. From the same synthetic pathway, hapten NP1 could be obtained, with a 20% overall yield, by cleavage of the methyl ether of NP2. Similarly, NP3 was obtained with a 34% total yield by simple hydrolysis of the ethyl ester of NP_b.

The synthesis of the type B competitor hapten NP4 was carried out as reported (38) and gave the product with an overall yield of 77% (see Scheme II in Figure 1). Finally, the preparation of type C haptens was accomplished through a pathway similar to that used for the preparation of the immunizing hapten NPVA but using anisole as starting material (see Scheme III in Figure 1). Acylation occurred preferentially at the para position if an equimolar anisole/acetylating agent ratio was used. NP6 was obtained using glutaric anhydride as acylating reagent with a 37% overall yield. Removal of the protecting group of the corresponding methyl ester led to NP5. NP9 was obtained using the corresponding acid chloride of the nonanedioic acid mono-methyl ester in the Friedel-Crafts reaction with a 16% overall yield.

Immunochemistry. NPVA was covalently attached to HCH and BSA and the conjugation verified by MALDI-TOF-

MS analysis (i.e., a hapten:protein molar ratio of 5 was estimated for the NPVA–BSA conjugate). As105–107, As110, and As111 were raised in white New Zealand rabbits. Initial attempts to develop a direct ELISA format failed because of the poor recognition of most of the competitor haptens, in this case, coupled to HRP. Orientation of the antibodies by first coating with goat anti-rabbit IgG did not enhance the signal. Insufficient conjugation to HRP or hindering of the haptens into the protein structure, due to their high hydrophobicity, were contemplated as some of the potential reasons to explain the low recognition observed (see Figure 1 for log *P* values). Only NP5–HRP and NP9–HRP were sufficiently recognized to proceed with our goal; however, very small or absolutely no recognition of the free analyte was observed. Due to its particular physicochemical properties, the behavior of the NP in this type of aqueous assays, usually performed in plastic labware, became one of our concerns. Thus, NP has already lost the surfactant properties of the parent NPEO, it is highly lipophilic (its log *P* value reaches values around 4.48 (17)), its solubility in water is low (around 5 mg L⁻¹), and, apparently, the rate of dissolution is also a slow process (39, 40). Although, according to data found in the literature (40), NP seems not to form micelles (its formation would be expected to occur at concentrations around 13 mg L⁻¹, which is higher than the solubility), a particular behavior due to this fact (i.e. formation of aggregates) could not be rejected, especially if the calibration curve was prepared by serial dilution of the most concentrated standard in aqueous media. On the other hand, nonspecific adsorption of nonpolar substances to the labware, especially to plastic, is more favored in an aqueous (i.e. immunoassay buffer) than in an organic media. Furthermore, NP is used in plastic manufacture, and some studies have revealed that a certain release from these type of containers can take place (41–43), producing a potential source of errors. As result of all these considerations, small but crucial modifications were introduced into our common immunochemical protocol. Thus, the use of plasticware was minimized as much as possible, and contact of the labware with detergents derived from nonionic surfactants was completely avoided. Moreover, the use of a certain percentage of organic solvent in the buffer was investigated with the goal to reduce nonspecific adsorption to the surfaces while improving the analyte solubility in the assay media. This strategy had shown to improve performance of immunochemical methods for other nonpolar substances (44, 45). Thus, the NP standards were always prepared in organic solvent (MeOH, EtOH, or DMSO) and then diluted 200 times in PBST buffer (0.5% of organic solvent in the buffer) immediately before using them for the assay. Using these new conditions, it was possible to observe competition of the free analyte, but reproducibility was very low (day-to-day variation can be found as Supporting Information). The use of several types of blocking agents (as a blocking step or by adding them to the assay buffer), with the goal to reduce potential nonspecific adsorption, detergents, and/or organic solvents in order to improve analyte solubility in the aqueous media, did not significantly improve immunoassay performance.

We hoped to overcome these problems by using an indirect ELISA format, but the first problem derived was the preparation of the haptenized proteins (BSA and CONA) used as coating antigens. Standard conjugation conditions (hapten:lysine residues molar ratio 2:1) led to quite insoluble protein conjugates. Because of the lipophilicity of these haptens (see log *P* values in Figure 1), conjugation of a certain number of residues (maximum 35 lysine residues in BSA) with lipophilic haptens probably modified the hydrophilic nature and conformation of the native protein and prevented it from being soluble in aqueous media (see predicted conformation of the hapten in Figure 2). Slightly more, although not

completely, soluble protein conjugates were obtained by reducing the hapten:lysine molar ratio to 1:1. Nevertheless, we decided to proceed by working with the soluble fraction of these conjugates. The hapten density determined by MALDI-TOF-MS analyses of these conjugates revealed values between 11 and 20 depending on the conjugate (see Supporting Information). In contrast to the direct format, all these conjugates were highly recognizable, and recognition of the free analyte was possible under competitive configurations; however, day-to-day variation of the immunoassay parameters remained very high, although the amount of organic solvent in the buffer (10% of EtOH) was increased.

The poor solubility of the immunoreagents and the consequent insufficient stability of their stock solutions were pointed out as the most likely reasons for this lack of reproducibility. Thus, the possibility that the orientation of the hapten molecules on the surface of the protein would change with time toward more hydrophobic pockets in the protein tertiary structure, diminishing antibody recognition or changing the immunoassay features, is feasible. In fact, the unsuitability of hydrophobic hapten–protein conjugates as coating antigens due to their poor adsorption properties had been reported before (46). On the other hand, some authors (47) have suggested using highly hydrophilic carriers to raise antibodies against hydrophobic haptens to diminish potential physicochemical interactions of those with the carrier. In our particular case, despite the encountered assay reproducibility problems, the antisera raised appeared to recognize nonylphenol sufficiently (eventually the assay was able to reach acceptable IC₅₀ values). However, the arguments of Fasciglione et al. (47) prompted us to try to improve the reproducibility of our assays by using hydrophilic coating antigen conjugates. The use of homopolymers of lysine (poly-L-lysine, PLL, i.e. refs 48, 49), gelatin (i.e. ref 50), and dextran (i.e. ref 51) to prepare coating antigen conjugates for immunoassay had been described before, although for different reasons.

Because of the reasons listed above, nonylphenol–hapten conjugates were prepared using PLL (MW, 39 000–48 100) and aminodextran (AD; MW, 70 000). When these conjugates were assayed, a high level of background noise, due to nonspecific binding of the secondary antibody, was observed if NPVA–PLL was employed as a coating antigen. Previous reports (49) have attributed this behavior to ionic interactions of the immunoglobulins with the highly charged amino-polymers. In contrast, a significant contribution of the specific signal was found when testing the avidity of the different antisera produced toward the different H–ADs (hapten–aminodextran conjugates: NPVA–, NP1–NP6–, and NP9–AD) on noncompetitive two-dimensional checkerboard titration experiments. Moreover, several reproducible competitive ELISAs for NP could be developed (see Table 1). Modification of the ELISA performance by changing the nature of the carrier protein of the coating antigen is a phenomenon often observed. The reasons for this behavior were obscure until some modern studies demonstrated the effect of the so-called secondary interaction forces (52, 53). These forces are the interaction between the external face of the antibody-binding region and the molecular carrier of the supported hapten. As result of these interactions, mainly of electrostatic nature, the affinity of the antigen–antibody binding can be significantly modified.

In all the combinations we studied, the presence of an even higher amount of organic solvent (10% of EtOH) was decisive to obtain competitive assays with high levels of reproducibility. This behavior has been previously observed in other immunoassays for hydrophobic substances (44) and it is not surprising since an increase in the availability of the analyte and in its solubility should be expected. These results

TABLE 1. Features of the NP Indirect ELISAs Obtained When Using H-AD Conjugates as Coating Antigens^a

As	antigen	As ^b dilution	[CA] ^b , $\mu\text{g mL}^{-1}$	A_{max}	A_{min}	S/N	IC_{50} , $\mu\text{g L}^{-1}$	slope	R^2
110	NPVA-AD	1/1000	0.125	1.213	0.209	5.8	1060	-0.81	0.983
	NP1-AD	1/500	0.125	0.794	0.290	2.7	326	-0.73	0.991
111	NPVA-AD	1/500	0.031	0.992	0.449	2.2	726	-0.98	0.958
	NP1-AD	1/500	0.125	0.844	0.360	2.3	97	-0.98	0.976
	NP3-AD	1/500	0.500	0.984	0.447	2.2	875	-0.98	0.973
	NP4-AD	1/500	0.250	0.823	0.570	1.4	398	-1.35	0.803

^a Only those As/coating antigen combinations showing acceptable immunoassay features are shown in the table. The analyte is the commercial mixture of the technical nonylphenol. The NP standards were prepared in DMSO and diluted 200 times in PBST-EtOH. The features are extracted from the four-parameter logistic equation used to fit the standard curves. ^b Antisera dilution and coating antigen concentration were obtained from the noncompetitive two-dimensional checkerboard titration assays.

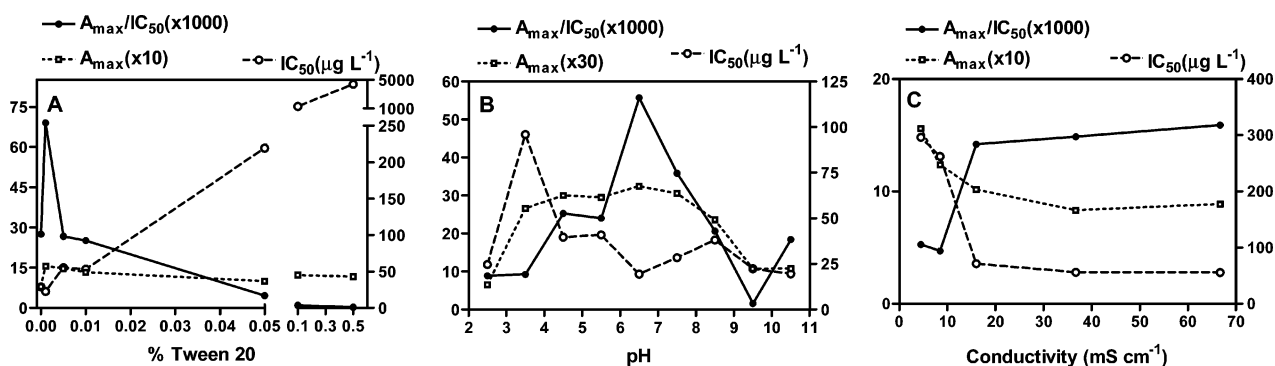


FIGURE 3. Graphs showing the effect of several parameters (A, concentration of Tween 20; B, pH; and C, ionic strength) in the indirect As110/NP1-AD ELISA. The effects of these parameters in the maximum absorbance (A_{max}), the IC_{50} value, and the ratio of both parameters are represented. Right axis shows IC_{50} values expressed in $\mu\text{g L}^{-1}$. Left axis shows A_{max} and $A_{\text{max}}/\text{IC}_{50}$. The data presented are extracted from the four-parameter equation used to fit the standard curve. Standard curves were prepared using two well replicates.

differ from those obtained in other immunochemical methodologies developed for the detection of NP, where it could be observed that an increase in the amount of organic solvent led to a decrease in the sensitivity (15, 55).

As shown in Table 1 only the quasi-homologous NP1 competitor (different from the immunizing hapten only on the keto group; see chemical structures in Figure 1) led to competitive assays with an acceptable detectability. Hapten densities of the AD conjugates were determined by UV since MALDI-TOF-MS spectra could not be appropriately recorded. About 16 mol of hapten NP1 was found to be linked to 1 mol of AD (73% conjugation yield). Assay As110/NP1-AD was selected for further investigation because, although it did not show the best detectability, experiments performed on different days were very reproducible.

Optimization and Evaluation of the As110/NP1-AD Immunoassay. Several blocking solutions (1% PVP in PBST, 1% BSA, 1% OVA, 1% gelatin, and 1% skimmed milk in PBS), used in both a blocking step and the assay buffer, were evaluated with the goal to reduce the nonspecific signal of the assay (see A_{min} in Table 1). When the blocking agent was added to the assay buffer, a slight improvement of the background noise was observed, but it was accompanied with a significant increase of the IC_{50} value (poor detectability). When these solutions were used as a blocking step, prior the competitive assay, only the skimmed milk decreased the nonspecific signal, without affecting the IC_{50} value, below 0.1 unit of absorbance. Lowering the concentration of Tween 20 in the assay buffer considerably improved the assay detectability (by about 1 order of magnitude, see Figure 3a). The same effect has been reported on ELISAs for other hydrophobic analytes (i.e., refs 44, 45), which supports the hypothesis proposed by some authors (45) regarding the establishment of hydrophobic interactions between the analyte and the detergent, thus hindering the interaction with the antibody. The assay could work in the absence of Tween; however, as, reported in refs 44 and 45, the coefficient

of variation was observed to be slightly higher, for which reason a low concentration (0.001%) of Tween 20 was kept in the buffer. The assay tolerated media with a broad range of pH values, with the best performance around pH 6.5 (see Figure 3b). From pH 4.5 to 8.5 the immunoassay showed acceptable features, although the background noise slightly increased below pH 7. Outside this interval, both detectability and maximum assay signal (A_{max}) decreased. Similarly, the assay remained very stable independent from the ionic strength of the media (see Figure 3c). Almost constant features were found (only a slight decrease of the A_{max} was observed) when the assay was performed in media with conductivity values from 15 to 67 mS cm^{-1} (10–50 mM in terms of PBS concentration). In contrast, the IC_{50} increased drastically if the assay was performed in low ionic strength media, which does not compromise the assay since this drawback can easily be solved by adding saline solution to reach a superior conductivity value.

Attending to the above-mentioned parameters and to the physicochemical parameters of NP, a protocol for the As110/NP1-AD assay for nonylphenol was established that included a 30 min blocking step with 1% skimmed milk. The assay takes 2 h and many samples can be processed simultaneously. The NP standards were prepared in DMSO, 200-fold more concentrated, and diluted prior to the assay with 10 mM PBST-EtOH (PBS containing 10% EtOH and 0.001% of Tween 20, the conductivity is 15 mS cm^{-1} , and the pH is 7.5). The use of plasticware was minimized by substituting it with glassware whenever possible. Under these conditions, a sigmoid calibration curve was obtained (see Figure 4) and an IC_{50} value of $29 \pm 5 \mu\text{g L}^{-1}$ ($N = 16$ assays) with a LOD (at the 10% of inhibition of the maximum signal) of $2.3 \pm 0.9 \mu\text{g L}^{-1}$ ($N = 16$ assays) was achieved (see Table 2 for immunoassay parameters). As can be seen from the standard deviation values, the reproducibility of the assay is very good. The IC_{50} value of standard curves measured on the same microtiter plate was found to be $33 \pm 1 \mu\text{g L}^{-1}$ ($\text{CV} = 2\%$, N

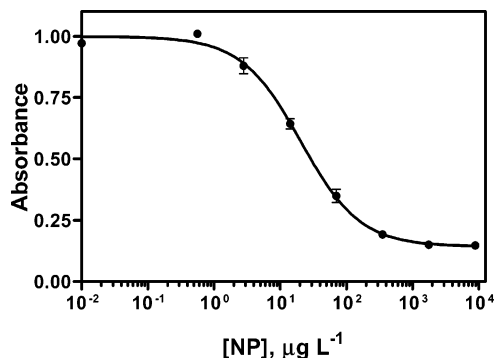


FIGURE 4. Calibration curve of the optimized NP immunoassay As110/NP1-AD run according to the established protocol described in the Experimental Section. The data presented correspond to the average and standard deviation of 16 assays, run on different days. The curves were prepared using two-well duplicates. The features of the assay are summarized in Table 2.

TABLE 2. Features of the Optimized Immunoassay As110/NP1-AD^a

A_{\max}	1.069 ± 0.069
A_{\min}	0.148 ± 0.019
S/N	7 ± 1
$IC_{50}, \mu\text{g L}^{-1}$	29 ± 5
dynamic range, $\mu\text{g L}^{-1}$	5.9 ± 1.4 to 131.8 ± 15.9
LOD, $\mu\text{g L}^{-1}$	2.3 ± 0.9
slope	-0.9 ± 0.1
R^2	0.997 ± 0.002
N	16

^a Data correspond to the average and standard deviation of the parameters extracted from the logistic equation used to fit the standard curves. Assays were performed using two-well replicates in 10 different days.

= 2); $28 \pm 2 \mu\text{g L}^{-1}$ (CV = 7%, $N = 4$) in the case of curves measured on the same day but on different microtiter plates, and $30 \pm 3 \mu\text{g L}^{-1}$ (CV 11%, $N = 5$) in the case of experiments performed on different days. Employing highly hydrophilic hapten conjugates has been critical to ensure immunoassay reproducibility and accuracy. The results obtained in this work suggest that using hydrophilic carriers may favor immunochemical determination of other hydrophobic molecules in aqueous media.

Immunoassay specificity was evaluated by preparing standard curves of the competitors under the same conditions as those for NP and measuring them with the optimized indirect ELISA. As predicted by the molecular modeling studies (see Figure 2), the nonyl isomeric mixture of alkyl chains seems to be the more important epitope since they are highly recognized by these antibodies. As can be observed in Table 3, there was a lower recognition (53%) of octylphenol (OP), with only one less carbon atom but with a rigid and unique structure (only one isomer present) instead of the mixture of different conformations present in the nonylphenol technical mixture. This recognition greatly decreased when phenol or short chain *p*-alkylphenols (i.e., *p*-cresol, ethylphenol) were measured (CR < 0.1%), highlighting the importance of the alkyl chain. In addition, when the hydroxyl group of the benzene was substituted by a methyl group, as in NP_a and NP₃ (hapten homologous) (see Figure 1 for chemical structures), recognition also dropped (5% and <0.1%), supporting previous data obtained by our group regarding the strong effect that the acid-base equilibrium has on the charge distribution of the molecule (33, 34). In contrast, unexpectedly parent compounds with different numbers of ethoxylate units were recognized in this assay. More surprisingly, a higher recognition was observed for

TABLE 3. Specificity of As110/NP1-AD ELISA^a

compound ^b	IC_{50} (nM)	% CR
NP	123	100
NPEO (long chain)	201	62
NPEO (medium chain)	288	43
NPEO (short chain)	366	35
NP1EC	4652	3
OP	249	53
OPEO (Triton X-100)	934	13
NP _a	2612	5
NP ₃	205 000	<0.1
NP _c	>1000 000	<0.01
phenol	>125 000	<0.1
<i>p</i> -cresol	>125 000	<0.1
ethylphenol	>125 000	<0.1
LAS	60 000	0.2

^a The percentage of recognition has been expressed as cross-reactivity (CR%) according to the expression $\{IC_{50}(\text{NP})/IC_{50}(\text{cross-reactant})\} \times 100$. ^b Abbreviations: NPEO, nonylphenol ethoxylate (long chain corresponds to a mixture of compounds with an average length of 10 ethoxylate units; medium chain corresponds to an average length of 7 ethoxylate units; short chain corresponds to a mixture 1:1 of mono- and diethoxylate compounds); NP1EC, nonylphenol monoethyl carboxylate; OP, 4-octylphenol; OPEO, octylphenol ethoxylate with an average length of 9.5 ethoxylate units; NP_a, methoxy-4-nonylbenzene; NP₃, 5-(2-methoxy-5-nonylphenyl)-pentanoic acid; NP_c, 5-(4-methoxyphenyl)-5-oxo-pentanoic acid; LAS, linear alkylbenzenesulfonates.

compounds with a long ethoxylate chain (Tergitol NP10EO units average, 62%) than for medium (Tergitol NP7EO units average, 43%) or short ethoxylate chain (only one or two ethoxylate units, Imbestin, $n = 1.5$, 35%). This recognition pattern could not be explained considering the chemical structure of the immunizing hapten and the marked decrease of the recognition produced by just converting the phenol group (NP, 100%) into a methyl ether (NP_a, 5%). At present, we do not have solid arguments to explain this recognition pattern, although we suspect that the reason could be related to the particular physical properties of the NP and its ethoxylate counterparts (solubility in aqueous media, possibility to form micelles and aggregates, etc.). In fact, a high degree of recognition of NPEO in most of the ELISAs previously reported (20, 22) has been observed, even if the immunizing hapten keeps the hydroxyl group free. Thus, as mentioned before, in the ELISA recently developed by Mart'ianov et al. (36) all the NPEO were highly recognized (NP1EO, NP2EO, NP(9-10)EO, and NP1EC with CR values of 188%, 279%, and 79%, and 48% respectively). The assay developed by Zeravik et al. (15) using the linear alkyl chain of NP did not recognize NPEO with 1-3 EO units and does not provide data regarding recognition of NPEO of medium and long EO chains.

Immunoassay accuracy was initially evaluated using spiked blind samples prepared in organic solvents (MeOH or DMSO) and subsequently diluted 200 times in PBST-EtOH. Prior to the ELISA measurements, samples were further diluted 2, 4, and 8 times in PBST-EtOH to ensure a reliable measure within the working range of the assay. As can be observed in Figure 5a, the correlation initially obtained between spiked and measured values was not very good. A slope around 0.7 indicated a clear underestimation of the real values. Considering the physicochemical properties of the NP and the behavior observed in the ELISA when trying to prepare the standard curve by serial dilutions, it was likely possible that the analyte could be lost on the consecutive serial dilutions performed to place the samples within the working range. To prove this hypothesis, similar experiments were conducted using only on-step dilution factors. Recoveries found in this case ranged between 96 and 100%, regardless of the dilution factor (i.e. 1:20 or 1:40 000). Different samples were tested on different plates and days and these

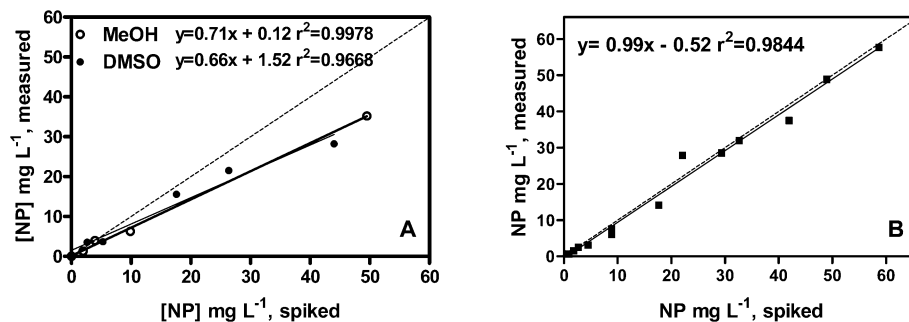


FIGURE 5. Results from the accuracy studies performed with the immunoassay As110/NP1-AD. The graphic shows the correlation between the spiked concentrations and the values measured by ELISA. Spiked blind samples were prepared in DMSO or MeOH and then diluted 200 times with PBST-EtOH (A). Immunochemical measurements were made on samples serially diluted with PBST-EtOH (B). Immunochemical determinations were made on samples prepared after on-step dilution of the blind sample. The data shown correspond to the average of two well replicates. The dotted line corresponds to a perfect correlation (slope = 1).

TABLE 4. Concentration of NP in WWTP Effluents (mg L⁻¹)^a

sample	ELISA	LC-ESI-MS	recovery %
effluent Martorell	2.19	1.66	132
effluent Igualada	1.70	1.94	88
effluent Igualada II	1.76	1.86	95
effluent Igualada III	1.77	1.90	93

^a LC-ESI-MS data obtained by a personal communication.

results are shown in Figure 5b. The excellent coefficient of regression and a slope close to 1 demonstrate the accuracy of the assay. Thus, the lack of accuracy observed before can clearly be attributed to the sample preparation method employed. These studies indicate that direct measurements of aqueous samples could be possible attending to a careful manipulation of the samples and diminishing as much as possible the number of steps involved, although preparation of organic extracts may be more convenient due to the lipophilic nature of NP and all the related problems involved in its immunochemical determination. Thus, under these conditions a preliminary accuracy study with real water samples from effluents of WWTPs was performed. Several dilutions of each methanol extract were prepared with assay buffer applying a single-step dilution factor and then analyzed in the ELISA. Only those diluted samples that could be measured within the linear range of the ELISA were used. The data shown in Table 4 indicate that, although a slightly underestimation is still observed, these results and those recorded by LC-ESI-MS match very well. A more extensive and complete study increasing the number of real environmental samples is currently being performed in order to evaluate, with objective data, the reliability and real potential of the methodology here described.

Acknowledgments

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Supporting Information Available

Synthetic methods, spectroscopic (¹H and ¹³C NMR, IR) and spectrometric data, and elementary analysis of the compounds described in the paper and the MALDI-TOF-MS results of the protein conjugates as well as results obtained with the direct ELISA. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Literature Cited

- Renner, R. European bans on surfactant trigger transatlantic debate. *Environ. Sci. Technol.* **1997**, *31*, 316A-320A.
- Staples, C. A.; Naylor, C. G.; Williams, J. B.; Gledhill, W. E. Ultimate biodegradation of alkylphenol ethoxylate surfactants and their biodegradation intermediates. *Environ. Toxicol. Chem.* **2001**, *20*, 2450-2455.
- Ahel, M.; Giger, W.; Koch, M. Behavior of alkylphenol polyethoxylate surfactants in the aquatic environment. I. Occurrence and transformation in sewage-treatment. *Water Res.* **1994**, *28*, 1131-1142.
- Ying, G.-G.; Williams, B.; Kookana, R. Environmental fate of alkylphenols and alkylphenol ethoxylates—a review. *Environ. Int.* **2002**, *28*, 215-226.
- Amaral Mendes, J. J. The endocrine disruptors: a major medical challenge. *Food Chem. Toxicol.* **2002**, *40*, 781-788.
- Knudsen, F. R.; Pottinger, T. G. Interaction of endocrine disrupting chemicals, singly and in combination, with estrogen-, androgen-, and corticosteroid-binding sites in rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* **1998**, *44*, 159-170.
- Van der Putte, I.; Groshart, C.; Okkerman, P. European Commission DG ENV: Towards the Establishment of a Priority List of Substances for Further Evaluation of Their Role in Endocrine Disruption. M0355008/1786Q710/11/00. BKH, Consulting Engineers and TNO, Nutrition and Food Research: Delft, The Netherlands, 2000.
- Muller, S.; Schmid, P.; Schlatter, C. Evaluation of the estrogenic potency of nonylphenol in non-occupationally exposed humans. *Environ. Toxicol. Pharmacol.* **1998**, *6*, 27-33.
- Muller, S.; Schmid, P.; Schlatter, C. Pharmacokinetic behavior of 4-nonylphenol in humans. *Environ. Toxicol. Pharmacol.* **1998**, *5*, 257-265.
- Jobling, S.; Sumpter, J. P. Detergent components in sewage effluent are weakly estrogenic in fish: An *in vitro* study using rainbow trout. *Aquat. Toxicol.* **1993**, *27*, 361-372.
- Calafat, A. M.; Kuklennyik, Z.; Reidy, J. A.; Caudill, S. P.; Ekong, J.; Needham, L. L. Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population. *Environ. Health Perspect.* **2005**, *113*, 391-395.
- Petrovic, M.; Eljarrat, E.; Lopez de Alda, M. J.; Barcelo, D. Recent advances in the mass spectrometric analysis related to endocrine disrupting compounds in aquatic environmental samples. *J. Chromatogr. A* **2002**, *974*, 23-51.
- Kojima, M.; Tsunoi, S.; Tanaka, M. Determination of 4-alkylphenols by novel derivatization and gas chromatography-mass spectrometry. *J. Chromatogr. A* **2003**, *984*, 237-243.
- Franek, M.; Zeravik, J.; Eremin, S. A.; Yakovleva, J.; Badea, M.; Danet, A.; Nistor, C.; Ocio, N.; Emneus, J. Antibody-based methods for surfactant screening. *Fresenius J. Anal. Chem.* **2001**, *371*, 456-466.
- Zeravik, J.; Skryjova, K.; Nevorankova, Z.; Franek, M. Development of direct ELISA for the determination of 4-nonylphenol and octylphenol. *Anal. Chem.* **2004**, *76*, 1021-1027.
- Goda, Y.; Kobayashi, A.; Fukuda, K.; Fujimoto, S.; Ike, M.; Fujita, M. Development of the ELISAs for detection of hormone-disrupting chemicals. *Water Sci. Technol.* **2000**, *42*, 81-88.
- Ahel, M.; Giger, W. Partitioning of alkylphenols and alkylphenol polyethoxylates between water and organic solvents. *Chemosphere* **1993**, *26*, 1471-1478.
- Mart'ianov, A. A.; Zherdev, A. V.; Eremin, S. A.; Dzantiev Boris, B. Preparation of antibodies and development of enzyme-linked

- immunosorbent assay for nonylphenol. *Int. J. Environ. Anal. Chem.* **2004**, *84*, 965–978.
- (19) Samsonova, J. V.; Rubtsova, M. Y.; Franek, M. Determination of 4-n-nonylphenol in water by enzyme immunoassay. *Anal. Bioanal. Chem.* **2003**, *375*, 1017–1019.
- (20) Yakovleva, J.; Zeravik, J.; Michura, I.; Formanovsky, A.; Franek, M.; Eremin, S. Hapten design and development of polarization fluorimmunoassay for nonylphenol. *Int. J. Environ. Anal. Chem.* **2003**, *83*, 597–607.
- (21) Yakovleva, J.; Lobanova, A. Y.; Shutaleva, E. A.; Kourkina, M. A.; Mart'ianov, A. A.; Zherdev, A. V.; Dzantiev Boris, B.; Eremin, S. Express detection of nonylphenol in water samples by fluorescence polarization immunoassay. *Anal. Bioanal. Chem.* **2004**, *378*, 634–641.
- (22) Badea, M.; Nistor, C.; Goda, Y.; Fujimoto, S.; Dosho, S.; Danet, A.; Barcelo, D.; Ventura, F.; Emnéus, J. A flow immunoassay for alkylphenol ethoxylate surfactants and their metabolites—questions associated with cross-reactivity, matrix effects, and validation by chromatographic techniques. *Analyst* **2003**, *128*, 849–856.
- (23) Rose, A.; Nistor, C.; Emneus, J.; Pfeiffer, D.; Wollenberger, U. GDH biosensor based off-line capillary immunoassay for alkylphenols and their ethoxylates. *Biosens. Bioelectron.* **2002**, *17*, 1033–1043.
- (24) Stewart, J. J. P. Optimization of parameters for semiempirical methods. 1. Method. *J. Comput. Chem.* **1989**, *10*, 209–220.
- (25) Ballesteros, B.; Barceló, D.; Camps, F.; Marco, M.-P. Enzyme-linked immunosorbent assay for the determination of the antifouling agent Irgarol 1051. *Anal. Chim. Acta* **1997**, *347*, 139–147.
- (26) Gascón, J.; Oubiña, A.; Ballesteros, B.; Barceló, D.; Camps, F.; Marco, M.-P.; González-Martínez, M.-A.; Morais, S.; Puchades, R.; Maquieira, A. Development of a highly-sensitive enzyme-linked immunosorbent assay for atrazine. Performance evaluation by flow-injection immunoassay. *Anal. Chim. Acta* **1997**, *347*, 149–162.
- (27) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
- (28) Cespedes, R.; Petrovic, M.; Raldúa, D.; Saura, U.; Piña, B.; Lacorte, S.; Viana, P.; Barcelo, D. Integrated procedure for determination of endocrine-disrupting activity in surface waters and sediments by use of the biological technique recombinant yeast assay and chemical analysis by LC-ESI-MS. *Anal. Bioanal. Chem.* **2004**, *378*, 697–708.
- (29) Gonzalez, S.; Petrovic, M.; Barcelo, D. Simultaneous extraction and fate of linear alkylbenzene sulfonates, coconut diethanol amides, nonylphenol ethoxylates and their degradation products in wastewater treatment plants, receiving coastal waters and sediments in the Catalanian area (NE Spain). *J. Chromatogr. A* **2004**, *1052*, 111–120.
- (30) Omelyanenko, V. G.; Jiskoot, W.; Herron, J. N. Role of electrostatic interactions in the binding of fluorescein by anti-fluorescein antibody 4-4-20. *Biochemistry* **1993**, *32*, 10423–10429.
- (31) van Oss, C. J. Hydrophobic, hydrophilic and other interactions in epitope-paratope binding. *Mol. Immunol.* **1995**, *32*, 199–211.
- (32) Zeder-Lutz, G.; Rauffer, N.; Altschuh, D.; Van Regenmortel, M. H. V. Analysis of cyclosporin interactions with antibodies an cyclophilin using BiAcore. *J. Immunol. Methods* **1995**, *189*, 131–140.
- (33) Galve, R.; Camps, F.; Sanchez-Baeza, F.; Marco, M.-P. Development of an immunochemical technique for the analysis of trichlorophenols using theoretical models. *Anal. Chem.* **2000**, *72*, 2237–2246.
- (34) Nickkova, M.; Galve, R.; Marco, M. P. Biological monitoring of 2,4,5-trichlorophenol (I): Preparation of antibodies and development of an immunoassay using theoretical models. *Chem. Res. Toxicol.* **2002**, *15*, 1360–1370.
- (35) Goda, Y.; Kobayashi, A.; Fujimoto, S.; Toyoda, Y.; Miyagawa, K.-I.; Ike, M.; Fujita, M. Development of an enzyme linked immunosorbent assay for detection of alkylphenol polyethoxylates and their biodegradation products. *Water Res.* **2004**, *38*, 4323–4330.
- (36) Mart'ianov, A. A.; Dzantiev, B. B.; Zherdev, A. V.; Eremin, S. A.; Cespedes, R.; Petrovic, M.; Barcelo, D. Immunoenzyme assay of nonylphenol: study of selectivity and detection of alkylphenolic non-ionic surfactants in water samples. *Talanta* **2005**, *65*, 367–374.
- (37) Newman, M. S.; Mekler, A. B. Synthesis of 7-methyl-2,3,4,5-tetrahydro-1-benzoxepin and 4-methyl-5,6,7,8-tetrahydronaphthol by alkaline cyclations. *J. Org. Chem.* **1961**, *26*, 336–338.
- (38) Galve, R.; Sanchez-Baeza, F.; Camps, F.; Marco, M. P. Indirect competitive immunoassay for trichlorophenol determination: Rational evaluation of the competitor heterogeneity effect. *Anal. Chim. Acta* **2002**, *452*, 191–206.
- (39) Ahel, M.; Giger, W. Aqueous solubility of alkylphenols and alkylphenol polyethoxylates. *Chemosphere* **1993**, *26*, 1461–1470.
- (40) Brix, R.; Hvidt, S.; Carlsen, L. Solubility of nonylphenol and nonylphenol polyethoxylates. On the possible role of micelles. *Chemosphere* **2001**, *44*, 759–763.
- (41) Kataoka, H.; Ise, M.; Narimatsu, S. Automated on-line in-tube solid-phase microextraction coupled with high performance liquid chromatography for the analysis of bisphenol A, alkylphenols, and phthalate esters in foods contacted with plastics. *J. Sep. Sci.* **2002**, *25*, 77–85.
- (42) Inoue, K.; Okumura, H.; Higuchi, T.; Oka, H.; Yoshimura, Y.; Nakazawa, H. Characterization of estrogenic compounds in medical polyvinyl chloride tubing by gas chromatography-mass spectrometry and estrogen receptor binding assay. *Clin. Chim. Acta* **2002**, *325*, 157–163.
- (43) Loyo-Rosales, J. E.; Rosales-Rivera, G. C.; Lynch, A. M.; Rice, C. P.; Torrents, A. Migration of nonylphenol from plastic containers to water and a milk surrogate. *J. Agric. Food Chem.* **2004**, *52*, 2016–2020.
- (44) Sanvicens, N.; Varela, B.; Marco, M.-P. Immunochemical determination of 2,4,6-trichloroanisole as the responsible agent for the musty odor in foods. 2. Immunoassay evaluation. *J. Agric. Food Chem.* **2003**, *51*, 3932–3939.
- (45) Manclus, J. J.; Montoya, A. Development of enzyme-linked immunosorbent assays for the insecticide chlorpyrifos .1. Monoclonal antibody production and immunoassay design - 2. Assay optimization and application to environmental waters. *J. Agric. Food Chem.* **1996**, *44*, 4063–4070.
- (46) Vyjayanthi, V.; Capoor, A. K.; Sashindhar, R. B. Binding characteristics of bovine serum albumine aflatoxin B1 to polystyrene microtiter plates: Importance of hapten to carrier protein molar ratio. *Indian J. Exp. Biol.* **1995**, *33*, 329–332.
- (47) Fasciglione, G. F.; Marini, S.; Bannister, J. V.; Giardina, B. Hapten-carrier interactions and their role in the production of monoclonal antibodies against hydrophobic haptens. *Hybridoma* **1996**, *15*, 1–9.
- (48) Pauillac, S.; Naar, J.; Branaa, P.; Chinain, M. An improved method for the production of antibodies to lipophilic carboxylic hapten using small amount of hapten-carrier conjugate. *J. Immunol. Methods* **1998**, *220*, 105–114.
- (49) Gegg, C. V.; Etzler, M. E. Directional coupling of synthetic peptides to poly-L-lysine and applications to the ELISA. *Anal. Biochem.* **1993**, *210*, 309–313.
- (50) Gunja-Smith, Z. An enzyme-linked immunosorbent assay to quantitate the elastin crosslink desmosine in tissue and urine samples. *Anal. Biochem.* **1985**, *147*, 258–264.
- (51) Xiao, H.; Clarke, J. R.; Marquardt, R. R.; Frohlich, A. A. Improved methods for conjugating selected mycotoxins to carrier proteins and dextran for immunoassays. *J. Agric. Food Chem.* **1995**, *43*, 2092–2097.
- (52) Mummert, M. E.; Voss, E. W. Effects of secondary forces on the ligand binding properties and variable domain conformations of a monoclonal anti-fluorescyl antibody. *Mol. Immunol.* **1966**, *33*, 1067–1077.
- (53) Leckband, D. E.; Kuhl, T.; Wang, H. K.; Herron, J.; Müller, W.; Ringsdorf, H. 4-4-20 Anti-fluorescyl IgG Fab' recognition membrane bound hapten: direct evidence for the role of protein and interfacial structure. *Biochemistry* **1995**, *34*, 11467–11478.
- (54) Michura, I. V.; Formanovsky, A. A.; Nikitin, A. O.; Yakovleva, J. N.; Eremin, S. A. Synthesis of ω -(4-hydroxyphenyl)alkanecarboxylic acids. *Mendeleev Commun.* **2000**, *5*, 193–194.
- (55) Samsonova, J. V.; Uskova, N. A.; Andreyuk, A. N.; Franek, M.; Elliott, C. T. Biacore biosensor immunoassay for 4-nonylphenols: assay optimization and applicability for shellfish analysis. *Chemosphere* **2004**, *57*, 975–985.

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