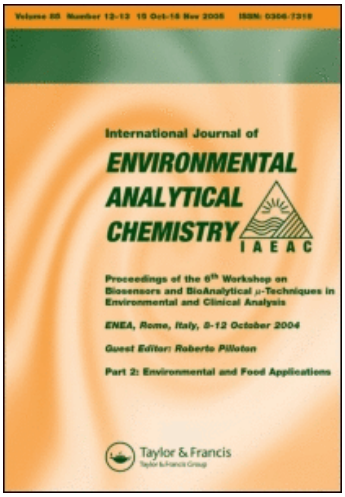

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Immunoassays for alkylphenolic pollutants with endocrine disrupting activity

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Among the numerous pollutants with endocrine/estrogenic activity, several detergents and plasticizers are found both in the environment and in food. Particularly, phenolic compounds such as alkylphenols (AP) and bisphenol A (BPA) may enter the food chain as a result of the application as emulgators in pesticides and plasticizers in plastic materials, respectively. In order to be able to measure and/or monitor these residues we designed several ELISAs. Antibodies were raised in rabbits and used in direct and indirect competitive ELISA, in IAC columns as well as in an immunosensor. Alkylphenolic compounds included octylphenol and nonylphenol. Validation of the ELISA revealed a detection limit of 1 and 2 nM for these compounds, respectively. Two of the antibodies showed a relatively broad cross-reactivity spectrum for 4-*n*-octylphenol (100%), nonylphenol (90%) and BPA (10%), whereas one other antibody was highly selective for 4-*n*-octylphenol. Further validation parameters included linearity of the assays ($R^2 \geq 0.9998$), recovery (110–130%) and matrix effects. The same antibodies were coupled onto wide porous activated silica and off-line columns were evaluated with HPLC. The capacity of the columns was 1.3 nmol of nonylphenol and 1.0 nmol of octylphenol (reproducibility 9 and 3% RSD, respectively). Polyclonal anti-BPA antibodies performed very well in the indirect as well as the direct competitive ELISA, showing a detection limit of 0.035 nM. Linearity ($R^2 \geq 0.9996$), intra-assay and inter-assay variation (about 14 and 8%, respectively), cross-reactivity and matrix effects were determined. IAC columns containing antibodies coupled onto wide porous activated silica were designed in both off-line and on-line (Guard) format coupled to HPLC. They were validated in terms of capacity, detection limit (0.03 nM), re-usability, and recovery (95–100% for spiked samples). An immunosensor assay for BPA was developed on the Biacore 3000 wherein either antibody or BPA-derivative was directly coupled onto the chip surface. A detection limit of 2 nM could be achieved and the assay appeared specific for BPA showing no cross-reactivity for other related and unrelated compounds. Real water samples (surface water and effluent) were spiked with varying concentrations of BPA with or without potentially interfering substances. A recovery of around 100% was found comparable to the ELISA. An attempt was also made to transfer the Biacore assay to the Spreeta^{TM} using the same chip and polyclonal antibody. Herein an IC50 for BPA of 5 ng mL^{-1} was found, whereas again the antibody cross-reacted mainly with BPA and BVA.

Keywords: Environment; ELISA; Immunosensor; IAC

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1. Introduction

Among the numerous pollutants in the environment those exhibiting endocrine disrupting (ED) activity currently are of particular interest [1, 2]. Risk assessment and human exposure studies involve both bioassays for the determination of ED activity and analytical procedures to detect, monitor and quantify suspected compounds [3–5]. Endocrine disrupting compounds (EDC) may exert their effects via various hormonal pathways and mechanisms as has been extensively described [6–8]. Surprisingly, it was found that most of the EDC show no structural relationship to their endogenous counterparts. This implies that each individual substance should be tested in a battery of bioassays, an almost impossible task given the tens of thousands of pollutants. Besides, many methods are still in development or have to be developed. EDC with estrogen mimicking properties pose a special problem due to their potential effects on human reproduction and the evolvement of sex-hormone (estrogens, androgens) related cancers. Many compounds displaying estrogenic activity have been found, including natural and synthetic hormones, pesticides, food additives, industrial chemicals, cosmetic ingredients, by-products, metabolites, etc. [9–13]. It has been reported that phenolic compounds constitute a group to which the human population is exposed continuously and in relatively high levels. For example, bisphenol A (BPA) is a well-known plasticizer used in industry for the fabrication of all kinds of plastic materials and articles. Nonylphenol (NP) and octylphenol (OP) are predominantly derived from ethoxylates used as detergents. The estrogenic properties of these phenolics have been determined [14–20]. In order to be able to assess emission rates, environmental loading and human exposure levels, cost-effective specific analytical methods are needed. The immunoassay is known as a suitable method for monitoring purposes. Therefore, we generated antibodies against BPA, NP and OP and using these antibodies we designed immunoassays and immunoaffinity chromatography (IAC) columns. Furthermore, attempts were made to develop an immunosensor assay for BPA on the Biacore as well as the Spreeta^{TM}. The results of our study are presented and future optimization and application are discussed.

2. Experimental

2.1 *Reagents*

Bovine serum albumin (BSA), ovalbumin (OVA), goat anti-rabbit HRP-conjugated antibody, caprylic acid, and various cross-reactant compounds were from Sigma (Zwijndrecht, The Netherlands), bisphenol A [2,2′-bis(4-hydroxyphenyl)propane), BVA [4,4-bis(4-hydroxyphenyl)valeric acid], 4-*n*-nonylphenol (Aldrich), 4-octylphenol, 4-*n*-octylphenol were purchased from Omnilabo (Breda, The Netherlands). Medium binding microtiter plates were from Greiner Bio-One BV (Alphen a/d Rijn, The Netherlands). Derivatives for the production of protein conjugates for the alkylphenols, HO-phenyl-$(CH_2)_8$-COOH (C8-AP) and HO-phenyl-$(CH_2)_6$-COOH (C6-AP), were kindly provided by Dr Eremin (Moscow University, Russia). Silica with a pore size of 700 Å and a particle diameter of 35–70 μm was kindly provided by Grace Vydac GmbH (Worms, Germany) and used to produce IAC columns. All reagents and solvents were of analytical grade.

2.2 *Buffers*

PBS was generally used for the ELISA and consisted of 8 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 2.68 mM KCl, pH 7.4. Antibodies were diluted in PBS-BSA (PBS + 0.1% BSA). Blocking buffer was PBS + 1% Marvel (skimmed milkpowder) and wash buffer was 0.01 M of PBS + 0.05% Tween 20.

2.3 *Equipment*

Biacore 3000 (Biacore; Uppsala, Sweden) was used for the immunosensor assay; microtiterplates were read with an ELISA reader (EAR SLT340; Beun de Ronde, Abcoude, The Netherlands); the HPLC system consisted of model HP 1100 High Pressure Liquid Chromatograph (Agilent, Amstelveen, The Netherlands) equipped with a low pressure quaternary pump, degasser, column oven and variable UV-Vis detector. Injection was performed by a model Rheodyne 7725i sample injector (Agilent, Amstelveen, The Netherlands) equipped with a 200 µL sample loop. When on-line sample preparation was performed by IAC, a model Rheodyne 7000 Switching valve (Bester BV, Amsterdam, The Netherlands) was used to switch the IAC column on- and off-line with the HPLC. With a model LC10AD isocratic pump (Shimadzu, Den Bosch, The Netherlands), sample was introduced onto the IAC column. Peaks were recorded by a model HP3395 integrator (Agilent, Amstelveen, The Netherlands).

2.4 *Preparation of conjugates*

Immunogens consisted of BSA-conjugates of BVA for bisphenol A and of C6-AP and C8-AP for the alkylphenols using the well-known mixed anhydride method. OVA-BVA was made by the coupling reaction with NHS and DCC, whereas C6/8-AP OVA-conjugates were produced using the same starting materials and procedures as for the immunogens. After synthesis the conjugates were purified using Biorad Econo-Pac 10DG columns (BioRad, Veenendaal, The Netherlands) according to the manufacturers instructions.

2.5 *Generation of antibodies*

New Zealand White rabbits were immunized by injecting 250 µL of a 1 : 1 (v/v) mixture of Freunds complete adjuvans and BSA-conjugate (0.5 mg mL^{-1}). Boosters were given every 3 weeks in Freunds incomplete adjuvans. After the 22nd week, the boosters were given every 6 weeks and then each time 40 mL of blood was drawn. The serum was used for titration of antibody levels and finally pooled, extracted using the caprylic acid method before use in the experiments. In short, extraction of antibodies from serum samples was performed by adding 4 mL of serum to 8 mL of 0.06 M acetate buffer, pH 4.0, followed by the dropwise addition of 0.3 mL of caprylic acid under stirring for 30 min. The solution was then centrifuged at 7800 × *g* at RT and the supernatant transferred into a fresh tube. The pellet was resuspended in 4 mL of 0.015 M acetate buffer, pH 4.8 and treated as above. Both supernatants were combined, filtrated and dialysed against PBS.

2.6 *ELISAs*

Indirect competitive ELISAs were set up using purified antibody. Microtiter plates were coated with OVA-conjugate (200 or 400 ng mL^{-1}, as appropriate) in PBS, pH 9.6, at 4°C overnight. After washing with PBS/Tween-20, the plates were blocked using 1% Marvel in PBS. Following removal of fluids, standard or sample and antibody were added and incubated 2 h at room temperature (RT). Washing (five times) with PBS/Tween-20 was followed by the addition of goat anti-rabbit HRP-conjugated antibody, incubation for 2 h at RT and the addition of hydrogenperoxide and TMB solution (15 min at RT). Colour development was measured at 450 nm on the microtiter plate reader. Standard curves were calculated using the four-parametric logit-log transformation of the absorption data. Detection limit was calculated at 3 S.D. from zero binding.

Direct competitive ELISAs were performed by coating microtiter plates with antibody and incubating overnight at 4°C. After washing five times with PBS/Tween-20 the plates were incubated with standard or sample and HRP-BVA for 2 h at RT. Following washing as above, hydrogen peroxide and TMB solution was added for colour development and absorbance readings.

2.7 *IAC columns*

IAC was performed both off-line and on-line and for that reason two types of IAC columns were constructed, i.e. conventional SPE columns (500 mg) and guard columns (1000 mg). The following antibody-containing solid phases were made: for BPA, 3 mL of antibody solution (0.7 mg mL^{-1} in PBS, pH 7.2) was placed into a flask and 160 mg sodium cyanoborohydride and 1 g of activated silica were added. The reaction suspension was agitated during the night at room temperature. Then the solid phase was washed with PBS, pH 6.8. For the AP, the same procedure was followed using 1.5 mL of a mixture of several anti-C6/C8-AP antibodies.

Several parameters of the produced columns were assessed. First, the affinity for its target compound(s) was determined by passing a standard solution in PBS at pH 6.8, made from a stock in acetonitrile, through the column and analysing the eluates by HPLC with UV-Vis detection. In the case of IAC-SPE for both AP and BPA, the conditions were as follows: loading of the samples, rinsing with 2×3 mL of PBS at pH 6.8, elution with 2 mL of 35% v/v acetonitrile in water, rinsing with 2 mL of 35% v/v of acetonitrile in water and 2×3 mL of PBS at pH 6.8. The eluates were analysed by HPLC using a Zorbax XDB-C8 analytical column, 5 μm, 4.6×250 mm (Agilent, Amstelveen, The Netherlands) and 75% v/v methanol in water as mobile phase at a flow rate of 0.8 mL min^{-1}, whereas detection took place at UV-Vis 230 nm. In the case of IAC-guard, the columns were loaded with 300 μL to 30 mL of sample in PBS at pH 6.8 at a flow rate of 0.5 mL min^{-1}, rinsed with the same buffer, eluted with 35% acetonitrile in water onto the analytical column and analysed accordingly. The capacity of the respective IAC columns was determined using an excess amount of target compounds.

Furthermore, the cross-reactivity of the respective columns was determined by using various mixtures of related and unrelated compounds and performing the IAC runs as above. In addition, PBS buffer and effluent were spiked with target compounds to determine matrix effects and recoveries.

2.8 *Immunosensor assay*

An inhibition assay was developed on the Biacore 3000 using anti-BPA antibody. Details of the design of this assay have been described [21]. In short, the surface of a CM5 chip was loaded with OVA-BVA and OVA (reference channel) at $50\,\mu g\,mL^{-1}$ in acetate buffer at pH 4.5. The chip was washed with methanol and dried under nitrogen gas. For the assay, diluted antibody solutions were mixed with standard (1 : 5) and 50 µL were injected at a flow rate of $20\,\mu L\,min^{-1}$. The surface was regenerated by injecting 20 µL NaOH (0.2 M) containing 20% acetonitrile. Peak responses were obtained 10 s after the injections.

Further, an attempt was made to transfer the Biacore assay to the Spreeta™ (Texas Instruments, Dallas, USA), a comparable simplified, experimental embodiment of an SPR-based immunosensor. This device was provided with the same CM5 chip directly coated with BVA.

3. Results

3.1 *Antibodies*

Two antibody preparations, K66 and K67, were obtained for BPA which showed a titer, defined as the dilution of antibody at 50% of the maximum absorption, of 500 and 1000, respectively. The serum was purified using the caprylic acid extraction method. In a checker board set-up the optimal dilutions of antibody, OVA-conjugate and HRP-tracers were found and the antibody preparations were used to prepare standard curves in direct and indirect ELISA format. From these the detection limit and working range were calculated and the results are shown in table 1. For the same antibody preparations cross-reactivities indicated that they were rather specific for BPA (see table 2). Linearity of the assay was determined by diluting a high concentration sample (316 nM) and plotting the measured *versus* expected concentration and applying linear regression resulting in a line of $y = 0.8194x + 0.10$, $r^2 = 0.9996$ and $y = 0.9133x + 20$, $r^2 = 0.9967$, respectively. The precision was calculated as the intra-assay variation by analysing a sample several times in one run and was 12.3% (8.6 ± 1.1 nM) for antibody K66 and 16.9% (124 ± 21 nM) for antibody K67. Inter-assay variation was determined from three different standard curves and varied from 1.1 to 8.2% depending on the concentration. The results for K67 are displayed in figure 1.

Four rabbits were used for the generation of antibodies against alkylphenols. Rabbits M32 and M33 were immunized with BSA-C6-AP, and M34 en M35 with BSA-C8-AP.

Table 1. Anti-bisphenol antibodies K66 en K67 were characterized for the main ELISA parameters using the indirect competitive format and four-parametric logit-log transformation for calculations. Detection limit was calculated at 3 S.D. from zero binding.

				Intra-assay variation ($n = 6$)		
Rabbit No.	Detection limit	Working range	Titer	Conc.	STD	% CV
K66	0.15 nM	0.1–100 nM	1000	8.6 nM	1.1 nM	12.3
K67	0.035 nM	0.1–1000 nM	500	124 nM	21 nM	16.9

Table 2. Cross-reactivities for anti-BPA antibodies K66 and K67 were calculated at the 50% binding point of the standard curves.

	K66% C.R.		K67% C.R.	
Compound	dcELISA	icELISA	dcELISA	icELISA
Bisphenol A	100	100	100	100
Bis-(4-hydoxyphenyl)-methane	1	1	1	n.d.
4-Cumylphenol	20	100	1	10
4,4′-(Ethylidene)bisphenol	10	100	10	10
Nonylphenol	1	n.d.	0.1	n.d.
Vinclozolin	0.1	< 0.1	0.1	< 0.1
17β-Estradiol	< 0.1	n.d.	< 0.1	n.d.
Sulfadimidine	< 0.1	n.d.	< 0.1	n.d.
Pirimiphos-ethyl	< 0.1	< 0.1	< 0.1	< 0.1
2,4-D	0.1	< 0.1	n.d.	< 0.1
Fenitrothion	n.d.	< 0.1	n.d.	< 0.1
Chlorpyrifos-methyl	n.d.	< 0.1	n.d.	< 0.1
Erythromycine	n.d.	< 0.1	n.d.	< 0.1

dcELISA is the direct competitive format; ic is the indirect competitive format; n.d. = not determined.

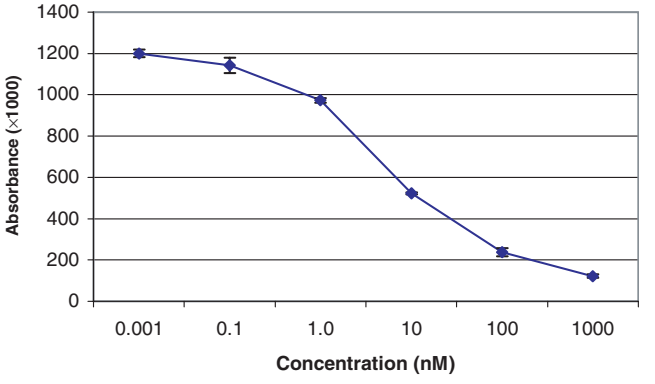

Figure 1. The mean ± SD of three standard curves for BPA using antibody K67 in the indirect ELISA format performed on different plates.

Since it appeared that the performance of the assay on OVA-conjugates was too low, each antibody preparation was tested on both BSA-C6-AP and BSA-C8-AP as coating conjugate. A difference in reaction to these two conjugates already was observed when following the evolution of the antibody titer up to immunization week 64. An example for M35 is given in figure 2. Since rabbit M32 died before the end of the immunizations, the antibody preparation was not included in the determination of the various parameters of the assays.

From indirect competitive standard curves a detection limit of about 1 nM for 4-*n*-octylphenol, about 20 nM for technical nonylphenol and about 2 nM for 4-*n*-nonylphenol was found. Using a high-level spiked sample the linearity in the assay with each antibody and coating conjugate was determined and for each case an R^2 of 0.999 or higher was found. To assess matrix effects and recovery rates, both demineralized water (demi) and a real water sample (WTP effluent) were spiked with various concentration of NP and OP. Initially, the recoveries were highly variable

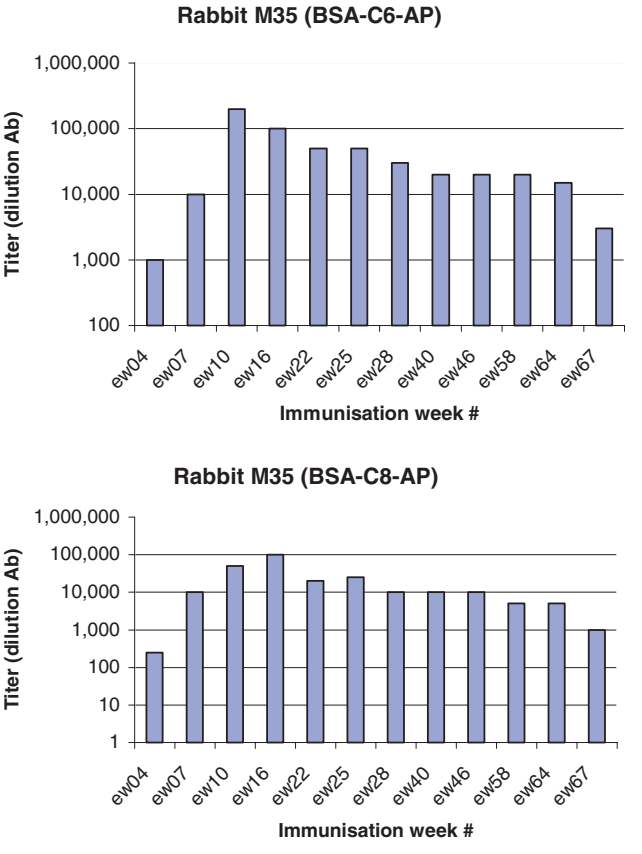

Figure 2. Example of the time course of anti-BPA antibody M35 measured in microtiter plates coated with BSA-C6-conjugate or BSA-C8-conjugate.

and low. Therefore, new samples were spiked, added with 10% MeOH and measured again, resulting in increased recovery data for 4-*n*-octylphenol and 4-*n*-nonylphenol, but not for technical nonylphenol. The results are shown in figure 3 and table 3, respectively. Furthermore, the cross-reactivities varied with antibody preparation and coating conjugate. A summary is given in table 4.

3.2 *IAC of BPA and AP*

For BPA both an SPE and a Guard column were produced and validated using BPA in PBS, pH 6.8. For these columns an absolute capacity of 0.35 nmol (80 ng) and 0.44 nmol (100 ng), respectively, was found. The reproducibility was 6 and 2% RSD with each sample measured six times and showing linearity within the capacity of the column. Limit of detection was determined at 0.02 nM (4 ng L^{-1}) for a 100 mL volume sample and 0.06 nM (15 ng L^{-1}) for a 25 mL volume sample, respectively. Both types of columns could be used several times without decrease in performance. Effluent of a WWT plant was spiked with BPA in a concentration range of 250–1000 ng L^{-1}. Initially, recoveries varied between 18 and 25%. However, by adjusting the pH of the samples to pH 6.8 and adding NaCl to 150 mM, the recoveries increased significantly to 95–100%.

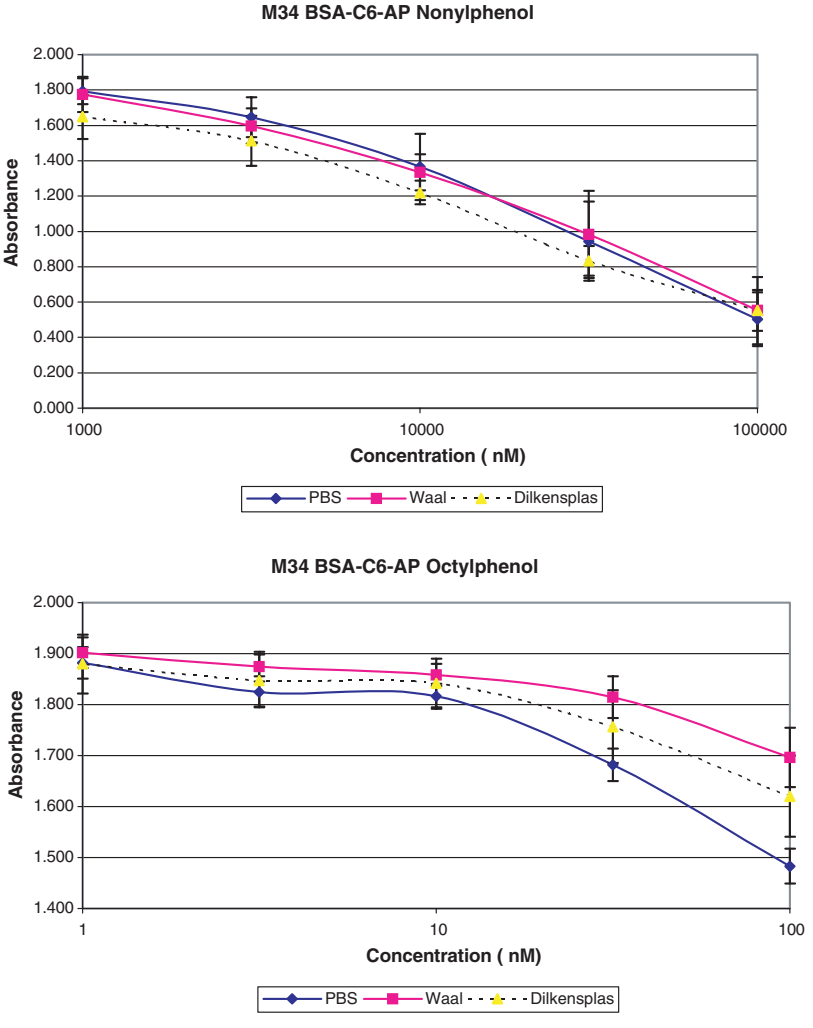

Figure 3. Comparison of standard curves in various matrices. NP was added to PBS (normal standard curve), Waal water (river) and Dilkensplas water (lagoon). The indirect competitive ELISA was performed on microtiter plates coated with BSA-C6-AP.

Similarly, an SPE-column for the AP, OP and NP, was validated using solutions containing various concentrations of nonylphenol and octylphenol. The capacity of the column was determined at 1.3 nmol for NP and 1.0 nmol for OP, showing a reproducibility of 9 and 3% RSD, respectively, being linear within the capacity range of the column. Again, the column may be used several times without deterioration.

3.3 *Immunosensor*

An assay on the Biacore 3000 was designed with either antibody or BVA coated onto a CM5 chip. The highest detection limit was 2 nM and cross-reactivity was displayed only for BPA and BVA. Matrix effects were determined by adding BPA to various water samples; the results are shown in table 5. In order to transfer this

Table 3. Demineralised water (demi) and WWTP effluent (WTP) were spiked with NP or OP, with or without 10% methanol. For OP the ELISA was also performed including a pre-incubation step of sample and antibody. Results are expressed in % recovery ± SD and % CV.

	Demi-water			WTP effluent		
4-*n*-Octylphenol						
Direct						
Concentration (nM)	1000	3200	10,000	1000	3200	10,000
Recovery mean (%)	92	95	84	42	40	49
SD	46	26	33	39	17	18
% CV	50	27	39	93	43	37
n	9	9	9	9	9	9
Pre-incubation						
Concentration (nM)	1000	3200	10,000	1000	3200	10,000
Recovery mean (%)	106	122	138	74	64	77
SD	37	13	7	25	12	3
% CV	35	11	5	34	19	4
n	3	3	3	3	3	3
4-*n*-nonylphenol						
Direct						
Concentration (nM)	1000	3200	10,000	1000	3200	10,000
Recovery mean (%)	60	48	22	44	34	16
SD	4	12	1	14	10	4
% CV	7	25	5	32	29	25
n	3	3	3	3	3	3

Table 4. Cross-reactivities for anti-AP antibodies M33, M34 and M35 were calculated at the 50% binding point of the standard curves. BSA-C6-conjugate was used for coating and the ELISA was performed in the indirect competitive format.

Compound	M33	M34	M35
4-Octylphenol	100	100	100
Nonylphenol	20	0.5	10
Bisphenol A	200	5	1
Phenol	< 0.1	2.5	< 0.1
Bis-(4-hydroxyphenyl)-methane	200	250	10
4-Cumylphenol	200	2.5	10
4,4′-(Ethylidene)bisphenol	100	25	10
BPA-diglycidyl ether	500	< 0.1	10
4,4′-Cyclohexylidene bisphenol	200	< 0.1	1
Vinclozolin	< 0.1	1	1
Pirimiphos-ethyl	< 0.1	1	1
Estradiol	< 0.1	1	< 0.1
2,4-D	< 0.1	< 0.1	< 0.1
Sulfadimidine	< 0.1	1	< 0.1

assay to a Spreeta format, the same CM5 chip coated with OVA-BVA was used to construct standard curves for BVA, BPA and stilbenes (DES, hexestrol, dienestrol) for comparison in the Biacore assay. The initial sensorgram showed binding of antibody to the OVA-conjugates, but not to OVA alone. Various standard concentrations were mixed with antibody K66 and K67 and injected according to the protocol as described above. Under these conditions, as shown in figure 5, 50% inhibition values obtained with K67 were approximately 3.5 ng mL^{-1} for BVA, 5 ng mL^{-1} for BPA

Table 5. Various samples were spiked at 4, 22, 44, 110 and 220 nM of BPA and measured at the Biacore for calculation of mean values for recovery and precision.

Source		Tap water	Rhine	Canal	Lagoon
Intra-assay variation	Recovery %	85	68	118	121
	CV %	2	2	1	2
Inter-assay variation	Recovery %	80	78	97	109
	CV %	14	7	19	40

and 1500 ng mL^{-1} for the stilbenes (0.2% cross-reactivity). Results using antibody K66 were similar. In the Spreeta assay 50% inhibition for BPA was found to be 1.2 ng mL^{-1} (LOD = 0.7 ng mL^{-1}). Further optimization of the conditions could lower the sensitity to 0.2 ng mL^{-1}.

4. Discussion

Polyclonal antibodies against BPA, NP and OP were raised and used to develop ELISAs for the target compounds that have been shown to possess estrogenic activity. The detection limits were around 1 nM for each compound. Literature data indicate that in water bodies such as waste water and effluents concentrations of these alkylphenols in the range of 0.002–0.04 μM may be found [22, 23], and BPA in a range of up to 4 nM. Generally, the applied analytical methods are HPLC, GC-MS, and LC-MS. However, although such conventional methods are highly sensitive, they are cumbersome and expensive and not suitable for measuring many samples in monitoring studies. According to a study of the RIWA in The Netherlands, 4-nonylphenol has even been detected in drinking water in a range of 0.1–1.0 μg L^{-1} (0.45–4.5 nM) [14] despite extensive chemical and biological purification. Gascon [12] reported about the presence of at least 20 different alkylphenolic compounds in drinking water and referred to possible additive or even synergistic effects. It has been reported that pollutants with estrogenic activity may have an effect on biological treatment system so that the efficiency of the purification is lowered [11]. ELISA and PFIA (polarization fluorescence immunoassay) have been developed for nonylphenol, but the sensitivity was in the range of 50–1000 μg L^{-1}, although PFIA performed somewhat better [24–27]. Remarkably, the recovery of spiked demi and effluent samples was increased when 10% MeOH was added to the samples, in accordance with the results of Ying *et al.* [28] who measured endo- and exo-estrogens with HPLC after SPE pre-treatment. However, the recoveries of technical nonylphenol were undectectable and overall the variation was quite high. A pre-incubation step increased the performance, but this item needs further optimization. In those cases that the sensivity of an assay is not enough for the particular water samples, an IAC column may be used for pre-treatment and concentration of the compounds. IAC columns with our antibodies were designed for off-line and on-line use. Eluates may be used for HPLC measurement or used in an ELISA. The performance of the columns was determined in terms of capacity, reproducibility and re-usability. Overall these columns appeared to meet the general conditions for pre-treatment of samples, whereby BPA could be captured in a concentration of 0.43 nmol and the alkylphenols at about 1.0 nmol. IAC columns in Guard column configuration

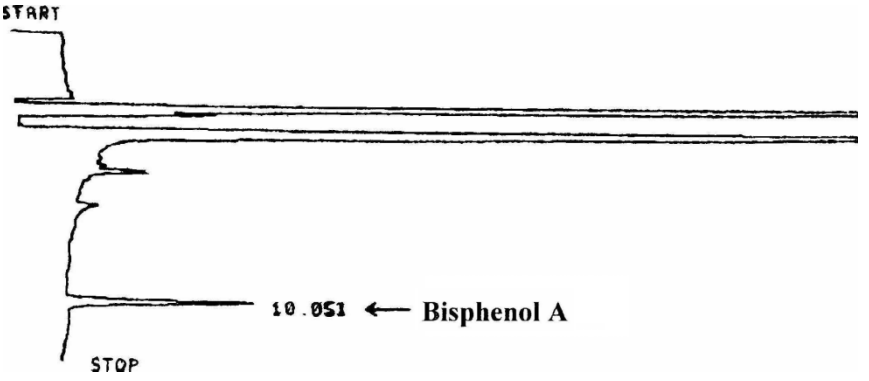

Figure 4. Chromatogram of BPA in waste water after purification using a column coupled with anti-BPA antibody using RP-HPLC and UV-Vis detection at 230 nm.

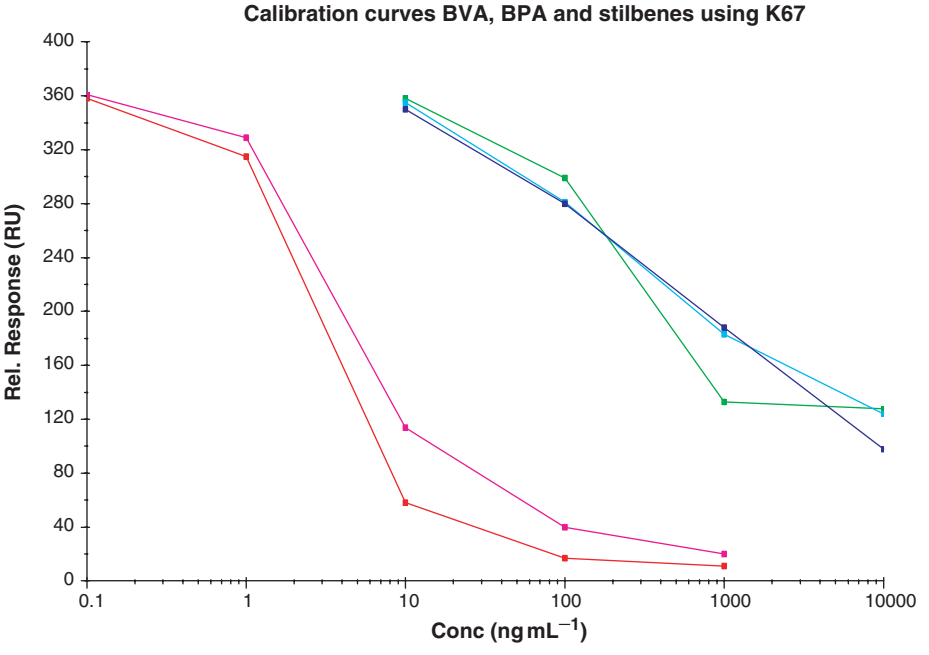

Figure 5. Comparison of the signal in a immunosensor assay at the Biacore 3000, in order from left to right for BVA, BPA and various stilbenes. It can be seen that stilbenes cross-react to a low extent.

may be used on-line with HPLC and provide chromatograms that are easily to interpret because of the absence of contaminating substances. An example of a chromatogram for BPA added to effluent and measured with HPLC after IAC pre-treatment is shown in figure 4. It is clear that a very clean chromatogram is obtained with this procedure.

Testing strategies for EDC haven been reviewed by Combes [3] and the estrogenic capacity of BPA, NP and OP has been demonstrated in both *in vitro* and *in vivo* assays [13, 14, 18, 19, 29, 30]. To assess the load in water bodies, in particular

drinking water resources, and potential risks for organisms including humans, monitoring studies have to be performed to quantify particular compounds. The ELISA described herein, optionally combined with corresponding IAC columns seem highly suitable for such purposes. For real-time measurements an immunosensor may be used. An assay on the Biacore has been developed and described before [21, 31]. However, such equipment is quite expensive and an assay on the Spreeta may be a good alternative. Our preliminary results indicate that such an immunosensor assay is feasible, although some optimization is needed. Future studies should also include food samples because of the presence of phenolic compounds that can migrate from all kinds of plastic packaging.

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