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Immunoenzyme assay of nonylphenol: study of selectivity and detection of alkylphenolic non-ionic surfactants in water samples

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Abstract

Immunoenzyme assay (ELISA) is proposed and characterized for determination of alkylphenol ethoxylates, a primary class of manufactured non-ionic surfactants. The assay is based on the obtained polyclonal antibodies against nonylphenol (NP), the main stable intermediate of the decomposition of nonylphenol ethoxylates. A mixture of non-modified branched isomers of NP was applied as hapten coupled to protein carriers by Mannich reaction with the use of formaldehyde. The proposed ELISA format is based on immobilized NP–(soybean trypsin inhibitor) conjugate as a competitor of antigen molecules contained in the tested sample for binding with specific antibodies indirectly labeled via an anti-species immunoperoxidase conjugate. The developed ELISA allows to reveal NP with the limit of detection about 10 ng ml^{-1} and NP-related compounds such as octylphenol, alkylphenoletoxylates, alkylphenolcarboxylates and their halogenated derivatives. The ELISA was applied for assaying polluted water samples, namely influents and effluents from different wastewater treatment plants (WWTP) and tap water. ELISA and chromatographic data demonstrate good correlation (r = 0.94), while ELISA gives higher values. Due to endocrine disrupting and other toxic activities of some metabolites of alkylphenolic non-ionic surfactants, the developed assay may be effectively used in ecological monitoring and sanitary control.

Keywords: Immunoenzyme assay; Non-ionic surfactants; Nonylphenol; Endocrine disruptors; Water pollution monitoring

1. Introduction

Wide use of non-ionic surfactants such as alkylphenolethoxylates (APEOs) in industrial and domestic sectors results in accumulation of their decomposition products in the environment. The main components of APEOs technical preparations are isomers of nonyl- and octylphenolethoxylates. Persistent intermediates of APEOs decomposition, alkylphenols (APs), short ethoxy chain alkylphenol ethoxylates (APE_nOs, n = 1–2) and their halogenated derivatives are characterized by substantial endocrine disrupting activity [1–7]. They constitute a menace to aquatic organisms above all, but other animals and humans receive these compounds along food chains and also undergo endocrine disrupting exposure [8]. The given reasons stipulate actuality of APEOs monitoring in various matrices.

Standard technique for APs assay is chromatography [9,10], either gas chromatography combined with mass spectrometry or liquid chromatography combined with various detection techniques. However, the shortcoming of such analyses is the necessity to use expensive sophisticated equipment, which in turn requires highly skilled analysts to operate it. The net result is that the measurements are performed at specialized laboratories, and the information is obtained at a considerable cost, days after the samples are taken [11].

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Immunoenzyme assays have demonstrated their effectiveness for detection of various substances in ecology, medicine and biotechnology [12,13]. A large number of microplate immunoenzyme assays (ELISA) were proposed for monitoring pesticides, dioxins and other environmental pollutants; the results of these investigations are summarized in [14,15]. Development of ELISA techniques for surfactants started in 1982 [16], but most of the systems were described recently [17–22] and were based on a limited set of antibodies.

The main problem confining further development of surfactant immunodetection is lack of antibodies with acceptable spectra of specificity. The necessary antibodies should allow to detect different mixtures of surfactants and their toxic metabolites with comparable effectiveness and at the same time not to bind pollutants from other chemical groups. Since technical non-ionic surfactants consist of a mixture of APEOs, nonylphenol (NP) as their common structural part and stable decomposition intermediate seems to be the most suitable hapten for immunoassays.

Traditional approach to couple a hapten without own reactive groups to a protein carrier is to obtain its amino or carboxylic derivative. In the NP case, a carboxylic derivative of a linear isomer is usually used [18,20]. However, this hapten does not correspond to the real technical non-ionic surfactants, and the induced antibodies typically do not interact with them [18]. We have proposed earlier to apply Mannich reaction for the formation of $–CH_2–$ bridges between the benzene ring of non-modified NP and a NH-group of a protein carrier [23]. This technique allows to use a real mixture of NP isomers as the hapten in immunogenic hapten–protein conjugate. By means of immunization using this conjugate, specific anti-NP rabbit antibodies were obtained, and their effectiveness in the NP ELISA was shown [23]. In the present investigation, the ELISA was characterized in terms of cross-reactions with alkylphenols, alkylphenoletoxylates, alkylphenolcarboxylates and their halogenated derivatives. Finally, the ELISA was compared with chromatography in the analysis of real wastewaters.

2. Experimental

2.1. Materials

Nonylphenol (technical mixture of isomers) was from Aldrich, Milwaukee, USA. Dimethylsulfoxide (DMSO), Freund's complete and incomplete adjuvants, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), 2-hydroxybenzoic acid (salicylic acid), *p*-hydroxyphenylacetic acid, 3(*p*-hydroxyphenyl)propionic acid and 5-amino-2,3-dihydro-1,4-phthalazinedione were from ICN Biomedicals, Aurora, USA. 4-Aminophenol, 4-chlorophenol, 2-amino-4-chlorophenol, 2,4-dinitrophenol, 4-chloro-3-methylphenol and Triton X-305 were from Merck, Darmstadt, Germany. Gelatin (60 bloom), 2,4-dimethylphenol, 5-aminosalicylic acid, dodecylbenzenesulfonic acid and 3,3′,5,5′-tetramethylbenzidine (TMB) were from Sigma, St. Louis, USA. 4-*n*-Nonylphenol, 4-tert-octylphenol, 4-hydroxybenzoic acid and nonylaldehyde (pelargonaldehyde) were from Fluka, Buchs, Switzerland. Nonylphenol-(1-2)-ethoxylate and octylphenol-(3)-ethoxylate were from ChemService, West Chester, USA. L-β-Phenyl-α-alanine was from Reanal, Budapest, Hungary, Triton X-100 from Serva, Heidelberg, Germany and Tween-80 from Koch-Light Laboratories, Colnbrook-Bucks, UK. Bovine serum albumin (BSA) from Sigma and soybean trypsin inhibitor (STI) from ICN Biomedicals were used as the carriers for hapten–protein conjugates. Peroxidase-labeled goat anti-rabbit immunoglobulins were from Medgamal (Moscow, Russia). Nonylphenolmonoethoxylate (NPE_1O), nonylphenoldiethoxylate (NPE_2O), nonylphenol carboxylate (NPE_1C), nonylphenol ethoxy carboxylate (NPE_2C), brominated nonylphenol (Br-NP), chlorinated nonylphenol (Cl-NP), brominated nonylphenol carboxylate (Br-NPE_1C) and chlorinated nonylphenol carboxylate (Cl-NPE_1C) were synthesized according to the method described elsewhere [24,25]. 7-*p*-Hydroxyphenyl heptanoic acid was obtained as described in [26]. Components of buffer solutions and other chemicals were of analytical grade.

Enzyme-linked immunosorbent assays were carried out in optically transparent polystyrene microtiter plates from Nalge Nunc International (Rochester, USA) or Medpolymer (Moscow, Russia).

2.2. Synthesis of nonylphenol–protein conjugates

Nonylphenol was conjugated with BSA and STI by Mannich reaction (modification of the protocol for D-3-methoxy-4-hydroxyphenylglycol described in [27]). NP was dissolved in DMSO to concentration 33 mg ml^{-1} (0.15 M) and added in 116 μl aliquot to 2.0 ml of a BSA solution (10 mg ml^{-1}) in 0.1 M sodium carbonate buffer, pH 10, reaching hapten/carrier molar ratio 50:1. (In the case of STI, the ratio was 30:1.) Then, 0.2 ml of 35% formaldehyde water solution was added dropwise. The reaction mixture was incubated for 30 min at room temperature with stirring and then kept for 5 days at 37 °C with periodic shaking. The resulting products were separated from low molecular weight compounds by exhaustive dialysis against 0.05 M potassium phosphate buffer solution, pH 7.4, with 0.1 M NaCl, (PBS) during 2 days.

2.3. Antisera obtaining

Chinchilla rabbits weighting 3–4 kg were immunized by the NP–BSA conjugate. For the first immunization, the immunogen was dissolved in PBS and emulsified with an equal volume of Freund's complete adjuvant to a final concentration of 1.0 mg ml^{-1}. 1.0 ml of this mixture was injected subcutaneously to seven sites on the back of the animal's body. Then rabbits received booster injections by one-half dose of the immunogen subcutaneously (43rd day, in PBS with Freund's incomplete adjuvant, v/v (1:1) and intravenously (46th

day, in PBS). After one week (53rd day), they were bled. Reimmunization cycles were repeated twice (subcutaneous immunizations: 60th and 95th days; intravenous immunizations: 81st and 116th days; bleeding: 88th and 123rd days).

Blood samples were settled for 30 min at 37 °C and then for 2 h at 4 °C. Upper layers were separated by centrifugation for 15 min at 3000 *g*, carefully collected, dispensed into 0.5–1.0 ml aliquots and stored at −20 °C.

2.4. Nonylphenol ELISA

Two variants of ELISA protocol were used.

Variant 1: NP–STI conjugate was immobilized in Medpolymer microplates from 1 μg ml^{-1} solution (100 μl per well) in PBS by overnight incubation at 4 °C. Then, the microplate was washed four times by PBS containing 0.05% Tween-80 (PBST). Thirty-minute incubation at 37 °C with 150 μl of 0.1% gelatin solution in PBS leads to blocking potential sites of non-specific sorption. After repeated washing, 50 μl of rabbit anti-NP-antiserum (1:10,000 dilution in 25 mM potassium phosphate buffer, pH 7.4, with 0.05 M NaCl and 0.05% gelatin) and 50 μl of NP (concentrations from 0.5 ng ml^{-1} to 200 μg ml^{-1}, in methanol/water or ethanol/water mixture, v/v, 1:4) were added into wells and incubated for 1 h at 37 °C. After washing, the peroxidase-labeled goat anti-rabbit antibodies were added into the wells (dilution of the commercial product was 1:6000) and incubated for 1 h at 37 °C. One more washing cycle was carried out (four times by PBST and then one by distilled water) and peroxidase activity of the formed immobilized complexes was measured as described below.

Variant 2: NP–STI conjugate was immobilized in Nunc microplate wells from 0.05 μg ml^{-1} solution in PBS by overnight incubation at 4 °C. Next steps of the assay were coincident to those described above but the dilution of rabbit anti-NP–BSA antiserum was 1:20,000 and all incubations were carried out at room temperature.

Measurements of the label's activity: Two substrate solutions were used. The first one contained ABTS (0.7 mM) and H_2O_2 (2.8 mM) in sodium–acetate buffer (30 mM), pH 4.5. Hundred microliters of the solution was added into microplate wells and incubated for 30 min at room temperature.

The alternate substrate solution contained TMB (0.4 mM) and H_2O_2 (1.2 mM) in 40 mM Na–citrate buffer, pH 5.5. Hundred microliters of the solution was added into the wells and incubated for 30 min at room temperature in darkness. Then 50 μl of 1 M H_2SO_4 (stop-solution) was added.

Optical density of the oxidation products was measured at 405 nm (ABTS) or 450 nm (TMB) by means of vertical photometers Multiscan EX (Labsystems, Helsinki, Finland) or Spectra Max Plus (Molecular Devices Corporation, Sunnyvale, USA).

Sampling: To analyze the influence of sample matrices on the ELISA parameters, the competitive curves for NP were obtained in tap, ground and drinking water and compared to that one in deionized water. The reaction mixture during the competition step of the ELISA contained 50 μl of the specific antiserum, 40 μl of water matrix and 10 μl of NP solution in methanol (thus assuring the same content of organics in the final volume).

Calculations: The obtained dependences of the detected signal (y) from the competing antigen concentration (x) were fitted according to four-parameter logistic equation:

$$y = \frac{A - D}{1 + (x/C)^B} + D$$

C value accords to antigen concentration inhibiting 50% of antibody–conjugate binding (IC_{50}). Cross-reactivity (CR) was calculated according to the formula:

$$\mathrm{CR} = \frac{100\% \times \mathrm{IC}_{50\,(\mathrm{NP})}}{\mathrm{IC}_{50\,(\text{cross-reactant})}}$$

2.5. Chemical analysis (LC–MS/(MS))

NPEOs and their halogenated derivatives were analyzed using LC–MS as described by Petrovic et al. [28], whereas NP, NPECs and their halogenated derivatives were analyzed using recently developed LC–MS/(MS) method [29].

Briefly, target compounds were isolated from water samples using solid phase extraction (SPE) on C18-silica (Accubond, J&W Scientific, Folsom, USA). Different volumes of sample (500 ml of tap water, 200 ml of WWTP effluents and 100 ml of WWTP influents) were loaded onto preconditioned cartridges. Afterwards, cartridges were air-dried under vacuum, and were eluted with 2 ml × 4 ml of methanol. The eluates were taken gently to dryness under a nitrogen steam and reconstituted in 1 ml of methanol.

LC–MS/(MS) analyses were performed on a Waters 2690 series Alliance HPLC (Waters, Milford, USA) with a quaternary pump equipped with a 120-vial capacity sample management system. The analytes were separated on a narrow-bore 3-μm, 55 mm × 2 mm i.d. C_{18} reversed phase column Purospher® STAR RP-18 endcapped (Merck, Darmstadt, Germany). The sample injection volume was set at 10 μl. A binary mobile phase gradient with methanol (A) and water (B) was used for analyte separation at a flow rate of 200 μl min^{-1}. The elution gradient was linearly increased from 30 to 85% A in 10 min, then increased to 95% A in 10 min and kept isocratic for 5 min.

A bench-top triple quadrupole mass spectrometer Quattro LC from Micromass (Manchester, UK) equipped with a pneumatically-assisted electrospray probe and a Z-spray interface was used for this study. Capillary voltage was set at −2.8 kV, extractor lens 7 V and RF lens 0.6 V. The source and desolvation temperatures were 150 and 350 °C, respectively. The nitrogen (99.999% purity) flows were optimized at 50 l h^{-1} for the cone gas and 540 l h^{-1} for desolvation gas. For MS–MS experiments, the argon collision gas was maintained at a pressure of 5.8×10^{-3} mbar.

Compounds detected under negative ionization conditions (NP, NPECs and corresponding halogenated analogs)

were analyzed using a quantitative LC–MS/(MS) in multiple reactions monitoring mode. Compounds detected under positive ionization conditions (NPEOs and corresponding halogenated analogs) were analyzed using a single stage MS in selected ion monitoring mode.

3. Results and discussion

3.1. Nonylphenol detection by the ELISA system

The obtained antibodies against NP were applied for competitive immunoenzyme assay with indirect labeling of antibodies. The assay conditions were optimized to reach maximal sensitivity of NP detection (technical mixture of NP isomers was used as antigen standard). The varied parameters included concentration of reactants (NP–STI conjugate and antisera), duration of incubation steps, the nature and content of organic solvent.

The chosen NP–STI concentration was 1 μg ml^{-1} for Medpolymer microplates and 0.05 μg ml^{-1} for Nunc ones; the antiserum dilution being 1:10,000 and 1:20,000, accordingly (see detailed description of the chosen protocols in the Table 1). The obtained calibration curves for two types of microplates are given in Figs. 1 and 2. At these conditions the maximal sensitivity was reached, and the assay accuracy was acceptable (the registered optical density in the absence of competitor ≥0.5).

To prevent cross-reactions of the antibodies, Tween-80 was applied as detergent instead of Tritons. Competitive step of the assay was carried out in diluted (25 mM) phosphate buffer without detergent to avoid masking of target antigen in micellar structures. Gelatin was used to block non-specific adsorption before and during the competitive step.

It was shown that preparation and storage of NP standards using plastic vials leads to false growth of detected content of NP. The followed titration of these preparations may cause seeming increase of the detection limit in an order or more.

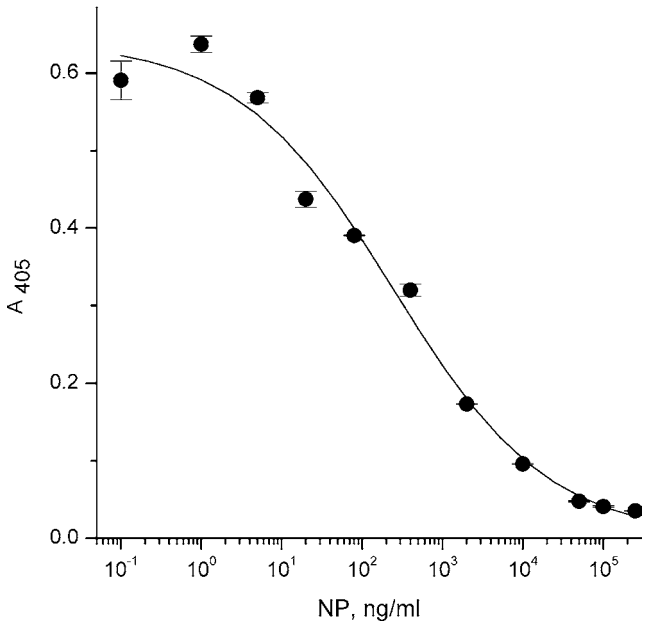


Fig. 1. Calibration ELISA curve for nonylphenol. Medpolymer microplate was used in combination with ABTS as chromogen.

The probable reason of this effect is a slow washing-out of some compounds similar to the studied surfactants from the vials surface. This assignment was confirmed by LC–MS in the course of storage of NP solutions in plastic vials additional peaks on the LC–MS curves appeared (data not shown). Similarly, seeming increase of sensitivity was observed in the case of direct titration of NP in microplates with its transfer from well to well by the same tips.

Based on these data, we have proposed to prepare standard solutions of NP for calibration curve in ethanol or methanol and to transfer them into microplate wells without subsidiary dilutions and tending to minimal duration of their contact with plastic tips.

Final content of organic solvent in the microplate wells was 10%, which makes it possible to work with extracts from

Table 1
Conditions and analytical characteristics for two variants of the developed ELISA

	Variant 1	Variant 2
Assay conditions		
Plate	Medpolymer	Nalge Nunc International
Concentration of NP–STI for coating (μg ml^{-1})	1.0	0.05
Antiserum dilution	1/10,000	1/20,000
Duration of immunochemical step of assay (min)	60	45
Anti-species conjugate dilution	1/6000	1/12,000
Duration of the second step of assay (min)	60	45
Chromogen used	ABTS	TMB
Duration of enzymatic step of assay (min)	30	30
Parameters of calibration curves		
A_{min}	0.001	0.028
A_{max}	0.641	0.919
Power	0.450	0.586
IC_{50} (ng ml^{-1})	246	291
Lower limit of determination, IC_{20} (ng ml^{-1})	11.2	27.3
Upper level of determination, IC_{80} (ng ml^{-1})	5400	3100

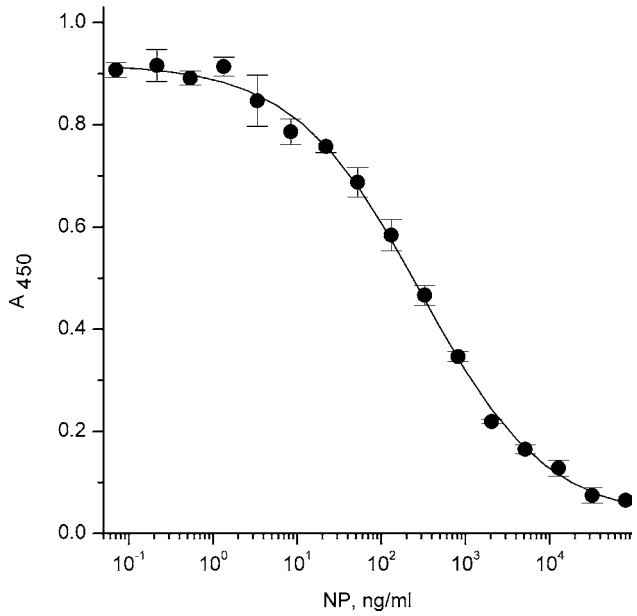


Fig. 2. Calibration ELISA curve for nonylphenol. Nunc microplate was used in combination with TMB as chromogen.

environmental samples. The chosen solvent content is in accordance with known tolerance of other immunoanalytical systems to 10–30% of the organics [30,31]. The earlier described ELISA system for the detection of linear NP [18] also presupposed the use of methanol at a final concentration of 10%.

Under the chosen conditions, about 10 ng ml^{-1} of NP could be quantitative determined (the case of Medpolymer microplates, see Fig. 1). The only known commercial AP ELISA kit of Takeda Chemical Industries for immunodetection of APs is characterized by an essentially worse sensitivity, IC_{20} = 70 ng ml^{-1} [32]. The sensitivity reached in our ELISA system allows to control NP in water samples that can exert acute toxic action [3].

3.2. Specificity of the ELISA system to different groups of pollutants

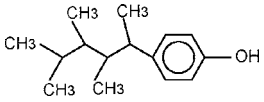
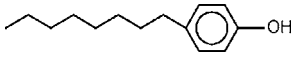
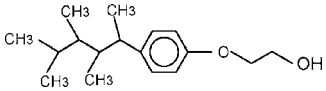
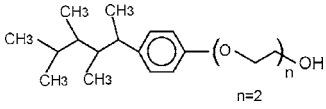
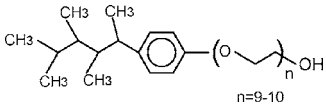
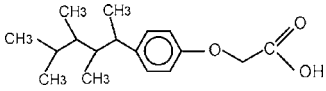
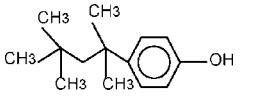
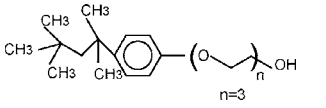
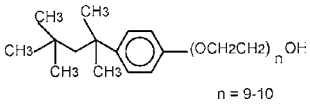
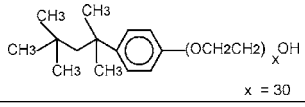
The proposed system was verified for its cross-reactivity with other classes of pollutants that potentially could be present in water samples. The assay is tolerant (CR < 0.05%) to such tested simple phenolic compounds as phenol, 4-chlorophenol, 2-amino-4-chlorophenol, 2,4-dinitrophenol, 4-chloro-3-methylphenol, 2,4-dimethylphenol, *p*-hydroxyphenylacetic acid, 2-hydroxybenzoic acid (salicylic acid), 5-aminosalicylic acid, 3(*p*-hydroxyphenyl)propionic acid, L-β-phenyl-α-alanine, 5-amino-2-3-dihydro-1,4-phthalazinedione, 4-hydroxybenzoic acid and nonylaldehyde (pelargonaldehyde). Weak binding values (0.05% ≤ CR ≤ 1%) were found for 4-aminophenol, dodecylbenzenesulfonic acid and hydroxyphenylheptanoic acid (HHA). The low levels of cross-reactions assure the absence of non-specific interactions in the course of the analysis of environmental samples.

Although HHA is a linear structural analog of NP, technical preparations of non-ionic surfactants contain predominantly branched isoforms of APs as a structural part [33]. Therefore, the recognition of linear NP is not required to characterize the degree of pollution.

3.3. Influence of APEOs and APs structure on the effectiveness of their detection

The recognition of native non-ionic surfactants by the proposed ELISA was initially studied for two OPEOs of common laboratory practice, Triton X-100 and Triton X-305 (Table 2). CR values for these compounds are similar if given in molar form (28.1 and 36.2%, correspondingly) though their essential difference in weight form (9.9 and 5.2%). In fact, cross-reactivity expressed in molar units reflects affinity of the antibodies to different compounds in terms of their molecular structure. The discovered phenomenon is stipulated by

Table 2
Cross-reactivities of APEOs in the developed ELISA

Compound	Structure[a]	CR values (%)	
		Mass	Mol
NP		100	100
4-*n*-NP (linear)		7.60	7.60
NPE_1O		156	188
NPE_2O		199	279
$NPE_{(9–10)}O$		27.3	79.0
NPE_1C		37.8	48.0
OP		51.9	48.5
OPE_3O		26.9	41.4
Triton X-100 ($OPE_{(9–10)}O$)		9.9	28.1
Triton X-305 ($OPE_{30}O$)		5.2	36.2

[a] Isomers for alkyl chains given here are chosen from several variants as examples.

the presence of same antigenic determinant in both Triton molecules and its similarity with the initial determinant of NP. The structure of the determinant may be described as benzene ring with branched uncharged C_8–C_9 chain. This element is the common structural part of APEOs and products of their decomposition.

For a more detailed description, representatives of primary classes of non-ionic surfactants (NPEOs and OPEOs) and their metabolites were analyzed. All tested compounds were characterized by high CR values (see Table 2), with the exception of the linear 4-*n*-nonylphenol.

Cross-reactivities for APs differing only in the length of $(–C_2H_4O–)_n$ chain were also compared for rows of (i) NPE_1O, NPE_2O and $NPE_{(9–10)}O$, and (ii) OP, OPE_3O, Triton X-100 ($OPE_{(9–10)}O$) and Triton X-305 ($OPE_{30}O$). The obtained data (see Table 2) confirm the above postulated statement about correlation of cross-reactivity levels with molar concentration of a unique antigenic determinant being common for non-ionic surfactants and their toxic metabolites. Thus, CR levels for OP derivatives vary in range 28–48% (in molar form) and increase for branched NP derivatives.

In this way, the ELISA system may be applied for the determination of both native surfactants and their stable metabolites belonging to two main groups, nonylphenol and octylphenol derivatives. The use of the obtained antibodies allows to accomplish group-specific immunodetection of alkylphenolic non-ionic surfactants.

3.4. ELISA detection of halogenated derivatives of non-ionic surfactants

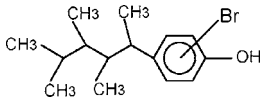
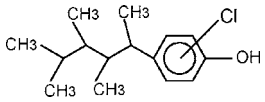
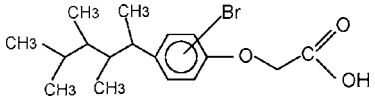
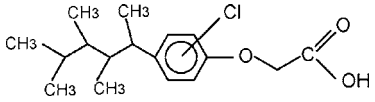
The developed system has been characterized for its availability to detect APEOs and APs halogenated derivatives. The formation of ring-halogenated alkylphenolic compounds during the chlorination process at drinking water treatment plants was reported in [25,28,29,34].

The proposed system is characterized by effective revealing of chlorinated and brominated derivatives of NP, such as Cl–NP, Cl–NPE_1C, Br–NP and Br–NPE_1C. Cross-reactivities for these compounds vary in the range 33–150% (in weight form), at that the brominated derivatives demonstrate higher affinity to the antibodies (Table 3). A possible reason of the found high CRs may be a rearrangement of charge distribution in benzene ring of the haptens that is caused by introduction of halogen atoms and following structural changes in branched alkyl chain. Thus, monitoring of alkylphenolic non-ionic surfactants by the developed ELISA technique may be realized also for the treated water containing halogenated derivatives of APEOs and APs.

3.5. Analysis of alkylphenolic non-ionic surfactants in polluted water samples with the use of the developed ELISA and chromatographic technique

The ELISA technique has been applied to determine levels of non-ionic surfactants and their derivatives in real water samples. WWTP influent and effluent, ground and tap water samples were studied. The determined values (Table 4) are in accordance with technological stages of wastewater purification (decrease of pollutant content in the row influent–effluent tap) and confirm effectiveness of the developed technique for ecological monitoring. NP can be detected effectively in environmental water matrixes, and the recovery for added NP is almost full under the stipulation that the content of own APs and APEOs in the matrixes used is negligible (near the detection limit of the assay) (Figs. 3 and 4). Reproducibility of repeated measurements (n = 7–8) is (14.2 ± 3.3)% that accords to characteristics of traditional ELISA techniques and is sufficient for needs of ecological monitoring.

Table 3
Cross-reactivities of halogenated APs in the developed ELISA

Compound	Structure	CR values (%)	
		Mass	Mol
Br-NP		74.4	101.2
Cl-NP		49.4	57.3
Br-NPE_1C		150	243
Cl-NPE_1C		33.3	47.4

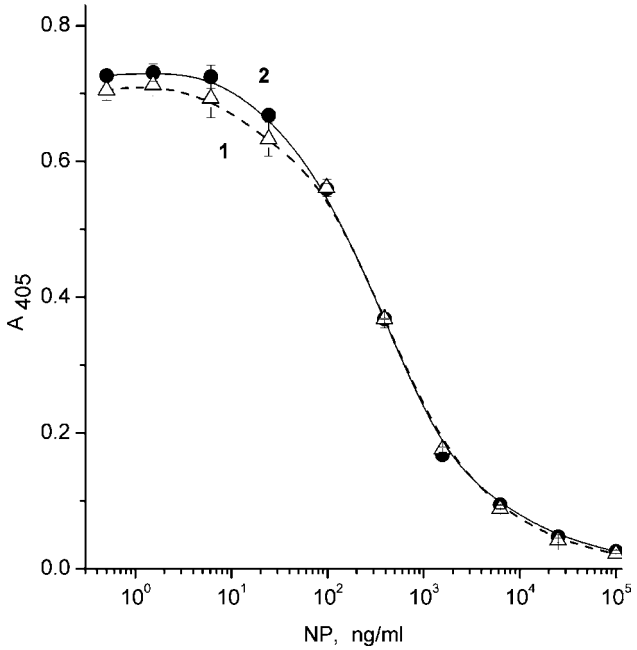


Fig. 3. Competitive curve for NP detection in spiked WWTP effluent A (1) in comparison with the data for deionized water (2).

Table 4
Determination of non-ionic surfactants in real water samples by the developed ELISA technique and by LC–MS

Samples	ELISA data	LC–MS data						
	Total APEOs + APs[a] (ng ml^{-1}) (Mean ± S.E.M., n = 7–8)	Concentrations (ng ml^{-1})						
		NPE_1O	NPE_2O	$NPE_{(3–14)}O$	NP	NPE_1C	NPE_2C	Sum
WWTP influent A	49.57 ± 10.97	4.0	<0.01	2.1	0.11	<0.01	<0.01	6.2
WWTP influent B	231.5 ± 18.6	7.4	5.1	71.2	0.22	0.35	0.21	84.5
WWTP influent C	359.8 ± 61.0	2.5	5.3	189	2.58	0.05	0.11	199.5
WWTP effluent A	7.14 ± 3.11[b]	<0.025	2.9	1.2	0.055	0.45	0.12	4.70
WWTP effluent D	37.13 ± 3.52	<0.025	0.5	1.4	0.38	0.82	0.18	3.29
Tap water A	2.85 ± 1.48[b]	<0.025	<0.01	0.1	<0.01	<0.01	<0.01	0.10

[a] The concentrations were calculated based on the calibration curve for NP.
[b] The mean values are given considering measurements above and below the limit of detection.

The above results of immunoenzyme determination were compared with levels of individual pollutants measured by chromatographic technique. The used LC–MS method yielded detection limits of 2 pg ml^{-1} for NP and NPECs and 10 pg ml^{-1} for NPEOs (25 pg ml^{-1} for NPE_1O). Recoveries from water ranged from 72 to 98%, with standard deviations below 7%.

The characterized compounds (Table 4) were NP, NPEOs and carboxylated NPEOs with different chain length. Levels of dicarboxylated compounds (with carboxylation of both ethoxy- and alkyl-chains) were not determined although they were known as substantial products of the NPEO biotransformation [35]. OP, OPEO, OPEC and halogenated derivatives were regarded as minor compounds that can contribute not more than 10% of the total pollution. Nevertheless, the common content of the determined types of non-ionic surfactants and their metabolites may be considered as a parameter reflecting the level of pollution.

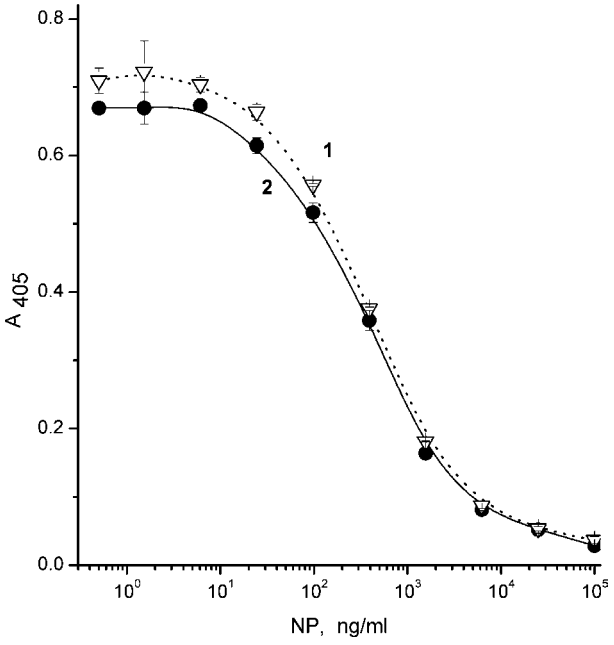


Fig. 4. Competitive curve for NP detection in spiked tap A water (1) in comparison with the data for deionized water (2).

The results of ELISA measurements correlate well with total values of chromatographically determined pollutants content data. The coefficient of correlation (r) was 0.88 for linear presentation of the data and 0.94 for logarithmic one. The values of the measured concentrations were higher in the ELISA that agreed with the absence of some compounds in the calculated content of surfactants based on chromatographic data. On the whole, the obtained results show that the proposed ELISA technique can be used for a group-specific monitoring of alkylphenolic non-ionic surfactants and their metabolites in water samples.

4. Conclusion

The preparation of immunogen by coupling of non-modified nonylphenol to protein carriers has been found to be an effective approach in the elaboration of immunodetection technique for non-ionic surfactants. The obtained set of reactants (antibodies + nonylphenol–protein conjugates) may be used for further development of different immunotechniques for surfactants detection. The developed ELISA allows to recognize specifically real surfactant compounds (in manufactured forms) and decomposition products such as alkylphenols, alkylphenoletoxylates and their carboxylates. The new ELISA protocol was applied to the trace determination of non-ionic surfactants in wastewater samples. Although the ELISA and LC–MS/(MS) results did not completely correspond with each other, the order of magnitude could be estimated that allows to propose this ELISA as screening method for nonylphenols in WWTPs. In this respect, it can be used to decide whether a wastewater sample contains nonylphenols below or above a certain threshold limit value indicating the correct operation of the WWTP.

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