Sensitive immunoassay for simultaneous determination of tetrabromobisphenol A bis(2-hydroxyethyl) ether and tetrabromobisphenol A mono(hydroxyethyl) ether: An effective and reliable strategy to estimate the typical tetrabromobisphenol A derivative and byproduct in aquatic environments[☆]

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ARTICLE INFO

Article history:

Received 10 January 2017

Received in revised form

16 May 2017

Accepted 27 May 2017

Available online 14 June 2017

ABSTRACT

Tetrabromobisphenol A bis(2-hydroxyethyl) ether (TBBPA DHEE), as one of the TBBPA derivatives, is frequently applied as a flame retardant. To date, however, little evidence has been gathered regarding its environmental behavior and fate. This is mainly attributed to the lack of an analytical approach available to measure it. Mass spectrometry cannot determine levels of TBBPA DHEE. LC-MS could not ionize TBBPA DHEE to yield enough fragments, while GC-MS could not be applied for the analysis either, due to the thermal degradation of TBBPA DHEE. We here designed a novel hapten, then conjugated it with a carrier protein and produced a type of polyclonal antibody capable of recognizing TBBPA DHEE. Based on this approach, a sensitive and high-throughput indirect competitive enzyme-linked immunosorbent assay (ELISA) was established. The respective detection limit (LOD, based on 90% B/B₀) and IC₅₀ calculated from the standard curve were 0.702 and 9.868 ng/mL for TBBPA DHEE. And the established method showed good tolerance to various matrix interferences. Using our ELISA, we measured the concentrations of these substances from environmental water samples with no pretreatment other than filtration. We found the presence of TBBPA DHEE in environmental water samples collected in Jiangsu province, and the detected concentrations ranging from 1.5 to 7.7 ng/mL. Our proposed method here could be applied to completely and systematically investigate TBBPA DHEE and TBBPA MHEE concentrations in environmental samples.

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1. Introduction

Tetrabromobisphenol A (TBBPA) has been considered one of the most important brominated flame retardants (BFRs) in the world, accounting for 60% of the total global BFRs market (Covaci et al., 2009; Qu et al., 2016a). It has been estimated that approximately 150,000 tons of TBBPA were produced worldwide in 2006, and its production volume increases year by year (Covaci et al., 2011; Qu et al., 2016a). According to data from the Bromine Science

Environmental Forum, approximately 18% of the TBBPA produced was used in manufacturing derivatives (Liu et al., 2017). The extensive use of these chemicals has led to their widespread occurrence in the environment: several derivatives of these substances have been found in water, sewage sludge, soils, and sediments, as well as in the herring gull eggs and marine animals (Letcher and Chu, 2010; Liu et al., 2016a, 2016b; Qu et al., 2016a, 2011, 2013; Shi et al., 2009).

Tetrabromobisphenol A bis(2-hydroxyethyl) ether (TBBPA DHEE), as one of the major TBBPA derivatives, was originally produced by Teijin Chemicals Ltd. (Japan) under the commercial name Fireguard 3600 (Covaci et al., 2011), and has mainly been used as a BFR additive in engineering polymers, epoxy resins, thermoplastic

[☆] This paper has been recommended for acceptance by Prof. von Hippel Frank A.

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polyesters, polyurethane and similar compounds (Bromine Science Environmental Forum, 1995; Covaci et al., 2009; Liu et al., 2015). Some researchers maintain that this chemical probably possesses typical features of persistent organic pollutants, such as environmental persistence and bioaccumulation, because of its high $\log K_{ow}$ values ~ 15 (Jonsson and Horsing, 2009). To date, no information has been reported on the accumulation, transport and environmental impacts of TBBPA DHEE. Recent evidence indicates that TBBPA DHEE exhibited higher neural toxicity when compared with the other TBBPA derivatives (Liu et al., 2016c). Considering TBBPA DHEE is currently being discharged into the environment, significant attention needs to be focused on the occurrence, distribution, and negative effects of this substance on ecosystems. Therefore, reliable analytical methods to investigate the chemical in environmental samples are urgently needed. However, due to the low proton affinity of TBBPA DHEE, ion sources based on ESI or APCI cannot generate enough specific ion clusters for quantitative determination. With derivatization with AgNO_3 , ESI-MS can trigger the TBBPA derivatives formed complexes ($[\text{M}+\text{Ag}]\text{NO}_3$) online, which could be effectively electrosprayed to generate ionic clusters ($[\text{M}+\text{Ag}]^+$) for mass analysis. But Ag^+ is necessary for this approach, and the introduction of large concentrations of AgNO_3 could increase instrument contamination (Tian et al., 2014, 2015).

Up to now, there has been little data reported on this chemical due to the lack of widely available analytical method capable of processing large numbers of samples in a short time. Recently, a method has been established based on ultra-high performance liquid chromatography-Orbitrap Fusion Tribrid mass spectrometer for determination of TBBPA-MHEE (Liu et al., 2015), as the byproducts or degradation products of TBBPA DHEE. This method however requires extremely expensive instruments and sophisticated sample pretreatment. Therefore, novel methods that use simple, low-cost and rapid procedures to measure TBBPA DHEE and TBBPA MHEE in environmental samples are urgently needed. Given that immunoassays are a sensitive, low-cost, simple and reliable method that has frequently been applied in the rapid screening of environmental contaminants, including antibiotics, pesticides, BFRs, and their degradation products in environmental compartments (Ahn et al., 2016; Zhang et al., 2010), a high-throughput enzyme-linked immunosorbent assays (ELISA) would be the optimal approach for TBBPA DHEE and TBBPA MHEE detection.

In this study, the synthesis of a hapten and the production of a polyclonal antibody to simultaneously recognize TBBPA DHEE and TBBPA MHEE is described. Accordingly, a sensitive indirect competitive ELISA (ic-ELISA) was established for determination of TBBPA DHEE and TBBPA MHEE in environmental samples with good sensitivity and high-throughput, and using the developed method, a variety of collected samples were validated. To the best of our knowledge, no similar method has yet been reported. Our ELISA has the potential to provide an alternative high-throughput method for extensive environmental investigation and risk assessment of these pollutants in further studies.

2. Materials and methods

2.1. Chemicals and materials

The standard of TBBPA bis(allyl) ether (TBBPA-DAE), TBBPA bis(2,3-dibromopropyl ether) (TBBPA-DBPE), TBBPA mono (allyl) ether (TBBPA-MAE), TBBPA mono (2,3-dibromopropyl) ether (TBBPA-MBPE), TBBPA-DHEE, TBBPA-MHEE were provided by State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. Bovine serum albumin (BSA), ovalbumin (OVA), N-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethyl

carbodiimide (EDC) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Complete and incomplete Freund's adjuvant, peroxidase-conjugated goat anti-rabbit IgG were purchased from Huamei Biotech Co. Ltd (Beijing, P. R. China). Other solvents and materials were bought from Sinopharm Chemical Reagent Beijing Co. Ltd (Beijing, P. R. China). All organic reagents were of analytical grade unless otherwise specified. Polystyrene microtiter plates were purchased from Costar Inc. (Cambridge, MA, USA), and the Infinite® M1000 PRO microplate reader was supplied by TECAN Inc. (Durham, NC, USA). The ^1H Nuclear Magnetic Resonance (NMR) Spectrometer was an Avance III 400 MHz instrument (Bruker, Switzerland) with CDCl_3 solution. Fourier transform infrared spectrometry was performed on a Nicolet 6700 FT-IR Spectrometer (Thermo, USA).

2.2. Buffers

Phosphate-buffered saline (PBS, 0.01M, pH7.4) (chemicals containing 8 g NaCl, 0.2 g KCl and 3.58 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ were dissolved in 1 L of water) and coating buffer (CB, 0.05M, pH9.6) (1.59 g Na_2CO_3 and 2.93 g NaHCO_3 were added to 1 L of water) were used. Blocking buffer (CB containing 1% gelatin), washing buffer (0.05% Tween-20 was diluted in PBS), and antibody dilution buffer (PBS with 0.01% tween-20 and 0.1% gelatin) were also used.

2.3. Hapten synthesis

1.08 g TBBPA and 0.828 g K_2CO_3 were dissolved in 10 mL dimethylformamide (DMF), then 0.306 g methyl bromoacetate was added dropwise to the mixture under stirring conditions at 90 °C. After 12 h reaction, 0.25 g 2-bromoethanol was added dropwise and stirred for 4 h. The solution was then washed with water (20 mL) and extracted with ethyl acetate (3×20 mL); the organic layer was dried over anhydrous Na_2SO_4 and concentrated, and then combined with 0.12 g NaOH into a mixed solution of MeOH- H_2O (20 mL, v/v = 1/1). After stirring for 2 h, the mixture was adjusted to pH < 7 using HCl, then extracted with dichloromethane (DCM, 3×20 mL); the combined extract was dried over anhydrous Na_2SO_4 , and enriched by flash silica column chromatography (petroleum ether/ethyl acetate, 10/1) to give **M3** (17%, 0.18 g): ^1H NMR (CDCl_3 , 400 MHz) δ (ppm): 1.62 (s, 6H), 3.76 (t, $J = 5.6$ Hz, 2H), 3.96 (t, $J = 5.6$ Hz, 2H), 4.49 (s, 2H), 4.91 (s, 1H), 7.51 (d, $J = 8$ Hz, 4H), 13.10 (s, 1H). ESI-MS (negative mode, m/z): calculated for $\text{C}_{19}\text{H}_{18}\text{Br}_4\text{O}_5$: 645.96; found: 644.88 ($[\text{M}-\text{H}]^+$). The synthesis procedures for hapten M3 are shown in Fig. 1.

2.4. Preparation of immunogen and coating antigen

The immunogens were prepared by coupling the hapten (M3) to BSA with the activated ester method, as previously reported (Adrian et al., 2009). In brief, 45 mg M3, 40 mg N-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC) were dissolved in 2 mL anhydrous (DMF) and reacted for 8 h at 4 °C under magnetically stirred conditions; the final supernate was then added into 1 mL BSA solution (17 mg/mL, carbonate buffer, pH 9.6), followed by continuous stirring overnight at 4 °C. The immunogens (BSA conjugates) were purified using dialysis solution (0.01M PBS, pH 7.4) for 3 days. In addition, the coating antigen was obtained using similar methods and only the conjugated protein was replaced with OVA. The value of hapten to protein conjugates was identified with MOLDI-TOF/MS by comparing the observed molecular weights of the prepared conjugates with the unreacted protein (Wang et al., 2015).

Fig. 1. Synthetic routes of hapten (M3).

2.5. Production of polyclonal antibodies

The immunization procedure for rabbit polyclonal antibody (pAB) was performed using the traditional method (Cui et al., 2011). Five female New Zealand white rabbits were immunized with the immunogens (M3-BSA), and antiserum was collected and separated by centrifugation after 5-month immunization, which could be used in the immunoassay without purification.

2.6. Immunoassay procedures

The polystyrene microtiter plates were coated with 100 μL /well coating antigen (M3-OVA, diluted in coating buffer) overnight at 4 $^{\circ}\text{C}$. The strips were washed 3 times with washing buffer, blocked with blocking buffer, then incubated for 2 h at 37 $^{\circ}\text{C}$. Antibody (50 μL /well, dissolved in assay buffer) was added to the solution, followed by TBBPA DHEE standards or targets. The plates were firstly incubated for 1.5 h at 37 $^{\circ}\text{C}$. After a washing step to remove the unbound compounds, Goat anti-rabbit IgG-HRP (1/2500, diluted in PBS) was added to 100 μL /well and incubated at 37 $^{\circ}\text{C}$ for 1 h. Following another washing step, the substrate solution TMB (100 μL /well) was applied to the strips, then stopped with H_2SO_4 (2 mol/L, 100 μL /well) after adequate enzymatic reaction. Absorbance values were measured at 450 nm with an ELISA plate reader.

2.7. Standard curves of immunoassay

The dose-response curves were obtained by plotting signal values against the log target concentrations and mathematically fitted to a four-parameter logistic equation with Origin 8.0 (Microcal, Studio City, CA):

$$Y = \frac{(A - D)}{1 + (X/C)^E} + D$$

Where A is the asymptotic maximum (OD values in the absence of targets, B_0), D is the asymptotic minimum (background signal), E is the slope factor at the inflection point, C is the concentration at the inflection point (corresponding to 50% specific binding of TBBPA DHEE, IC_{50}), and X is the calibration concentration.

2.8. Immunoassay optimization

In order to improve the sensitivity of the method, experimental parameters that could influence the performance of immunoassays

were evaluated in detail, including concentrations of coating antigen, antibody and tracer (goat anti-rabbit IgG-HRP conjugate), and necessary factors like organic solvents (methanol), ionic strength and pH values. B_0/IC_{50} was selected as important criteria to evaluate the performance of the immunoassays (Brun et al., 2005; Korpimäki et al., 2004). In addition, IC_{50} and B_0 also were regarded as primary physicochemical factors to estimate the performance of the methods (Li et al., 2014).

2.9. Selectivity and accuracy of ELISA method

The selectivity of the method was determined by cross-reactivity (CR), comparing the respective IC_{50} values against a group of structure analogs that could be calculated using the equation of $\text{CR}\% = [(\text{IC}_{50} \text{ value of TBBPA DHEE})/(\text{IC}_{50} \text{ value of competitors})] \times 100$.

The accuracy of the ELISA method developed was estimated using a recovery test performed with samples from different sources fortified with a variety of target concentrations (within the dynamic range). The recovery was estimated as follows:

$$\text{Recovery (\%)} = \text{Cd} / \text{Cs} \times 100$$

Where Cd and Cs were the detected concentration in spiked samples and spiked concentration, respectively.

In the present study, concentrations of 3, 12, 24 ng/mL TBBPA DHEE were spiked in environmental water samples; each sample was determined in triplicate.

2.10. Sample preparation

Samples of pond water, tap water, lake water, river water and paddy water were collected in Zhenjiang, Jiangsu province. Distilled water and tap water were taken from our lab. Real water samples (S1–S12) were collected from a river in Suzhou, Jiangsu province. Others were obtained from Liuyang River in Liuyang, Hunan, China. All water samples were filtered to remove particles larger than 0.22 μm .

3. Results and discussion

3.1. Preparation of hapten M3, its conjugates and antibody

TBBPA DHEE, with a molecular weight of 631.98, belongs to the

small molecule category and can not be used as an immunogen to elicit immunogenicity through direct injection of animals. The most common strategy is to conjugate the chemical with carrier proteins (e.g., bovine serum albumin, BSA) (Bu et al., 2014; Xu et al., 2012). Because there is no functional group (carboxy-, amino-, hydro-sulfuryl-) in its chemical structure to couple proteins, TBBPA DHEE has to be modified with a suitable reactive group. Theoretically, it is ideal to convert the hydroxyl group to an ether and subsequently convert it into a carboxylic acid. However, the failure of this approach indicated that attempting to obtain an acid through direct molecular structure adjustment did not work. An alternative method to generate suitable hapten is shown in Fig. 1; in the reaction, TBBPA was used as starting materials. Hapten M3 was identified by HPLC–MS/MS and HNMR (Fig. S1 supporting material). M3 has a reactive group (carboxyl-) that is used to form a covalent linkage with BSA, which resulted in the similarity on the other parts of its molecular structure with TBBPA DHEE and TBBPA MHEE (Fig. S2), therefore, M3 can mimic the structure of these target compounds to a large extent. In fact, the farthest parts of the M3 molecular structure coupling site (hapten and the carrier protein) are the same as TBBPA DHEE and TBBPA MHEE. According to the rule in immunology (Bucknall et al., 2003; Watanabe et al., 2002; Zhang et al., 2013), antibodies elicited from haptenic conjugates showed a preferential recognition to the molecule part that was farthest from coupling site. In this study, TBBPA DHEE and TBBPA MHEE (the structures of both chemicals are shown in Fig. S2) were probably simultaneously recognized by the potential antibody that may be produced using the hapten.

The hapten M3 was coupled to BSA and OVA using the activated ester method to produce immunogen and coating antigen, respectively (Wang et al., 2007). According to the UV–Vis spectra in Fig. S3, the characteristic absorption peaks of BSA–M3 (277 nm) were quite different when compared with those of BSA (280 nm), revealing that the hapten was successfully conjugated to the corresponding carrier protein. The displacement of the absorption peaks of OVA–M3 (276 nm) and OVA (280 nm) also showed similar results (Fig. S3). Our results measured using MALDI–TOF/MS indicated that immunogen and coating antigen were synthesized as expected, and the molar ratio of hapten to protein was 9:1. When comparing the observed molecular weights of BSA–M3 with the BSA (Fig. S4), the value fell within the range of 8:1 to 25:1, which was the best range for antibody production (Wang et al., 2015). So our synthesized immunogen would lead to suitable antibodies as anticipated in our original strategy.

The parameters of the antisera obtained from the rabbits are shown in Table S1. In general, five antisera were collected after immunizing six New Zealand white rabbits (one rabbit died) using the synthesized immunogens (BSA–M3); rabbit R3–4 had higher titers than the others with antisera dilution of 1/25,000 but worse sensitivity (IC_{50} value, 95 ng/mL). However, the antisera from the rabbit R3–2 showed the best affinity to TBBPA DHEE (IC_{50} value, 31 ng/mL) when the antisera were diluted to 12,000 times. The above-noted observations indicated that there was no certain relationship between antibody sensitivity and titer (Wang et al., 2015). Due to better sensitivity, the antisera from the rabbit R3–2 was used in the subsequent study.

The cross-reactivity of the antisera was estimated using ci-ELISA with a series of structure-related chemicals. As shown in Table 1, the pAB could simultaneously recognize TBBPA DHEE and TBBPA MHEE, manifesting low cross-reactivity to the other selected compounds. Meanwhile, although a linker arm of 3–5 carbon atoms was generally considered an optimal distance between hapten and the carrier protein (Vasylijeva et al., 2015), our hapten design showed that linker arms of less than 3 also met the requirements for generating the antibodies.

Table 1

Cross-reactivity of antiserum to TBBPA–DHEE analogs.

Chemicals	Structure	Cross-reactivity (%)
TBBPA–DHEE		100
TBBPA–MHEE		98.7
TBBPA		<0.1
TBBPA–DAE		<0.1
TBBPA–DBPE		<0.1
TBBPA–MAE		<0.1
TBBPA–MBPE		<0.1
DBDPE		<0.05
BTBPE		<0.05
TBB		<0.05
BPA		<0.05
BDE-28		<0.05
BDE-47		<0.05
BDE-99		<0.05
BDE-100		<0.05
BDE-153		<0.05

3.2. Optimization of ELISA conditions

In order to improve ELISA performance, several parameters were optimized in detail, including concentrations of the coating antigen, antibody and tracer, reaction time, ionic strength (Na^+), buffers and organic solvents. B_0/IC_{50} was considered a major criterion to estimate immunoassay performance. In addition, other important factors, including IC_{50} (50% B/B_0), B_0 and the limit of detection (LOD, 90% B/B_0), were also taken into account (Liang et al., 2007; Zhang et al., 2010).

The concentrations of coating antigen and antibody were the key parameters that influenced the sensitivity of the assay. When antisera and antigen were diluted 12,000 and 5000 times, respectively, the highest B_0/IC_{50} and the lowest IC_{50} were observed. In addition, considering Na^+ could strongly affect the interaction of the antibody and hydrophobic target (De Meulenaer et al., 2002), the concentration of Na^+ was also investigated. In our study, the best analytical performance was obtained when the concentration of Na^+ in the assay system was 0.2 mol/L. Other optimum ELISA parameters were as obtained follows: tracer dilution (1/5000); reaction time (30 min), antibody dilution buffer (PBS with 0.01% tween-20 and 0.1% gelatin).

Since TBBPA-DHEE belongs to a nonpolar chemical, methanol is useful to keep the hydrophobic targets in immunoassay solution and prevent nonspecific binding on the container walls (Vasylieva et al., 2015). In order to improve the performance of the ELISA method, methanol was added to the assay system. We tried different concentrations of methanol dissolved in assay buffer to evaluate the effect on ELISA performance. As shown in Fig. 2, IC_{50} distinctly decreased from 31 to 18 ng/mL when the amount of methanol increased from 0% to 20% (content of methanol, v/v); however, B_0/IC_{50} was the best when the buffer contained 30% methanol, although the IC_{50} (19 ng/mL) at that concentration was a little worse than 20% methanol (IC_{50} , 18 ng/mL). Therefore, the 30% methanol was considered the optimal concentration of the assay buffer. This could be explained by the findings from the previous

Fig. 2. Optimization of methanol (A) and Na^+ (B) in the assay system. At the competition step, antibody was prepared in an antibody dilution buffer with a serial dilution of methanol (A) or diluted with different Na^+ (sodium chloride) (B). Each point represents the average of three replicates.

Fig. 3. Indirect competitive ELISA standard curves for TBBPA-DHEE under optimized conditions. Each point represents the average values $\pm$ standard deviation for three replicates.

investigation (Liang et al., 2007): at the competition step methanol enhanced the polarity of the medium, improved antibody-analyte interaction and increased the conformational changes at the step of antibody capturing targets. In addition, side effects (higher IC_{50} , lower B_0/IC_{50}) were found with the addition of methanol concentrations exceeding 30% in the detecting system, which caused antibody denaturation due to excess organic solvent. Similar trends were also observed in acetonitrile and acetone estimation. All factors (B_0/IC_{50} , and LOD) considered, 30% methanol in the assay buffer offered the greatest improvements in ELISA performance.

Under the above-optimized conditions, the standard curves were established (Fig. 3); the LOD (calculated, based on 90% B/B_0 (Adrian et al., 2009)) and IC_{50} of this method were 0.702 and 9.868 ng/mL, respectively. With the help of enhancement through sample pretreatment, the ELISA we developed could be used for detection of trace TBBPA-DHEE in a variety of environmental samples.

3.3. Matrix and assay conditions effects

Because environmental samples are complex, containing numerous matrix interferences and vary in terms of assay conditions, such as organic solvents (acetonitrile and acetone), salinity (K^+ and Ca^{2+}) and pH value. These factors can influence the specific interaction between antibody and antigen, and as a consequence, affect the sensitivity and accuracy of the method. So matrix and assay condition effects were also evaluated in this study. The ELISA system showed relatively weak tolerance to samples containing acetonitrile and acetone (Fig. 4). This was different from our previously reported time-resolved fluoroimmunoassay (Zhang et al., 2010), where both of the organic solvents in our samples exceeded 15% (v/v), non-specific reaction happened, which can probably be attributed to the denaturation of antibodies by acetonitrile and acetone. As for K^+ effects, similar effects to Na^+ (seen in Fig. 2) were found after adding K^+ (potassium chloride) to the samples. The results indicated that the presence of K^+ could improve the analytical performance; when the concentration of K^+ in the samples reached 0.1 mol/L, better sensitivity was acquired. Furthermore, the system could endure as much as 0.5 mol/L K^+ (displayed in Fig. 4), which was higher than environmental levels. Compared to K^+ in the assay (as shown in

Fig. 4. Effects of acetonitrile (A) and acetone (B), K^+ (C), Ca^{2+} (D) and pH (E). At the ELISA competition step, a series of TBBPA-DHEE standards were prepared in distilled water with different concentrations of acetonitrile (A) and acetone (B), K^+ (C), Ca^{2+} (D) or varied pH (E). Each point represents the average of three replicates.

Fig. 4), the ELISA system can not tolerate more than 0.1 mol/L Ca^{2+} , although the lower IC_{50} and higher B_0/IC_{50} were observed with Ca^{2+} levels ranging from 0–0.1 mol/L in the samples. Excessive Ca^{2+} caused the intra-assay coefficient of variation to become too high in the standard curves leading to an inaccurate determination (data not be shown). However, due to the concentration of Ca^{2+} below 0.1 mol/L for water samples at the environmental level, the side effects to the assay system can be neglected.

3.4. Method validation and real samples analysis

The accuracy and precision of the proposed methods were assessed using a spike-recovery analysis using water samples from various sources fortified with a variety of TBBPA-DHEE concentrations (distilled water, pond water, tap water, lake water, river water, and paddy water). The target chemicals in the water samples can be directly detected after filtration (0.22 μ m) with no other form of

pretreatment. Generally, the intra-assay coefficient of variation (CV) values were within 0.2–6.5%, and the recoveries were acceptable. The recoveries from five water samples (distilled water, pond water, lake water, river water, paddy water) ranged from 83–121% (Table 2), which was satisfactory and indicated that the method was suitable for measuring these types of samples. In contrast, the recovery from tap water was between 119% and 133% (Table 2), which could be explained by the presence of chlorine in tap water: the recoveries in all three tap water samples spiked samples more than 100%. Even so, the measurement of the tap water samples was reliable enough to be used for real sample analysis.

The proposed ELISA was applied to investigate potential occurrences of TBBPA DHEE and TBBPA MHEE in environmental samples. Eventually, these contaminants were detected in river water samples collected in Suzhou, a well-known city in eastern China. After analysis of 12 water samples that were collected along the river at 10 m intervals, as shown in Table 3, TBBPA DHEE was positively

Table 2
Analysis of six TBBPA DHEE – spiked environmental water samples using ELISA.

Sample	Added (ng/mL)	Found (ng/mL)	Recovery (%)	CV ^a (%)
Distilled water	3.0	2.50	83	5.3
	12.0	11.3	94	6.4
	24	28.4	119	1.9
River water	3.0	2.7	90	4.6
	12.0	10.1	84	1.7
	24	25.2	105	0.2
Lake water	3.0	2.61	87	2.2
	12.0	13.1	109	0.8
	24	23.0	96	1.2
Pond water	3.0	2.8	93	6.5
	12.0	13.3	111	3.4
	24	29.0	121	1.2
Paddy water	3.0	2.5	82	4.7
	12.0	12.2	102	0.4
	24	27.6	115	0.7
Tap water	3.0	3.57	119	3.9
	12.0	15.9	133	2.1
	24	29.8	124	1.3

^a CV, intra-assay coefficient of variation obtained from six determinations performed in the same polystyrene microtiter plate.

Table 3
Real environmental water analysis using the established ELISA.

Sample NO.	Concentration of detected compound (ng/mL), n = 3	Sample NO.	Concentration of detected compound (ng/mL), n = 3
S1	<LOD	S16	<LOD
S2	<LOD	S17	<LOD
S3	<LOD	S18	<LOD
S4	7.7 ± 0.03	S19	<LOD
S5	7.2 ± 0.03	S20	<LOD
S6	2.6 ± 0.05	S21	<LOD
S7	1.7 ± 0.03	S22	<LOD
S8	1.5 ± 0.02	S23	<LOD
S9	<LOD	S24	<LOD
S10	<LOD	S25	<LOD
S11	<LOD	S26	<LOD
S12	<LOD	S27	<LOD
S13	<LOD	S28	<LOD
S14	<LOD	S29	<LOD
S15	<LOD	S30	<LOD

detected in five samples taken at locations near the factory, with concentrations ranging from 1.5–7.7 ng/mL. To judge the concentrations of TBBPA MHEE in the 5 positive water samples, they were measured using the HPLC-MS/MS method we reported previously (Liu et al., 2015), and the results indicated that the contamination level of TBBPA MHEE in these samples was lower than the LOD (0.0009 ng/mL). Water samples (marked from S13–S30, Table 3) collected from Liuyang River, Hunan, the chemicals were not found. These results suggested that TBBPA derivatives could be detected in a wide range of environmental compartments.

Since TBBPA DHEE can not be analyzed using widely applied mass spectrometry methods such as LC-MS or GC-MS due to ionization or thermal degradation of the retardant, the method developed here is a powerful tool for surveying the levels of TBBPA DHEE in environmental samples. The ELISA method

Table 4
LOD of TBBPA DHEE or TBBPA MHEE using ELISA or ESI¹⁰.

	ELISA	ESI with Ag derivization	ESI
TBBPA-DHEE	0.7 ng/mL	0.49 ng/mL	–
TBBPA-MHEE	0.7 ng/mL	0.33 ng/mL	0.0009 ng/mL

developed here responded to the presence of both TBBPA DHEE and TBBPA MHEE. Considering the extremely low or possible negligible levels of MHEE in comparison with TBBPA DHEE, this method could be widely applied for high-throughput and rapid analysis of TBBPA DHEE. In the context that the concentration of TBBPA MHEE is warranted determination, with the support of LC-MS/MS, which has proven to be a highly sensitive method for TBBPA MHEE detection (Table 4), both TBBPA DHEE and TBBPA MHEE could be analyzed in environmental samples using this technique.

In summary, our study is the first to develop a sensitive analytical method for determination of TBBPA DHEE and TBBPA MHEE in environmental samples based on the generation of antibodies using a synthesized novel hapten. The method we developed showed (i) high throughput; (ii) low detection limits (IC₅₀, 9.868 ng/mL; LOD, 0.702 ng/mL), which could be applied for the determination of a lower concentration of the contaminants from various samples after enhancement through sample pretreatment; (iii) good accuracy (recoveries ranged in 83–133%), which was acceptable for measurement of environmental samples; and (iv) low cost, good tolerance to potential environmental matrix interferences and no sample pretreatment other than filtration for waters. This method has been validated in real environmental samples, and we found the occurrence of TBBPA DHEE in environmental waters.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (Grants 21577051 and 31502118), the Natural Science Foundation of Jiangsu Province (Grant BK20140543) and the Jiangsu Collaborative Innovation Center for Technology and Water Treatment. We would also like to thank Professor Zhan Hui Wang at China Agricultural University for evaluating the hapten to protein conjugate values using MALDI-TOF/MS.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.envpol.2017.05.076>.

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