

## Detection of bisphenol A in water samples using ELISA determination method

J. Zheng, S. Q. Zhao, X. T. Xu and K. Zhang

### ABSTRACT

In order to study whether bisphenol A (BPA) can pass into drinking water from polycarbonate barrel and exist in the river and industrial effluent the indirect competitive enzyme-linked immunosorbent assay (ELISA) for the determination of BPA was established. The results presented an inhibition concentration at 50% absorbance (IC<sub>50</sub>) of 0.123 mg L<sup>-1</sup>, and the limit of detection (LOD) is 9.934 µg L<sup>-1</sup>. The specificity of antiserum was proved well because the cross-reactivity with benzene, tert-butylbenzene, hydroquinone and o-hydroxybenzoic acid were found lower than 0.01%, except phenol was 0.26%. The method was found to be reliable and repeatable. It was used for monitoring the concentration of BPA in the barreled drinking water. The results confirmed BPA can pass into barreled drinking water from the polycarbonate barrel and concentration increased as days went on. A certain content of BPA was found in industrial effluent. The results of ELISA were consistent with the results of UV spectrophotometry. BPA could not be found in the water samples obtained from Zhujiang River. The established method shows specific recognition of BPA and could be applied in detection of environmental BPA.

**Key words** | bisphenol A, ELISA, water

J. Zheng

S. Q. Zhao (corresponding author)

X. T. Xu

K. Zhang

Faculty of Chemical Engineering and Light Industry,  
Guangdong University of Technology,  
No.100 Waihuan Xi Road,  
Guangzhou Higher Education Mega Center,  
Panyu District,  
Guangzhou,  
P.R China

E-mail: [suqingzhao@hotmail.com](mailto:suqingzhao@hotmail.com);

[suqingzhao@yahoo.com](mailto:suqingzhao@yahoo.com);

[shinhwajay@hotmail.com](mailto:shinhwajay@hotmail.com)

### INTRODUCTION

Bisphenol A is mainly used as an intermediate in the preparation of epoxide resins, polycarbonate (PC) plastics and as an antioxidant or stabilizer in polyvinylchloride (Alexander *et al.* 1988). Extensive use of BPA has made it an environmental contaminant that is found in soil and river water (Matsumoto 1982; Fromme *et al.* 2002). In the meantime the waste landfill leachates and coatings on cans have also created the possibility of contact to BPA. The estrogenic effect of BPA has been shown both in vitro and in vivo experiments (Kim *et al.* 2001). BPA is able to mimic the effects of endogenous hormones, estrogens, and androgens (Krishnan *et al.* 1993), therefore, it influences the central nervous system (CNS) as well as the reproductive system in many animals (Colborn *et al.* 1993). Exposure to large quantities of BPA through the

skin, however, causes extensive damage to multiple organ systems including the kidney, liver, spleen, pancreas, and lungs (Atkinson & Roy 1955). High doses of BPA also cause reproductive toxicity and stimulate growth of the rodent uterus (Morrissey *et al.* 1987). In male rats and mice BPA is associated with an increased amount of cancers of the hematopoietic system (Ashby & Tennant 1988). It also caused other various kinds of cancers, such as prostate, testicular, and breast cancer, and has diverse pleiotropic actions in the brain and cardiovascular system (Davis *et al.* 1993; Steinmetz *et al.* 1998).

The studies mentioned above indicate it is important to detect BPA residues. The methods most frequently used for the determination of BPA currently are gas chromatography

(Olmo *et al.* 1997) and liquid chromatography (Sajiki 2001). ELISA, enzymatic biosensor, optical immunosensor (Zhao *et al.* 2002; Andreescu & Sadik 2004; Rodriguez-Mozaz *et al.* 2005) are also set up and allow sensitive detection of BPA. While the limit of detection may reach 4–6 ng L<sup>-1</sup> (Mol *et al.* 2000) by instrumental analysis, these instrumental methods require time-consuming complex pretreatment steps and are not suited in situ detection and batch determination. Hence, the ELISA test strips or kits should be developed for requirement. In this paper, following the successful synthesis of an artificial antigen of BPA (Zheng *et al.* 2008) an ELISA method for BPA is established. Barreled drinking water is used abroad in daily life. But the determination of BPA in barreled drinking water has not been reported before. In this paper, ELISA method was applied to determine BPA in barreled drinking water at different periods. In addition water samples from river and industrial effluent were also analysed. The ELISA results were corroborated by UV spectrophotometry method.

## MATERIALS AND METHODS

### Materials

Bisphenol A (2, 2-bis(4-hydroxyphenyl)propane) of chemical purity was purchased from chemical reagent co (Shang Hai China). Aluminium chloride anhydrous, dichloromethane and chloroacetic acid were purchased from Fuchen chemical reagent co (Tian Jing China). DMF (N,N-dimethylformamide) was purchased from Kermal chemical reagent co (Tian Jing China). NHS (N-hydroxysuccinimide) was purchased from Sinopharm chemical reagent co (Shang Hai China). BSA (Bovine Serum Albumin) was purchased from Bio Life Science & Technology Co (Shang Hai China). HRP-conjugated goat anti-mouse IgG was purchased from Boster Bioengineering co (Wu Han China). EDC·HCl (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride), FCA (Freund's complete adjuvant) and FIC (Freund's incomplete adjuvant) and OPD (o-dihydroxybenzene) were purchased from Sigma-Aldrich chemical co (USA). Phosphate buffer solution (PBS): pH 7.4 (8 g L<sup>-1</sup> NaCl, 0.2 g L<sup>-1</sup> NaH<sub>2</sub>PO<sub>4</sub> · 2 H<sub>2</sub>O, 2.9 g L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub> · 12H<sub>2</sub>O). PBS contained 0.05% tween-20 (PBST) was used for washing steps. Substrate buffer:

1.02 g citric acid, 3.6 g Na<sub>2</sub>HPO<sub>4</sub> · 12H<sub>2</sub>O in 100 ml distilled water. Coating buffer: 0.375 g Na<sub>2</sub>CO<sub>3</sub>, 0.733 g NaHCO<sub>3</sub> in 250 ml distilled water. Other reagents were of the highest grade available from local commercial chemical suppliers.

### Determination of BPA by ELISA

#### Production of anti-serum

The detailed procedure of preparation and characterization for the antigen was previously described by Zheng *et al.* 2008. Five 4 weeks old Balb/C mice were used for immunization. For each mouse, a total of four injections of 500 µg of the complete antigen in Freund's adjuvant were performed at days 0, 10, 25, and 35 before the final bleeding at day 45. The first injection of 200 µg of antigen was emulsified with FCA. The later three booster injections were emulsified with Freund's incomplete adjuvant (FIA) and contained 100 µg of the antigen. The titre of antiserum was determined with chequer-board titration using the microwell plates coated with the complete antigen solution in coating buffer.

#### ELISA test procedure

The ELISA tests procedure was carried out as following description. A 96-well microtiter plate was coated with 100 µL the complete antigen in carbonate buffer (pH 9.6) at the concentration of 10 mg L<sup>-1</sup> per well overnight at 4°C. Then the wells were washed three times with 370 µL of 0.01 mol L<sup>-1</sup> PBS containing 0.5% Tween 20 (PBST) and blocked using 200 µL of 20 g L<sup>-1</sup> skimmed milk for 2 h at 37°C. After washing three times with PBST, the wells were incubated with 50 µL of 1:4000 diluted antisera and BPA standard solution of known concentration for 1 h at 37°C. To the washed wells 100 µL of 1:20000 diluted HRP-conjugated goat anti-mouse IgG was added and incubated for another 1 h at 37°C. After washing three times with PBST and then once with pure water, the wells were filled with 100 µL of 0.4 g L<sup>-1</sup> OPD in water dissolved in citrate phosphate buffer (pH 6.0) containing 1 mL L<sup>-1</sup> of H<sub>2</sub>O<sub>2</sub>. After incubation for 20 min at 37°C without light, 50 µL of 2 mol L<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> was added to stop the enzyme reaction and the absorbance of each well was determined with the Bio-Rad Model 680 Micro plate Reader (Bio-Rad, USA) at 492 nm.

### BPA standard curve

The standard curve for BPA by ELISA was established using the method described above. And define the concentration range that BPA was tested over. ( $1$  to  $1 \times 10^6 \mu\text{g L}^{-1}$ ).

### ELISA specificity testing

The specificity of BPA ELISA was tested by determining whether the polyclonal antibody could cross react with compounds that possess analogues or part structures of BPA. Compounds tested include benzene, phenol, tert-Butylbenzene, hydroquinone and o-hydroxybenzoic acid were substituted of BPA followed the same assay. Each compound was tested following the ELISA test procedure as detailed above.

### Test reproducibility of the ELISA determination method

The test reproducibility was taken by adding BPA into the blank samples,  $50 \mu\text{L}$  BPA standard solution of  $2 \mu\text{g L}^{-1}$ ,  $5 \mu\text{g L}^{-1}$ ,  $10 \mu\text{g L}^{-1}$  were respectively added to  $50 \mu\text{L}$  blank solution, replicated three times. The assay was conformed to ELISA determination method.

### UV determination of BPA

For proving the dependability of ELISA method, ultraviolet spectra method was carried out with a TU 1901 UV-Vis spectrometer for comparison. BPA were dissolved in dichloromethane (DCM) of the concentration  $400 \mu\text{g L}^{-1}$ ,  $300 \mu\text{g L}^{-1}$ ,  $200 \mu\text{g L}^{-1}$ ,  $100 \mu\text{g L}^{-1}$ ,  $10 \mu\text{g L}^{-1}$ ,  $1 \mu\text{g L}^{-1}$ . DCM was used as the reference solution. UV spectrogram showed 3 peaks respectively existing at  $230 \text{ nm}$ ,  $277 \text{ nm}$ ,  $284 \text{ nm}$ . Considering the effect of impurities at  $230 \text{ nm}$  and the slight fluctuant at  $284 \text{ nm}$ , the peak value at  $277 \text{ nm}$  was chosen as the characteristic absorbance. The absorbance at  $277 \text{ nm}$  was recorded to set up the UV standard curve.

### Determination of BPA in water samples

In this experiment, drinking water was filled up in three polycarbonate barrels which were made of polycarbonate

and stored at room temperature. At days 10, 20 and 30, the water samples were taken out respectively for testing. The river samples were taken from the Zhujiang River (China) and industrial effluent samples were taken from the drain ditch near a factory which is related with PC production. Before testing, all the samples have been filtered by the medium speed filter paper to remove the precipitate. Both the competitive ELISA method and UV method were used for monitoring the concentration of BPA in barreled water at the mean time. For ELISA method, the water samples were directly determined without further treatment. For UV method,  $50 \text{ mL}$  water samples were extracted for three times with a total of  $50 \text{ mL}$  dichloromethane and bottom layer liquid was measured by the UV Spectrometer and the absorbance at  $277 \text{ nm}$  was recorded. According to the standard curves, concentration of BPA could be calculated.

## RESULTS AND DISCUSSION

### The development of an ELISA method for BPA

#### ELISA Standard curve for determination BPA

The chequer-board titration results show the titer of the antiserum is 1:128000, the working concentration of antiserum is diluted to 1:4000 as the OD value almost approached 1.0, respectively, and the optimal concentration for complete antigen as the coating antigen is  $10 \text{ mg L}^{-1}$ . Quantification of BPA was evaluated by measuring the OD value decrease with increasing concentration of BPA, and a 7-point calibration curve was constructed for indicating relationship between concentration of BPA and inhibitory rate. Figure 1 shows the plot of  $B/B_0\%$  as a function of the logarithm of BPA concentration. Each data have at least 4 replicates and were the mean value. The standard error was less than 0.08. As it can be seen, the curve presents an inhibition concentration at 50% absorbance ( $\text{IC}_{50}$ ) of  $0.123 \text{ mg L}^{-1}$ , and the limit of detection (LOD) is  $9.934 \mu\text{g L}^{-1}$ .

### Specificity of the ELISA test method

Cross-reactivity of the compounds with structure analogous to BPA was evaluated in the BPA ELISA and was calculated

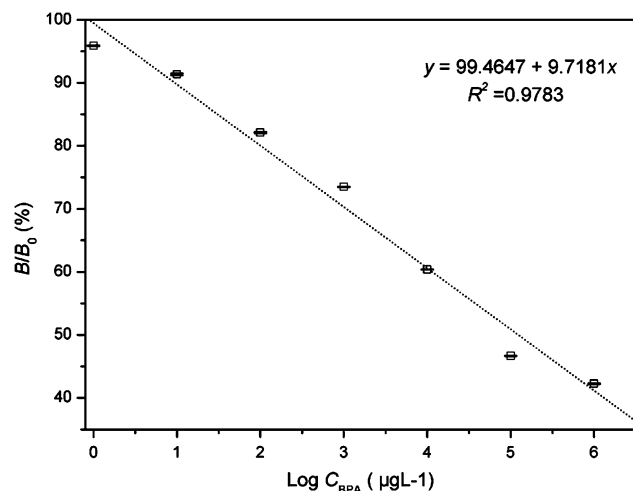


Figure 1 | The standard curve of antisera against BPA obtained by competitive ELISA.

with the equation  $CR (\%) = (BPA \text{ IC}_{50}) / (\text{Cross-reactant IC}_{50}) \times 100$ . The inhibition competitive curves of the five selected compounds were shown in Figure 2. Each data have at least 4 replicates and were the mean value. The standard error was less than 0.31. From Table 1, it shows the cross-reactivity to phenol of 0.26%, while the other four compounds have little influence on the reaction with cross-reactivity all lower than 0.01%. The results account for that structure of phenol is more analogous with the structure of BPA, correspondingly, the structures of other chemical has low correlation with BPA. This is suggested that the phenolic

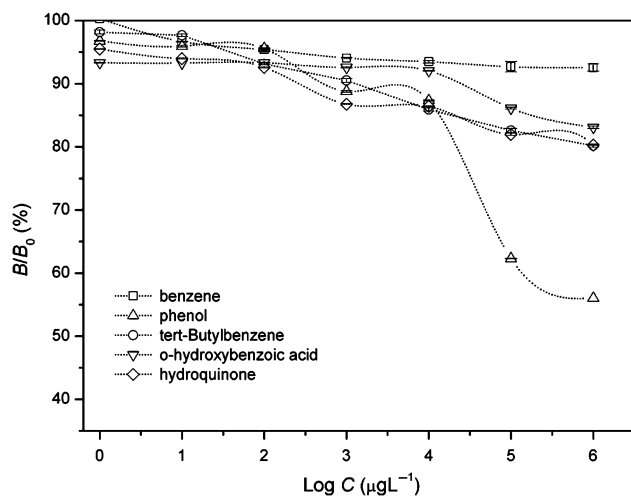


Figure 2 | The competitive inhibition of ELISA for antisera against the five analogous compounds.

Table 1 | Cross-reactivity of the assay with the selected compounds

Compound	Cross-reactivities (%)
Bisphenol A	100
benzene	<0.01
phenol	0.26
tert-Butylbenzene	<0.01
hydroquinone	<0.01
o-hydroxybenzoic acid	<0.01

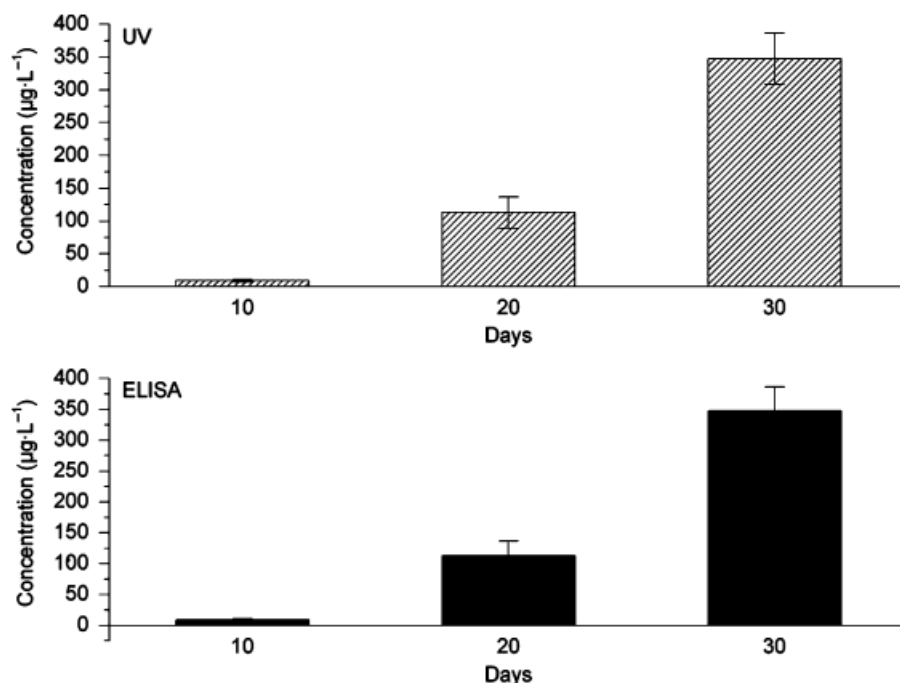
hydroxyl group was important discriminating site. According to the 50% displacement method (Pratt 1978), the results from the cross-reactivity test indicated the antiserum possessed good specificity.

### Reproducibility of the determination method

Table 2 showed the reproducibility of BPA from the blank solution respectively. The values are the mean of 4 replicates, and data means  $\pm$  SE. The deviations were below 1% and the results showed excellent reliability and reproducibility of ELISA method.

### Determination of BPA in water samples

The polycarbonate barrel was selected for checking whether BPA released from the barrel over time. By ELISA test, the decline of the OD value can show whether BPA release from the barrel. The data were the mean of 4 tests of the same sample and mean  $\pm$  SE. The results show that BPA can not be detected in the original barreled water. At 10th day about  $10 \mu\text{g L}^{-1}$  BPA has been found. However, 20 times and 30 times BPA were detected at 20th and 30th day respectively. It proves that BPA had released from the barrel and BPA concentration increased over time. At the mean time UV assay was carried out for comparison with ELISA method. The column plots were set up to present the change of BPA concentration detected by both ELISA and UV method (Figure 3). The results from the detection of river and industrial effluent samples were shown in Table 3. It was possible that the BPA in river was less than the LOD. Nevertheless BPA were found to exist in industrial samples obviously. Due to disturbance from the extraneous



**Figure 3** | The results of comparison between ELISA determination method and UV method.

component in river samples, UV methods can not give a definite result. In conclusion, the consistency of results from both the two methods proves the reliability of ELISA method.

## CONCLUSIONS

As a low molecular weight compound,  $M = 228.3 \text{ g mol}^{-1}$ , BPA itself isn't capable of arousing the immune response, so it should be conjugated with the carrier protein of high molecular weight. According to the previous reports about ELISA method (Mol *et al.* 2000; Zhao *et al.* 2002), BPA derivatives by modification of BPA phenolic hydroxyl group were utilized as hapten, and it results in the lost of the characteristic structure of BPA and may account for the low selectivity. In addition 4,4 bis-(4-hydroxyphenyl) valeric

acid (BVA) is not available for the expensive cost. In this experiment, an artificial antigen was used for ELISA establishment because it were easy to synthesize and cost less, moreover, the basic BPA structure was reserved. Results of ELISA test show that antiserum is capable of restraining BPA effectively and have good reproducibility. Cross-reactivity measurements show that antiserum possess good specificity and declare that phenolic hydroxyl group is an important discriminating site.  $IC_{50}$  of BPA is higher than the previous reports, and the LOD for BPA  $9.934 \text{ µg L}^{-1}$  is not as sensitive as the report (Goda *et al.* 2000) which showed the LOD was  $5 \text{ µg L}^{-1}$ , antiserum has not purified should be the main consideration. For achieving more sensitive effect, monoclonal antibody should be prepared in the future. This determination method has already been applied in detection of BPA in barreled drinking water in the Lab, the results show BPA

**Table 2** | Reproducibility test of BPA from blank solution

BPA Added/ $\text{µg L}^{-1}$	Reproducibility (%)
2	$94.2 \pm 0.8$
5	$91.5 \pm 0.5$
10	$98.8 \pm 0.2$

**Table 3** | The determination results of river and industrial effluent samples

Samples Method	River		Industrial effluent	
	ELISA	UV	ELISA	UV
Amount ( $\text{µg L}^{-1}$ )	<LOD	<LOD	114	127



can release from the polycarbonate barrel and the concentration will increase over time. The amount of BPA in river was lower than LOD. In addition, industrial effluent can produce BPA pollution by the confirmation of ELISA and UV methods and finally will be drained to river. For avoiding harm to human beings and animals from BPA, it is necessary to pay more attention on monitoring the content of BPA in environmental water and the barreled drinking water. Test strip should be developed in situ detection.

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