

# Immunoassays for 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 environment and the food chain as a result of the application as emulgators in pesticides, plasticizers in plastic materials, food additives as well as from cosmetics. In order to be able to measure and/or monitor these residues, polyclonal antibodies against octylphenol (OP), nonylphenol (NP) and BPA were raised in rabbits and with these we designed the corresponding ELISAs. The assays were validated in the usual way in indirect and/or direct competitive ELISA. The same antibodies were coupled onto solid support for use in IAC columns as well as in an immunosensor.

## Reagents

Two different derivatives for the production of protein conjugates for the alkylphenols, HO-phenyl-(CH<sub>2</sub>)<sub>8</sub>-COOH (C8-AP) and HO-phenyl-(CH<sub>2</sub>)<sub>6</sub>-COOH (C6-AP) were kindly provided by Dr. Eremin (Moscow University, Russia). OVA- and BSA-conjugates were synthesized using the coupling reaction with NHS and DCC. The immunogen for BPA was synthesized from BVA [4,4-bis(4-hydroxyphenyl)valeric acid] using the mixed anhydride method. Silica with a pore size 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.

## Equipment

Biacore 3000 (Biacore; Uppsala, Sweden) was used for the immunosensor assay; microtiterplates were read with an ELISA reader (EAR 340; Beun de Ronde); 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. In the on-line IAC-HPLC mode, a model Rheodyne 7000 Switching valve (Bester BV, Amsterdam, the Netherlands) is used to switch the IAC column on- and off-line with the HPLC. Peaks were recorded by a model HP3395 integrator (Agilent, Amstelveen, the Netherlands).

## Results

### ELISA

Alkylphenolic compounds included octylphenol and nonylphenol. Validation of the ELISA revealed a detection limit of 1 nM (working range 5 – 1000 nM) and 2 nM (3 – 600 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. Linearity of the assays was highly significant ( $R^2 \geq 0.9998$ ); the recovery appeared

dependent on the coating conjugate used and ranged from 110 – 130 %. Matrix effects of various surface water in these assays were moderate. An example of standard curves made in PBS and surface water is shown in Figure 1.

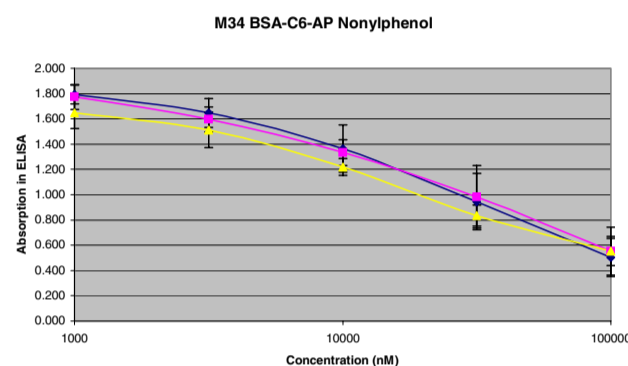


Figure 1. Matrix effects of anti-AP antibody M34

(NP was added to PBS (standard curve), Waal water (river) and Dilkensplas water (lagoon). The indirect competitive ELISA was performed on microtiter plates coated with BSA-C6-AP).

Polyclonal anti-BPA antibodies (K66 and K67) performed very well in the indirect as well as the direct competitive ELISAs. A detection limit of 0.035 nM could be achieved; working range 10 – 1000 nM. The linearity in the assay was highly significant ( $R^2 \geq 0.9996$ ); intra-assay and inter-assay variation were determined at about 14% and up to 8%, respectively. The antibodies showed a cross-reactivity with various other phenolic compounds of up to 10% and  $\leq 0.1\%$  with unrelated substances. The results are shown in Table 1. Matrix effects were moderate and the recovery of BPA in various matrices was around 100%.

Table 1. Cross-reactivities of antibodies K66 and K67

Compound	K66 % C.R.		K67 % C.R.	
	dcELISA	icELISA	dcELISA	icELISA
Bisphenol A	100	100	100	100
Bis-(4-hydroxyphenyl)-methane	1	1	1	n.d.
4-Cumylphenol	20	100	1	n.d.
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	<0.1
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.	<0.1a

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

### IAC of BPA and AP

For BPA both an SPE and a Guard column were produced and validated using BPA in PBS of pH 6.8, using HPLC UV-VIS at 230 nm. The chromatograms showed no contaminating peaks in addition to the target compounds. Absolute capacity was 0.35 nMol (80 ng) and 0.44 nMol (100 ng), respectively. The reproducibility was 6% and 2% RSD (n=6) and showing linearity within the capacity of the column. Limit of detection was determined at 0.02 nM (4 ng/l) for a 100 ml volume sample and 0.06 nM (15 ng/l) for a 25 ml volume sample, respectively. Both types of columns could be used several times without decrease in performance. Recovery of BPA in effluent of a WWT plant (range of 250 – 1000 ng/l), initially varying

between 18 and 25%, were increased to 95-100% by adjusting the pH of the samples to pH 6.8 by adding NaCl to 150 mM. Similarly, SPE-columns for OP and NP were validated. The capacity of the column was determined at 1.3 nM for NP and 1.0 nM 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.

### Immunosensor

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) 50 μg/ml in acetate buffer at pH 4.5. For the assay, diluted antibody solutions were mixed with standard (1:5) and 50 μl were injected at a flow rate of 20 μg/min. Peak responses were obtained 10 seconds after the injections.

Further, an attempt was made to transfer the Biacore assay to the Spreeta™, a comparable simplified, experimental embodiment of an SPR-based immunosensor. This device was provided with the same CM5 chip directly coated with BVA. Various standard concentrations were mixed with antibody K66 and K67 and injected according to the general protocol. Under these conditions, as shown in Figure 2, 50 % inhibition values obtained with K67 were approximately 3.5 ng/ml for BVA, 5 ng/ml for BPA and 1500 ng/ml 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 (LOD = 0.7 ng/ml). Further optimisation of the conditions could lower the sensitivity to 0.2 ng/ml.

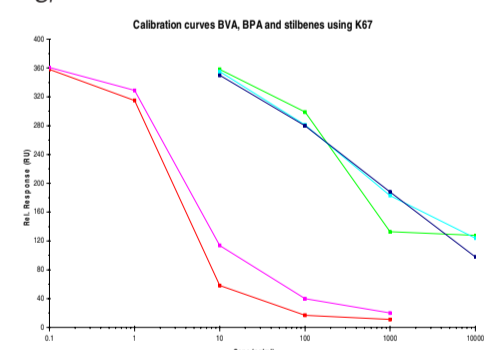


Figure 2. Specificity and sensitivity of the immunosensor assay for BPS. (Comparison of the signal in a immunosensor assay at the Biacore 3000 for BPA, BVA and stilbenes.)

## Discussion

The ELISAs developed for the detection of BPA, OP and NP in water are highly satisfying with regard to their general performance. They may be used in monitoring studies for various kinds of water types being superior to conventional methods in respect of cost-effectiveness, speed and sample volume. In case the levels are expected to be below the detection limit or samples are highly contaminated, the corresponding IAC columns be used for pre-treatment providing for both purification and concentration. Newer developments comprise immunosensor assays for real-time measurement as shown on the Biacore as well as the Spreeta, although these embodiments need further optimisation to enhance the sensitivity.