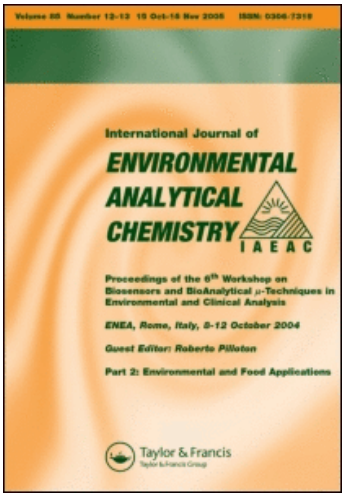

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HAPTEN DESIGN AND DEVELOPMENT OF POLARIZATION FLUOROIMMUNOASSAY FOR NONYLPHENOL

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Polyclonal antibodies to nonylphenol (NP), raised against two different haptenic derivatives were characterized by a rapid, homogeneous polarization fluoroimmunoassay (PFIA). The first hapten, ω-(4-hydroxyphenyl)nonanoic acid (NP9), was designed to mimic the linear NP isomer and contains hydroxyphenyl and linear alkyl chain moieties of the NP molecule. The second hapten, 4-aminophenol (4AP), contains the hydroxyphenyl moiety of NP molecule alone and thus potentially mimics various phenolic compounds with different side chain structures. A number of fluorescent labeled antigens (tracers) with various structures of the spacer arm between the antigen and the fluorescent dye was synthesized and used to optimize the competitor structure for NP-specific PFIA. The most sensitive assay with limit of detection (LOD) and IC_{50} values of 8 and 53 mg L^{-1}, respectively, was obtained when anti-NP9 antibody and NP9-labeled antigen were used. Anti-NP9 resulted in more specific assay, where the cross-reactivity toward the relative phenolic compounds did not exceed 5%. Anti-4AP displayed substantial recognition of several bis-substituted phenols, including 2-amino-4-chlorophenol and 2,4-dinitrophenol.

Keywords: Surfactants; Nonylphenol; Polarization fluoroimmunoassay; Hapten design; Crossreactivity

INTRODUCTION

Alkylphenols (APs) such as 4-nonylphenol (NP) and 4-octylphenol are stable degradation products of the widely used group of non-ionic surfactants, alkylphenolethoxylates (APEOs). NP was found to be toxic for aquatic organisms at the concentrations of 0.18–5 mg L^{-1} [1] and identified as an estrogenic substance, which interferes the natural hormone system and induces the proliferation of estrogen-dependent MCF_7 human breast tumor cells *in vitro* [2]. Surveys of APs in environmental samples performed in many countries revealed the presence of NP at concentrations of

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10–220 $\mu g\,L^{-1}$ in river water [3,4] and 20–30 $\mu g\,kg^{-1}$ in fish tissues [5,6]. Detection, identification and quantification of APs is carried out mostly by gas chromatography and mass spectrometry [7,8], and liquid chromatography with various detection techniques [9–11]. However, due to their complexity, consumption of large amounts of organic solvents, time- and cost-inefficiency, these traditional analytical methods are hardly suitable for a screening of large sample populations.

Immunochemical methods provide simple, powerful and inexpensive tools for analysis of environmental contamination and have been increasingly employed within the recent years for the analysis of pesticides [12], polychlorinated biphenyls [13], dioxins [14] and other toxic compounds. In regard to small phenolic antigens with a limited number of epitopes like APs, the production of high-affinity antibodies is a difficult task. Although some immunoassay kits for the simultaneous detection of benzene, toluene and xylenes in the environmental samples are available [15], there is a lack of information on appropriate hapten synthesis for these compounds. Careful hapten design enabled the production of functional antibodies against such substituted phenolic analytes as 4-nitrophenol [16,17], salicylic acid [18] and paracetamol [19]. Production of antibodies for detection of APs has not been reported in the literature so far, however, an ELISA kit based on monoclonal antibodies is available on market from Takeda Chemical Industries, allowing the detection of APEOs in the 50–500 $\mu g\,L^{-1}$ range [20]. The lack of specific antibodies toward APs may be due to the weak immunogenicity of these compounds and also for their heterogeneous composition. APs are complex mixtures of isomers with various structures of alkyl radical and positions of benzene ring in the aliphatic chain.

In this work NP-specific antibodies against two different immunogens were compared. The first approach is based on the NP derivative with an active carboxylic group at the end of the alkyl chain (NP9) [21]. The production and characterization of rabbit polyclonal antibodies against this hapten was described in our previous work [22]. Another approach explored 4-aminophenol (4AP) conjugated to a carrier protein by glutaraldehyde as an immunizing antigen. This allows realizing altered hapten spatial and electronic configuration in the vicinity of the benzene ring compared to NP. In the present study both anti-NP9 and anti-4AP in combination with different labeled competitor structures were used to develop homogeneous polarization fluoroimmunoassay (PFIA) for the screening detection of NP, and the assay sensitivity and specificity toward the target analyte were investigated.

EXPERIMENTAL

Reagents

Bovine serum albumin (BSA), *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), 4-(hydroxyphenyl)propionic acid (HPPA), glutaraldehyde (GA) 25% v/v aqueous solution, sodium borohydride and fluorescein isothiocyanate (FITC) isomer I were purchased through Sigma Chemical Co. (St. Louis, MO, USA). The analytical standards of 4-NP, phenol, and the technical mixture of NP ring and chain isomers were obtained from Aldrich (Steinheim, Germany). The compounds tested for cross-reactivity such as 4-aminophenol, 4-hydroxybenzoic acid, 4-chlorophenol, 2,4-dimethylphenol, 2,4-dinitrophenol, 2-amino-4-chlorophenol and

phenol were from Sigma Chemical Co. (St. Louis, MO, USA). Dimethylformamide (DMF) was purchased through Merck (Darmstadt, Germany). All other chemicals were analytical grade. The PBS buffer is 10 mM phosphate buffer, containing 0.8% w/v of saline, pH 7.5. Borate buffer is 25 mM sodium borate with 1% w/v NaN_3, pH 8.0. Pre-coated silica gel 60 F_{254} (0.25 mm) aluminum sheets for thin-layer chromatography (TLC) were acquired from Merck (Darmstadt, Germany). Carbonate buffer is 0.01 M sodium hydrocarbonate, pH 9.6.

Apparatus

An Abbott TDx analyzer (Abbott Laboratories, Irving, TX, USA) was used to measure the fluorescence polarization response in photo-check mode. Hapten density of protein conjugate was determined by matrix-assisted desorption ionization with a time-of-flight mass spectrometer (MALDI-TOF/MS) using a Kratos Kompact MALDI III instrument (Kratos Analytical, UK) equipped with a 337-nm nitrogen laser. Mass spectra of NP9 and NP7 haptens and the corresponding fluorescent tracers were obtained using an API 3000 MS/MS instrument in Turbo Ion Spray mode.

Synthesis of NP9–OVA Conjugate and Antibody Preparation

Haptens ω-(4-hydroxyphenyl)heptanoic acid (NP7) and ω-(4-hydroxyphenyl)nonanoic acid (NP9) were synthesized in six steps starting with α,ω-alkandicarboxylic acid as described in [21]. The NP9–OVA conjugate was obtained and used to immunize the rabbits as described elsewhere [23].

Synthesis of the 4AP–BSA Conjugate and Antibody Preparation

An amount of 22 mg (0.2 mmol) of 4AP dissolved in 1 mL of DMF was added dropwise to the mixture of 0.9 mL of distilled water and 0.1 mL (0.2 mmol) of 25% w/v glutaraldehyde. The reaction mixture was gently shaked for 10 min, then slowly added to a solution of 136 mg (2 μmol) of BSA in 10 mL of carbonate buffer and stirred for 15 min at room temperature (RT). To reduce the Schiff bases, 10 mg (200 μmol) of sodium borohydride was added and the reaction mixture was incubated for 3 h. After that the solution was dialyzed against 3 L of PBS (five changes of buffer) and lyophilized. Hapten densities of the 4AP–BSA conjugate were determined by MALDI-MS technique. To acquire MALDI-TOF/MS spectra, 0.5 μL of the crystal matrix *trans*-3,5-dimethoxy-4-hydroxycinnamic acid (10 mg mL^{-1} in 70% CH_3CN, containing 0.1% trifluoroacetic acid) mixed with 0.5 μL of conjugate or protein solution (3.5 mg mL^{-1} in 70% CH_3CN, containing 0.1% trifluoroacetic acid) directly on the MALDI target plate. To determine the hapten density, the molecular weight obtained for the BSA standard was compared with that of the conjugate. The 4AP-BSA hapten density determined in this way was 1 : 12.

The 4AP–BSA conjugate was used to immunize three female rabbits according to the following protocol. Animals were injected intradermally with 1 mL of the immunogen solution (0.3–1.2 mg in PBS: Freund's complete adjuvant, 1/1, v/v; the dose was gradually increasing for each rabbit) at multiple sites on the back and boosted at 7 day intervals for 5 weeks. For the next three months rabbits were injected intramuscularly (1 mg of the immunogen in PBS: Freund's incomplete adjuvant, 1/1, v/v) at 7 day

interval. After six months of the immunization the whole blood (35–40 mL) was obtained, the antiserum was isolated and stored at −18°C until the use.

Synthesis of NP Fluorescent Conjugates

Synthesis of 4AP-FITC. An amount of 4 mg (10 μmol) of fluorescein isothiocyanate isomer I (FITC) was dissolved in 0.5 mL of methanol, containing 10 μL of triethylamine. Then 4AP (4 mg, 40 μmol) was added and stirred overnight at RT. Next day the reaction mixture was applied to a preparative TLC plate using chloroform: methanol (4/1, v/v) as an eluent. The intense yellow band at R_f 0.1 was isolated and stored at 4°C.

Synthesis of NP3-EDF. NP7-EDF and NP9-EDF. Fluorescein thiocarbamyl ethylene diamine (EDF) was synthesized as previously described from fluorescein isothiocyanate isomer I[24]. The tracers NP3-EDF, NP7-EDF and NP9-EDF were synthesized according to NHS-ester method. NHS (8 mg, 80 μmol) and DCC (8 mg, 40 μmol) were added to a solution of NP3 (3.3 mg, 20 μmol), NP7 (4.4 mg, 20 μmol) or NP9 (5 mg, 20 μmol) in 0.2 mL of dimethylformamide, respectively. The reaction was allowed to proceed overnight, and the next day the precipitate was removed by centrifugation, and 5 mg (10 μmol) of EDF was added to the supernatant. After that the mixture was stirred for 3 h at RT until the total dissolution of EDF. Small portions of reaction mixture (50 μL) were separated by TLC using methylene chloride/methanol (4/1, v/v) as the eluent. The main yellow band at R_f 0.9 was isolated and stored in methanol at 4°C. The tracer concentrations were estimated spectrophotometrically at 492 nm, assuming the absorbance in borate buffer (25 mM, pH 8) to be the same as for fluorescein ($\varepsilon = 8.78 \times 10^4\ M^{-1}\,cm^{-1}$). The tracer solution was further diluted in 25 mM borate buffer and used for PFIA measurements. API-MS: NP7-EDF [M+H] 654.8 Da; NP9-EDF [M+H] 682.2 Da.

Polarization Fluoroimmunoassay (PFIA)

Antibody dilution curves were constructed by mixing of 500 μL of the fluorescein-labeled antigen (1 nM solution in BB) with 500 μL of antiserum in dilutions ranging from 1/80 to 1/2,560. Fluorescence polarization signal (mP) was measured using TDx glass cuvettes loaded into a "Photo Check" carousel. The competitive calibration curves were constructed with the technical mixture of NP chain and ring at the concentrations of 1, 10, 30, 100 and 300 mg L^{-1}. The NP standard (100 μL) was mixed with the labeled antigen and antiserum solution in a final volume of 1 mL, and the mP signal was measured. The mP values were plotted against the analyte concentration and a four-parameter equation was used to fit the experimental sigmoidal curve in Origin 6.0 for Windows. The assay limit of detection (LOD) was determined from the calibration curve using the blank signal and the three-fold confidence interval, and converted into the analyte concentration [25]. Cross-reactivity (CR) data for anti-NP9 and anti-4AP were calculated according to the following equation:

$$\%CR = 100 \times IC_{50}(LDS)/IC_{50}(\text{cross-reactant})$$

where IC_{50} is the concentration of analyte or cross-reacting compound that inhibits 50% of a fluorescent tracer binding.

RESULTS AND DISCUSSION

Design of NP Haptenic Derivatives

Specific antibodies are a key component in the development of any immunochemical technique. Since NP represents a mixture of isomers, differing in the structure of the alkyl chain moiety and the position of hydroxyphenyl ring, the strategy for antibody production against NP should focus on class detection.

Two different immunizing haptens were employed to produce antibodies toward NP (Fig. 1(a)). The first approach utilized a linear NP structure, comprising the hydroxyphenyl moiety and nine-carbon atom alkyl chain corresponding to the natural analyte molecule. In the NP9 derivative the active carboxyl group was incorporated at the end of the alkyl chain and kept distant from the hydroxyphenyl ring, an important antigenic epitope of NP.

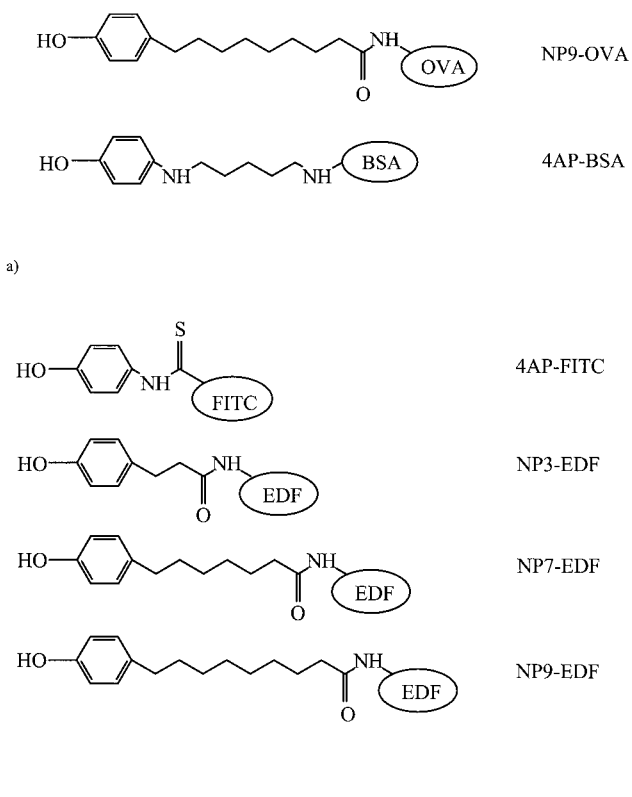

FIGURE 1 Chemical structures of the immunogens (a) and labeled antigens (b).

Another approach, based on 4AP, utilized the hydroxyphenyl moiety of the NP molecule alone. The change of an alkyl moiety in this haptenic derivative was intended to construct an immunogen that mimics the general NP structure and is suitable for a variety of molecules with different side chains. When using 4AP, the spacer arm between the hapten and a carrier protein was obtained as a result of cross-linking by glutaraldehyde. Based on the design of the two NP haptenic derivatives, one may expect that anti-NP9 antibodies will display a favorable recognition of linear NP, whereas anti-4AP will exhibit noticeable cross-reactivity with various phenolic compounds, substituted *para* to hydroxyl group.

Assessment of Antibody Binding Using a Set of Fluorescent Tracers

The principle of the homogeneous PFIA technique is based on the increase of fluorescence polarization signal of a small labeled antigen (tracer) when it is bound to the specific antibody [26]. PFIA offers a rapid detection of target analytes at $\mu g\,L^{-1}$ or low $mg\,L^{-1}$ level, and a number of assays for the detection of small organic pollutants have been developed in our laboratory in the recent years, including those for atrazine [27], 2,4-D [28]; acetochlor [29], metsulfuron-methyl [30], DDT [31].

The concentration of the labeled antigen (tracer) is an important parameter to be optimized for the development of a sensitive PFIA. The tracer concentration sets the intensity of the emitted light and, because PFIA involves the competition with the analyte for the antibody binding sites, the lowest possible tracer concentration should be used for sensitive assays. The optimal tracer concentration, corresponding to the total fluorescence intensity at least 10 times higher than the background signal from buffer was approximately 1 nM for all tracers. For the preliminary assessment of antibody binding to the labeled antigen, dilution curves for anti-NP9 and anti-4AP were recorded using NP9-EDF as labeled antigen (Fig. 2). As seen from the comparison of the curves obtained for anti-NP9 and anti-4AP with that of normal rabbit serum, sufficient binding of labeled antigen was achieved by both antisera, where the highest titre was exhibited by anti-NP9.

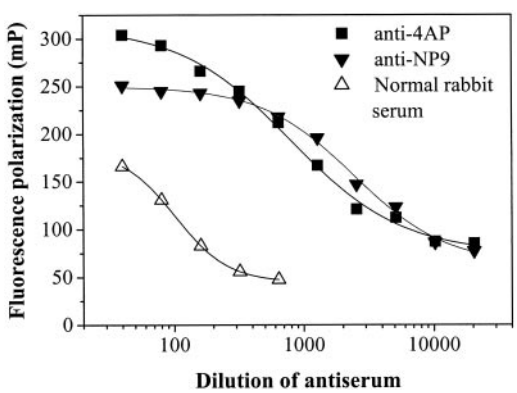

FIGURE 2 Anti-4AP and anti-NP9 dilution curves in a comparison with normal rabbit serum. NP9-EDF tracer (1 nM) was used for all experiments.

TABLE I Binding of labeled antigens with different spacers by anti-NP9 and anti-4AP. Antibody titre values corresponding to a 50% tracer and determined from the corresponding dilution curves are given

Antiserum	*4AP-FITC*	*NP3-EDF*	*NP7-EDF*	*NP9-EDF*
Anti-4AP	1/100	1/80	1/300	1/1000
Anti-NP9	1/120	1/100	1/900	1/2500

Recognition of the labeled antigen by antibody is an important factor, influencing the parameters of the resulting immunoassay. The assay sensitivity is determined in part by the relative affinity of antibody for analyte and labeled antigen. For this reason, at the next step we investigated the influence of tracer structure on binding to anti-NP9 and anti-4AP. Antibody dilution curves were recorded for both antisera using 4AP-FITC, NP3-EDF, NP7-EDF and NP9-EDF fluorescent tracers (see Fig. 1(b)), and antibody titre values corresponding to a 50% tracer binding were determined (Table I). The results in Table I demonstrate that antibody titre was significantly dependent on the tracer structure. Labeled antigens with a short spacer (4AP-FITC and NP3-EDF) resulted in a markedly reduced binding with both anti-NP9 and anti-4AP. Probably, the short spacer arms result in steric hindrance of tracer binding, when the large fluorescent dye residue is brought close to the antigen-binding site of antibody.

Tracers with long flexible spacer arms (NP7-EDF and NP9-EDF) were recognized by both antisera to a larger extent, while anti-NP9 displayed higher binding of NP9-EDF and NP7-EDF as compared to anti-4AP. Among the labeled antigens with long spacers, the highest antibody titre value was observed for the homologous combination anti-NP9 and NP9-EDF (1/2,500). The heterologous pairs anti-4AP and NP9-EDF, anti-NP9 and NP7-EDF resulted in lower titre values, which were 1/1,000 and 1/900, respectively. Thus, based on the optimization of antibody binding to the labeled antigen, three antibody–tracer combinations were chosen for further investigations: anti-NP9 and NP9-EDF, anti-NP9 and NP7-EDF, anti-4AP and NP9-EDF.

Assay Sensitivity

The assay sensitivity was investigated using NP standard (a technical mixture of NP chain and ring isomers). NP calibration curves were obtained under optimal antibody concentration, corresponding to a 70% tracer binding. Criteria to evaluate the PFIAs were the IC_{50} value (analyte concentration producing a 50% inhibition in fluorescence polarization), the assay dynamic range (defined by the analyte concentrations that inhibited tracer binding for 20% and 80%) and the slope of the linear range of the calibration curve (Table II). The limit of detection (LOD) was determined using the blank value and the three-fold confidence interval, converted into the corresponding concentration value. Figure 3 shows NP calibration curves obtained using anti-NP9 with NP7-EDF and NP9-EDF tracers and anti-4AP with NP9-EDF tracer. As seen in Fig. 3, anti-NP9 and anti-4AP resulted in different assay sensitivity. Practically no influence of the fluorescent tracer structure on assay sensitivity was observed when anti-NP9 and NP9-EDF or NP7-EDF were used. Table III shows the parameters of NP calibration curves seen in Fig. 3. The best assay sensitivity in terms of LOD value was observed for the combination of anti-NP9 and NP9-EDF or NP7-EDF

TABLE II Parameters of NP PFIA calibration curves using a technical mixture of isomers. IC_{50} values and slopes of the sigmoid were extracted from the four-parameter logistic equations used to fit the calibration curves (see Fig. 3 caption). LOD values and the dynamic range were determined as described in the experimental section

Antibody and tracer	*LOD* ($mg\,L^{-1}$)	*IC_{50}* ($mg\,L^{-1}$)	*Dynamic range* ($mg\,L^{-1}$)	*Slope*
Anti-PAP and NP9-EDF	32.2	157 ± 18	62–396	1.5 ± 0.2
Anti-NP9 and NP9-EDF	7.9	53 ± 9	18–157	1.3 ± 0.3
Anti-NP9 and NP7-EDF	9.2	42 ± 9	10–177	0.9 ± 0.1

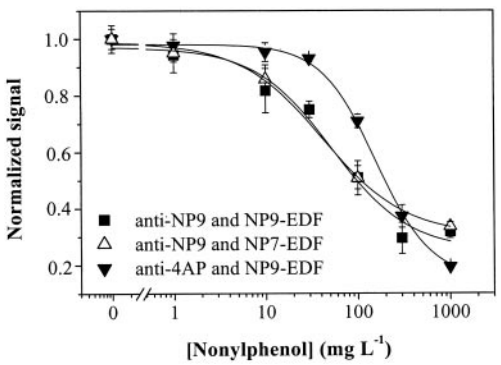

FIGURE 3 NP calibration curves using anti-NP9 and NP9-EDF, anti-NP9 and NP7-EDF, anti-4AP and NP9-EDF combinations. The normalized signals expressed as (B-F)/(B_0-F) (where B and B_0 are the fluorescence polarization intensities obtained for analyte solution and blank sample, respectively, and F is the background signal in the absence antibody) are plotted vs. NP concentration. A four parameter logistic equation was used to fit the calibration curve according to the following formula: $y = (A\text{-}D)/[I+(x/C)^B]+D$, where A and D are the maximal and minimal fluorescence polarization signals, B corresponds to the slope of the sigmoid and C is analyte concentration resulting in 50% inhibition of tracer binding (IC_{50}). Represented points correspond to the average of three replicates.

fluorescent tracers. Contrary to that, the highest slope of a linear range of the calibration curve was observed when a combination of anti-4AP with NP9-EDF was used, though the LOD and IC_{50} values were worse in this case. Dynamic ranges of all calibration curves fell within $mg\,L^{-1}$ level, which indicate low assay sensitivity. It may result from different antibody affinity for various isomers of the structurally heterogeneous NP mixture. The strategy of NP9 immunogen design was directed against the linear NP isomer, whose content in the NP mixture is typically low, not exceeding 5–10%. For another immunogen, 4AP, designed against 4-hydroxyphenyl moiety and expected to result in class specific antibodies for APs, low recognition of NP mixture may account for the structural dissimilarity compared to the target analyte.

Cross-reactivity Studies

To evaluate the assay specificity, anti-NP9 and anti-4AP were tested for cross-reactivity with *para*-substituted phenolic compounds. These compounds were investigated for their competitive binding with anti-NP9 and anti-4AP using NP9-EDF and NP7-EDF fluorescent tracers. The cross-reactivity (CR) data are presented in Table III, which illustrates different recognition patterns exhibited by anti-NP9 and anti-4AP.

TABLE III Anti-PAP and anti-NP9 cross-reactivities (CR) toward the phenolic compounds. CR is expressed as the percentage ratio between IC_{50} values for NP and cross-reactant calibration curves, respectively

Compound	*Anti-NP9 (CR, %)*	*Anti-4AP (CR, %)*
Technical NP mixture	100	140
4-Aminophenol	5.2	111
4-Hydroxybenzoic acid	0.2	1.7
4-Chlorophenol	3.5	n/d
2,4-Dimethylphenol	2.0	2.0
2,4-Dinitrophenol	2.8	24
2-Amino-4-chlorophenol	1.8	46
Phenol	0.8	3.0

As was expected, anti-4AP displayed a considerable degree of CR with the immunizing hapten 4AP (111%), whereas anti-NP9 demonstrated weak interaction with this compound (5%). In respect to other mono-substituted phenols (4-hydroxybenzoic acid, 4-chlorophenol) both anti-NP9 and anti-4AP showed minor cross-reactivity (less than 5%), which is probably due to the fact that the nature of *para*-positioned group of phenyl ring is not crucial for antigen binding.

Bis-substituted phenolic compounds (2,4-dinitrophenol and 2-amino-4-chlorophenol) were recognized by both antisera according to the different patterns. Low CR with these compounds was observed for anti-NP9 (2–3%), though more appreciable values were found for anti-4AP (CR 24–46%). High degree of 2,4-dinitrophenol and 2-amino-4-chlorophenol recognition by anti-4AP was surprising. It can be assumed that anti-4AP recognizes a specific ''electronic shape'' of these compounds formed by the interaction of π-electrons with aromatic ring and, perhaps, being similar to that of the immunizing hapten. However, the nature of such similarity cannot be clearly interpreted. In the case when the cross-reacting compound contains no π-electronic substitutions, the recognition by anti-4AP is negligible (2–3% for phenol and 2,4-dimethylphenol).

CONCLUSIONS

Technical nonylphenol is a mixture of chain and ring isomers and represents a difficult analyte for specific immunochemical quantification. In this work we have compared two strategies for the production of anti-NP antibodies and development of PFIA for a screening detection of NP. The first strategy was based on hapten mimic to the linear chain NP isomer (NP9), while the second one relied only on the hydroxyphenyl moiety of the NP molecule and was intended to obtain class-specific antibodies (4AP). Anti-NP9 was found to offer more sensitive detection of the technical NP mixture, resulting in the LOD of $8\,mg\,L^{-1}$ and IC_{50} of $53\,mg\,L^{-1}$. Such sensitivity is sufficient for the preliminary screening of industrial wastewater effluents when NP concentration may reach $mg\,L^{-1}$ or even $g\,L^{-1}$ values, but is not enough for the screening of environmental samples. Low sensitivity of PFIA based on anti-NP9 may be due to the low affinity of antibodies which are specific to the linear NP chain isomer to other isomers in the NP mixture. Anti-NP9 and anti-4AP were characterized by different cross-reactivity patterns toward phenolic compounds, where the former displayed specific detection of NP. Anti-4AP demonstrated relatively high recognition of some

bis-substituted phenols, such as 2-amino-4-chlorophenol and 2,4-dinitrophenol, probably, due to specific electronic configuration of these compounds, resembling that of 4AP. Based on the investigation of assay cross-reactivities reached by both antisera, anti-NP9-based assay can be used to detect specifically NP. Anti-4AP based assay was found to have approximately the same efficiency toward NP and 4AP (CR 111%) and, potentially, can be used for determination of both these analytes. The future work will be focused on the improvements of the assay sensitivities (e.g. by using solid-phase extraction procedure for a preconcentration step) for the analysis of low NP concentrations in the environmental samples. Another possibility is the exploration of anti-4AP cross-reactivity with phenolic compounds for multi-analyte detection.

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