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Biotin–streptavidin enzyme-linked immunosorbent assay for detecting Tetrabromobisphenol A in electronic waste

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ABSTRACT

Tetrabromobisphenol A (TBBPA) is a widely used brominated flame retardant. A sensitive and selective indirect competitive biotin–streptavidin-amplified enzyme-linked immunosorbent assay (BA-ELISA) was developed for detecting TBBPA. The optimal hapten of TBBPA was 2-(2,6-dibromo-4-(2-(3,5-dibromo-4-hydroxyphenly)propan-2-yl)) acetic acid. Several physiochemical factors that influence assay performance, such as optimal coupling concentration of immunogen and antibody, organic solvent, ionic strength, and pH, were studied and optimized. The limit of detection (IC_{10}) was 0.027 ng/mL and the median inhibitory concentration (IC_{50}) was 0.58 ng/mL. The BA-ELISA was highly selective, with low cross-reactivity with TBBPA analogs. Finally, the assay was used to detect TBBPA in electronic waste samples. The results are consistent with those using liquid chromatography, which proves that the proposed immunoassay is accurate and receptive. This BA-ELISA method is suitable for the rapid and sensitive screening of TBBPA in environmental monitoring.

1. Introduction

Tetrabromobisphenol A (TBBPA) is the most widely used brominated flame retardant (BFR), and it mixes with or covalently bonds to host materials. As an additive or reactive flame retardant, TBBPA is commonly used to produce brominated epoxy resins, polycarbonate resins, phenolic resins, unsaturated polyester, high-impact polystyrene, acrylonitrile–butadiene–styrene, and so on. The global consumption of TBBPA has increased from 64,000 t in 1994 to 119,700 t in 2001 [1]. The TBBPA production capacity of China was approximately 18,000 t in 2007 [2]. The worldwide use of TBBPA has transferred this compound from different processes and sources into the environment. Trace concentrations of TBBPA have been detected both in abiotic and biotic media, including indoor air (stationary air samples [3]: <3–180 ng/m^3; dust samples [4]: 2300–2900 pg/d), water [5–7] (wastewater [6]: 0.013–0.031 ng/mL), soil [8] (industrial soils: 3.4–32.2 ng/g *dw*; agricultural soil: 0.3 ng/g *dw*), human tissue (serum [9]: <1–3.4 pmol/g *lw*; breast milk [10]: 7000 ng/kg *lw*), food (Chinese total diet [11]: $<$LOD–2044 pg/g; eggs [12]: <0.1–940 ng/g *lw*) and wild animals (birds of prey [13]: 13 pg/g; dolphin fat [14]: 0.1–418 μg/kg *lw*).

TBBPA is listed in the convention for the protection of the marine environment of the North-East Atlantic as a hazardous substance. TBBPA is classified as an endocrine-disrupting chemical [15] because its molecular structure is similar to that of thyroxine. Other studies have shown that TBBPA is an immunotoxic and neurotoxic compound [16,17]. TBBPA is dehalogenated under anaerobic and aerobic conditions to yield bisphenol A (BPA).

Gas and liquid chromatography techniques have been used to detect TBBPA and its derivatives in different environmental samples [18–22]. However, these instrumental analytical methods are generally expensive, time-consuming, labor-intensive, and require complex pretreatment procedures, which restrict their widespread use for the rapid detection of TBBPA in environmental samples. Compared with instrumental analytical methods, enzyme linked immunosorbent assay (ELISA) is well suited for detecting trace pollutants in the environment because of its high specificity, sensitivity, and throughput. In the literature, ELISA methods have been used to measure polybrominated diphenyl ethers [23–27] and other brominated flame retardants.

This study aims to develop a modified indirect competitive ELISA for TBBPA using a biotin–streptavidin amplification system. Diverse TBBPA haptens, immunogens, and relative polyclonal antibodies were prepared. The established immunoassay procedures were optimized, and then their accuracy and sensitivity were compared with those of the liquid chromatography.

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The biotin–streptavidin-amplified ELISA (BA-ELISA) selectively and sensitively detected TBBPA in various environmental samples and it will be helpful in environmental studies.

2. Materials and method

2.1. Reagents and apparatus

The TBBPA standard and organic materials for hapten synthesis were purchased from J&K Chemical (Beijing, China). Hapten was purified through column chromatography using silica gel (40 μm average particle size) from Shanghai Sanpont Co. Ltd. (China). Bovine serum albumin (BSA), egg albumin (OVA), biotinylated *N*-hydroxysuccinimide ester (BNHS), *N*,*N*-dimethylformamide (DMF), *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC), dimethyl sulfoxide (DMSO), hydrogen peroxide, Coomassie Brilliant Blue G250, Tween 20, complete and incomplete Freund's adjuvant, and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sinopharm, China. Streptavidin–horseradish peroxidase (SA–HRP) was purchased from Sangon Biotech (Shanghai, China). All reagents were of analytical grade unless specified otherwise.

The ^{1}H 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 instrument (Thermo, USA). The Multiskan MK3 ELISA reader (Thermo, USA) used to determine absorbance in dual wavelength mode (450 nm/650 nm), with polystyrene 96-well microplates, was purchased from Sangon Biotech. TBBPA–protein conjugate was characterized on a UV-2012 PC spectrophotometer (UNICO, USA). Ultrapure water used was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).

HPLC analysis was performed on an Agilent LC1100 HPLC system equipped with an Agilent Eclipse Plus C18 (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of methanol and water in the ratio 80:20, v/v at a flow rate of 0.3 mL/min. The injection volume was 20 μL and detection was at 280 nm.

2.2. Buffers and solutions

Phosphate-buffered saline (PBS: NaCl 137 mmol/L, KCl 2.7 mmol/L, Na_2HPO_4 10 mmol/L, KH_2PO_4 2 mmol/L), carbonate buffer solution (CBS: 15 mmol/L Na_2CO_3, 34.9 mmol/L $NaHCO_3$), PBST (PBS with 0.05% Tween 20), phosphate–citrate buffer (0.1 mol/L citric acid, 0.2 mol/LNa_2HPO_4; pH = 4.3), and TMB substrate solution (0.4 mL, 2.5 g/L TMB ethanol solution, 10 mL phosphate–citrate buffer, 10 μL 30% H_2O_2) were used.

2.3. Synthesis of TBBPA hapten

TBBPA molecules do not contain functional groups that can connect with proteins directly. Therefore, TBBPA hapten must be synthesized first. The synthesis reactions are illustrated in Fig. 1. The results of the hapten synthesis and characterization are given below.

Bromoethanoic acid (0.5109 g, 3.677 mmol) dissolved in 5 mL of DMF was added dropwise to a mixture of TBBPA (2 g, 3.677 mmol) and NaOH (0.294 g, 7.354 mmol) in 10 mL of DMF with stirring. The mixture was allowed to react isothermally for 6 h at 80 °C, and the mixture was cooled to ambient temperature. Then, 30 mL of water was added to the reaction mixture and acidified to pH 3 using a HCl solution. The precipitates were obtained and extracted with ethyl acetate (20 mL × 3). The organic phase was washed with water and dried with anhydrous sodium sulfate. After evaporation under vacuum, the light yellow residue was purified via silica column chromatography (*n*-hexane:acetone = 4:1) and identified as 2-(2,6-dibromo-4-(2-(3,5-dibromo-4-hydroxyphenly)propan-2-yl)) acetic acid, $C_{17}H_{14}Br_4O_4$, m.w.: 601.92, yield: 71.5%, and m.p.:167–170 °C.

IR (KBr) ν (cm^{-1}): 3475.69 (O–H stretching vibration), 1733.86 (C–O stretching vibration), 1577.22 and 1472.99 (C–C framework vibration), 875.55, 778.72, and 734.95 (C–H flexural vibrations), and 621.08 (C–Br stretching vibration). ^{1}H NMR ($CDCl_3$) δ (ppm): 11.5 (1H, COOH), 7.31 (2H Aromatic H), 4.19 (2H–OCH_2COOH), 2.50 (1H, –OH), and 1.60 (6H, $2CH_3$).

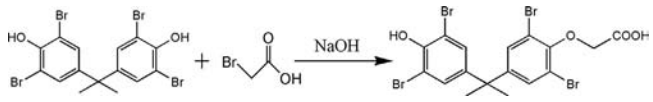


Fig. 1. Synthesis route of TBBPA hapten.

2.4. Preparation of immunogen and coating antigen

A contact portion between hapten and carrier protein is called linking arm [28], which could become an antigenic determinant and specifically conjugate with antibody. In order to reduce the specific binding caused by linking arm, different coupling methods are often used for preparing immunogen and coating antigen. So in this study, the activated ester method and the mixed anhydride method were employed to couple hapten to the carrier proteins using the terminal carboxyl group on a TBBPA hapten molecule. The details were as follows: for the activated ester method, equimolar TBBPA hapten, NHS and DCC were dissolved in 1 mL DMF and magnetically stirred for 8 h at 4 °C. The obtained supernate was added dropwise into BSA solution (12 mg/mL, in PBS) and then stirred at 4 °C overnight. After complete reaction, the suspension was dialyzed against PBS (0.01 M, pH 7.4) for 3 d. For the mixed anhydride method, 300 mg (0.5 mmol) of TBBPA hapten was dissolved in 1 mL DMF. Equimolar isobutylamine and *n*-butyl chloroformate ester were sequentially added and magnetically stirred for 1 h at 4 °C. After reaction, 12 mg/mL of OVA suspension was added dropwise and was allowed to react for 5 h at 4 °C. After complete reaction, the suspension was dialyzed against PBS for 3 d. The prepared hapten–protein conjugates BSA–TBBPA and OVA–TBBPA were used as the immunogen and the coating antigen, respectively.

All protein conjugates were identified using a UV–vis spectrophotometer and the coupling ratios were estimated based on mole absorbance ε and calculated using the following equation [29,30]:

$$\text{coupling ratio} = \frac{\varepsilon_{\text{conjugate}} - \varepsilon_{\text{protein}}}{\varepsilon_{\text{hapten}}} = \frac{(\text{OD}_{\text{conjugate}} - \text{OD}_{\text{protein}})\, C_{\text{hapten}} M_{\text{protein}}}{\text{OD}_{\text{hapten}}\, M_{\text{hapten}}\, C_{\text{protein}}} \quad (1)$$

2.5. Preparation of biotinylated antibody

Rabbit polyclonal anti-TBBPA antibodies (pAb-TBBPA) were prepared as described in previous reports of our research team [31]. After immunization with TBBPA–BSA for 4 months, the polyclonal antibodies were separated and purified from rabbit serum. Biotinylated TBBPA antibodies (Bi-pAb-TBBPA) were prepared as follows: 5.0 mg of purified pAb-TBBPA was dissolved in 0.1 mol/L sodium carbonate buffer (pH 9.6) at concentrations of 1.0–2.0 mg/mL. The antibody solution was mixed with 1.0 mg/mL BNHS in DMSO in the mass ratio of 1:10. The mixture was stirred for 4 h and then dialyzed against PBS for 3 d. The as-obtained biotinylated antibodies were stored at 4 °C before use.

2.6. Heterologous indirect competitive BA-ELISA

Indirect BA-ELISA determinations were performed in 96-well microplates. The microplates were coated overnight with 100 μL/well of the coating solution at 4 °C. After washing (three times), the unbound active sites were blocked with 200 μL/well of blocking reagent, and were incubated at 37 °C for 60 min. After another washing step, the standard or the sample (50 μL/well) and the biotinylated antibodies (50 μL /well) were added and the mixture was incubated for 90 min at 37 °C. After an additional washing step, 100 μL/well SA–HRP conjugate (dilution 1:1000) was added and incubated for 60 min. After a final washing step, 100 μL/well of the substrate solution TMB was added. After sufficient color development (15 min), the enzymatic reaction was stopped by adding 50 μL/well of 2 mol/L H_2SO_4. The absorbance of each well was observed in dual-wavelength mode, with 450 nm as the test and 650 nm as the reference.

2.7. Immunoassay optimization

Several parameters were optimized to develop a sensitive immunoassay method. First, the concentrations of Bi-pAb-TBBPA and the coating antigen were determined using a checkerboard assay. In the immunoassay, the optimal blocking solution should exhibit the lowest background value. Different blocking solutions, such as gelatin, OVA, milk powder, $PEG_{20,000}$, and poly(vinyl alcohol) (PVA), were dissolved in PBS buffer and their background values were compared. The effects of different ionic strengths, pH, matrix effects, and competitive curves were determined from the TBBPA standards and the relative antibody titers in PBS buffer. All determinations were performed in triplicate and the mean absorbance values were calculated. Under optimal conditions, the calibration curve of BA-ELISA is determined by plotting inhibition (%), calculated as follows, against the logarithm of the standard concentration:

$$inhibition(\%) = \frac{(A_{\max} - A_{\min}) - (A_s - A_{\min})}{A_{max} - A_{\min}} \times 100\% \quad (2)$$

where $A_{\max}$ is the absorbance in the absence of TBBPA, $A_{\min}$ is the absorbance of the blank sample, and A_s is the absorbance of TBBPA at the standard concentration.

The IC_{50}, the concentration at which a compound inhibits a particular phenomenon by 50%, is used to evaluate the sensitivity of the method. Analogously, the limit of detection (LOD) is evaluated in terms of IC_{10}.

2.8. Cross-reaction

The specificity of the optimized assay was determined by measuring cross-reactivity (CR) using a group of structural analogs. The CR values were calculated as follows:

$$CR(\%) = \frac{IC_{50}\ of\ TBBPA}{IC_{50}\ of\ ana\log\ s} \times 100\% \quad (3)$$

2.9. Sample preparation

TBBPA is widely used as a brominated flame retardant in electronic products, especially in plastic assemblies. Consequently, some electronic waste samples, such as computers, cell phones, and printer, were collected from local electronic waste recycling market in Shanghai. The plastic portion was disassembled for determination. Before analysis, the samples were broken down into small sizes (5 mm × 5 mm). The detailed procedures are as follows [8,32]: the samples (0.5 g) were ground into powder after freezing in liquid nitrogen. The powdered samples were added into 25 mL test tubes containing 20 mL of dichloromethane and ultrasonicated for 1 h. The extract liquor was removed and the residue was washed with methanol. Under a gentle nitrogen stream, the organic phase was evaporated to near dryness and the residue was redissolved in 5 mL of methanol. After filtering, the filtrate was subjected to immunoassay and HPLC analyses. Prior to the immunoassay, the extracts of the solid plastic particulates were diluted at least 20-fold in PBS with Tween 20 (0.05% Tween 20).

2.10. HPLC analysis

The BA-ELISA detection results were verified. The TBBPA concentrations in different samples were detected using HPLC (Agilent LC1100) with a high pressure pump, a column thermostat (Model 201TP5415), a variable wavelength ultraviolet detector, and a vacuum de-air machine. The temperature of the C18 chromatographic column (250 mm × 4.6 mm, 5 μm) was maintained at 35 °C. The mobile phase consisted of methanol and water in the ratio 80:20, v/v. The sample solution of 20 μL was injected at a flow rate of 0.3 mL/min. The detection wavelength was 280 nm.

3. Results and discussion

3.1. Characterization of immunogen, coating antigen, and antibody

To develop a sensitive and specific immunoassay, TBBPA molecules must be derived to be coupled with the protein. The phenolic group of TBBPA was the most available for coupling and its acidity would facilitate the generation of the corresponding alkoxide with sodium hydroxide. The usual strategy would be to convert the phenolic group into an ether and subsequently convert it into a carboxyl group. The carboxyl group was then coupled with proteins to synthesize BSA–TBBPA and OVA–TBBPA for use as the immunogen and the coating antigen, respectively. The prepared hapten, protein, and conjugates were characterized using a UV–visible spectrophotometer, and the results are shown in Fig. 2. Meanwhile, the coupling ratio was calculated using Eq. (1).

As shown from Fig. 2, several characteristic absorption peaks of TBBPA hapten and proteins appeared at 287 nm (for hapten), 227

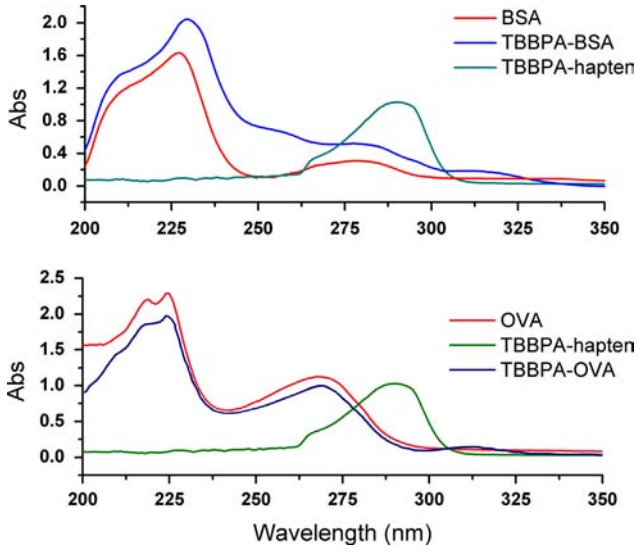


Fig. 2. The UV spectra of hapten, protein and conjugate; absorbance value at characteristic peak, 317 nm: $OD_{TBBPA-BSA}$=0.145, OD_{hapten}=0.045, OD_{BSA}=0.102, 312 nm: $OD_{TBBPA-OVA}$=0.173, OD_{hapten}=0.035, OD_{OVA}=0.131; C_{BSA}: 0.23 g/L, C_{OVA}: 0.27 g/L, C_{hapten}: 0.05 g/L; protein and conjugate were dissolved in 0.01 M pH 7.4 PBS buffer; hapten was dissolved in DMSO.

and 273 nm (for BSA), and 219, 224, and 268 nm (for OVA). Different from carrier proteins, new characteristic peaks of TBBPA–BSA and TBBPA–OVA were exhibited at 317 nm and 312 nm, respectively. The displacement of the absorption peaks indicated that the hapten was successfully conjugated into the protein. The coupling ratio was 23 for BSA–TBBPA and was 16 for OVA–TBBPA.

The BSA–TBBPA artificial antigen was injected into rabbits, which developed immunity for 16 weeks, with the highest antibody titer at 1:120,000. The concentration of immunogen is 7.69 mg/mL, the coating antigen is 4.24 mg/mL, and pAb-TBBPA is 17.57 mg/mL, as determined using Coomassie Blue staining.

Table 1
Optimal concentrations of biotinylated antibody and TBBPA–OVA in terms of absorbance.

Dilutions of biotinylated antibody[a]	TBBPA–OVA concentration (μg/mL)					
	42.4	21.2	8.48	4.24	2.12	1.06
200	2.544	2.58	2.537	2.378	2.3605	2.482
500	1.583	1.752	1.082	1.728	1.651	1.7915
1000	1.05	1.263	1.455	1.3335	1.338	1.409
1500	0.890	1.018	1.193	1.071	1.282	1.305
2000	0.675	0.845	0.959	0.95	1.142	1.122
3000	0.508	0.696	0.695	0.7895	0.846	1.0105
4000	0.464	0.572	0.646	0.7195	0.8135	0.889
Blank	0.021	0.025	0.033	0.0455	0.0605	0.049

[a] The concentration of biotinylated anti-TBBPA antibody was 16.23 mg/mL.

3.2. Suitable operating conditions of the immunoassay method

The optimum concentrations for Bi-pAb-TBBPA and the coating antigen were used as the primary influencing factor to improve the sensitivity of the immunoassay. According to checkerboard titration, the optimum reagent concentrations were those that resulted in the maximum absorbance (A_0) of approximately 1.0 and the lowest antibody and coating antigen concentrations. The results are shown in Table 1. The optimal concentration of the coated TTBPA–OVA was 21.2 μg/mL and the Bi-pAb-TBBPA was at 1:1500 dilution (10.8 μg/mL).

Blocking is necessary in immunoassay procedures because it helps eliminate unoccupied sites on the plates. Otherwise, the unoccupied sites may interact with components such as Bi-pAb-TBBPA and SA–HRP during subsequent steps. Different blocking solutions, such as gelatin (0.1% and 0.5%), 0.5% $PEG_{20,000}$, 2% glucan, 1% PVA, 0.5% OVA and 0.5% milk powder in PBS, were compared, and the results are shown in Fig. 3a. An optimum blocking reagent should achieve the lowest background value. The 0.5% gelatin blocking agent achieved the lowest background value (0.09). The other blocking agents had similar background values: 0.1% gelatin (0.13), 0.5% $PEG_{20,000}$ (0.17), 0.5% OVA (0.15), 2% glucan (0.16), 1% PVA (0.12), and 0.5% milk powder (0.16). Therefore, 0.5% gelatin was selected as the blocking agent in the subsequent experiments.

Heterologous immunosorbent assay is based on the combination of the coating conjugate, the conjugate absorbed on the surface of the solid phase, and the relative antibodies in the liquid phase, which are greatly affected by the ionic strength, the pH, and other organic molecules in the liquid phase. The IC_{50}, maximal

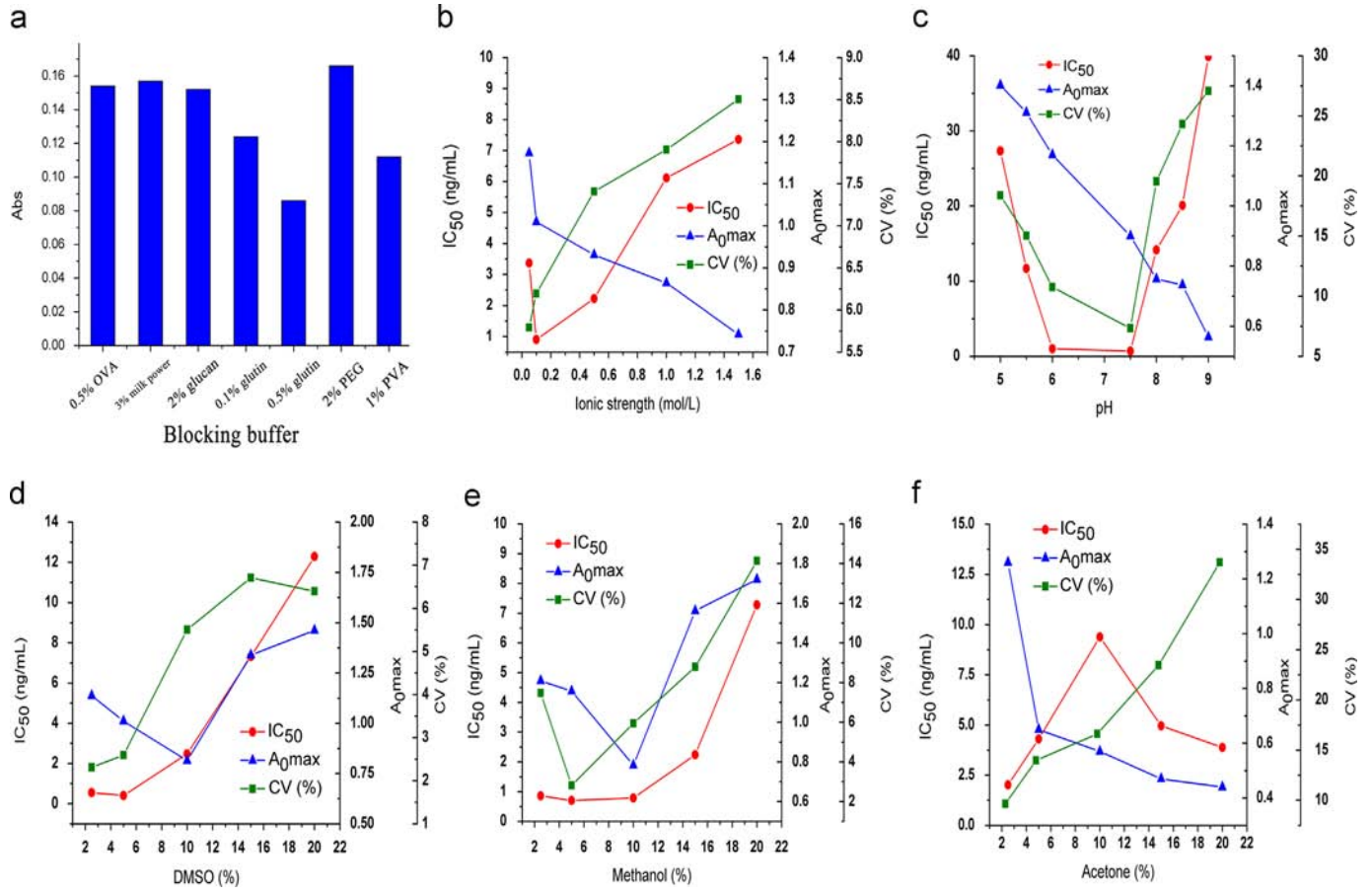


Fig. 3. Suitable operating conditions of the immunoassay method: (a) the blocking reagent, (b) ionic strength in PBS buffer, (c) pH of buffer, and (d)–(f) influence of different volume percentages of DMSO, methanol and acetone on PBS buffer.

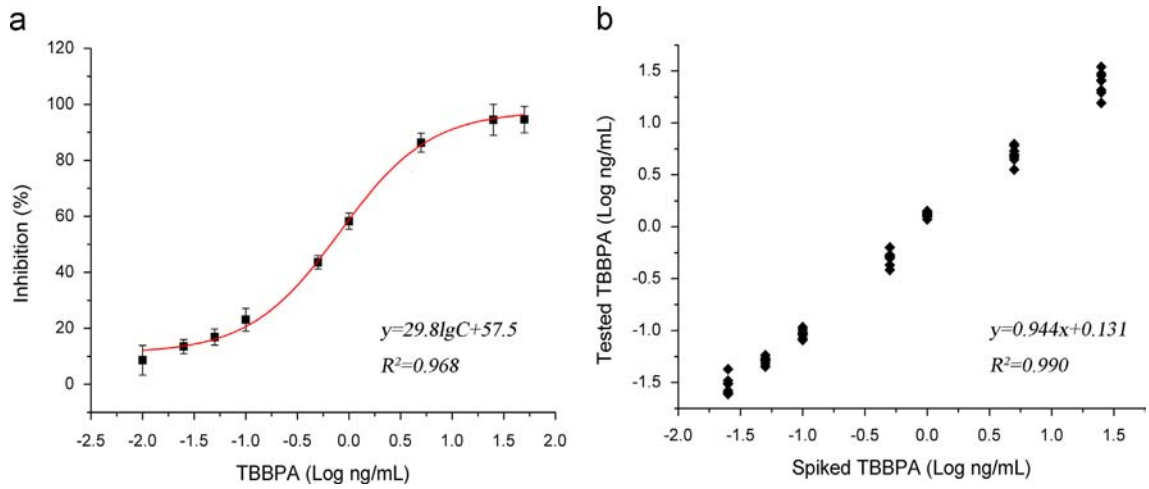


Fig. 4. (a) Standard curve for detecting TBBPA concentration by the BA-ELISA method. (b) The working range was analyzed by duplicate tests, plotting theoretic TBBPA concentrations against TBBPA concentrations measured by the assay, $n=8$.

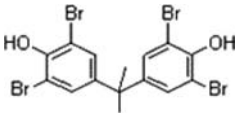
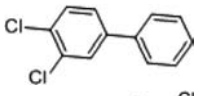
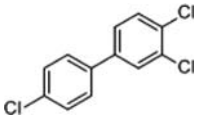
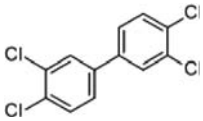
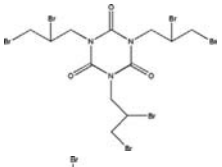
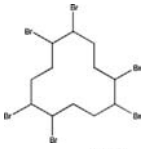
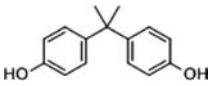
absorbance, and coefficients of variation (CV, %) from the competitive curves were determined under diverse operating conditions, and the results are displayed in Fig. 3b–f.

Immunoassay performance was determined under different ionic strengths (NaCl concentrations ranging from 0.05 mol/L to 1.5 mol/L; Fig. 3b). A_{0max} decreased from 1.17 to 0.74 with increasing salt concentration. Salt concentration slightly affected the sensitivity, with the IC_{50} ranging from 0.62 ng/mL to 7.33 ng/mL. The lowest IC_{50} (0.62 ng/mL) was obtained at an ionic strength of 0.1 mol/L. Hence, a salt concentration of 0.1 mol/L was selected for the buffer in the competition assay.

The antibody–antigen binding reaction is under a dynamic balance, is characterized by weak intermolecular bonds, and is easily affected by pH. The pH of optimum assay buffer was adjusted to 5–9. The IC_{50}, A_{0max}, and CV (%) were determined under varying pH; the results are shown in Fig. 3c. The A_{0max} values decreased with increasing pH. However, CV (%) increased from 5.7% (pH 7.5) to 24.33% (pH 9), which indicates that the stability of the immunoassay decreased with increasing pH. The appropriate IC_{50}, 0.59 ng/mL, was obtained at pH 7.5. Thus, pH 7.5 was used in the competitive immunoassay.

The effects of common organic solutions used in preparing standard solutions and sample extraction were determined to evaluate the resulting interference. The matrix effects of DMSO, methanol, and acetone were compared (Fig. 3d–f). In the DMSO and methanol trials, sensitivity clearly decreased with increasing amounts of organic solvents in the buffer. The lowest IC_{50} values, 0.58 and 0.60 ng/mL, were obtained with 5% DMSO (v/v) and 5% methanol (v/v), respectively. The sensitivity and stability of the assay were lower with acetone than with other solvents. The matrix effects indicate that lower amounts of organic solvent negatively affect the performance of the assay; thus, sample extracts containing organic solvents should be appropriately diluted in assay buffer to obtain reliable results.

Table 2
The cross-reactivity of anti-TBBPA antibody with structural analogs.

Analogs	Structure	IC_{50}(ng/mL)	Cross-reactivity(%)
TBBPA		0.58	100
PCB12		41.06	1.4
PCB37		14.54	3.9
PCB77		6.29	9.2
TBC		> 1160	< 0.05
HBCD		> 5800	< 0.01
BPA		9.56	6.1

3.3. LOD and working range

Complete indirect ELISA was used to detect TBBPA under optimum conditions; the calibration curves are reported in Fig. 4a. The linear working range, which is determined as the concentration range that causes 20–80% color inhibition [33], was 0.06–5.89 ng/mL, with the linear regression equation $y=29.8\log C+57.7$, $R^2=0.968$. The LOD of the TBBPA assay, represented as the IC_{10}, was 0.03 ng/mL and the sensitivity of the TBBPA assay, represented as the IC_{50}, was 0.58 ng/mL. In order to denote the accuracy of the immunoassay, the working range was analyzed by duplicate tests, and the theoretical TBBPA concentrations were plotted against the results of the BA-ELISA, with an excellent correlation coefficient ($R^2=0.990$, $n=8$; Fig. 4b).

3.4. Specificity

Assay specificity indicates the ability of the antibodies to combine with only the target molecule. CR (%) is an important

Table 3
The variability of intra-assay and interassay test.

Object	TBBPA concentration (ng/mL)							
	0.025	0.05	0.1	0.5	1	5	25	Mean
Intra-assay coefficient of variation (%)	10.51	8.64	8.35	6.56	5.48	3.65	4.06	6.75
Interassay coefficient of variation (%)	13.23	11.27	10.24	9.01	8.09	6.45	5.19	9.07

Table 4
Concentration of TBBPA in electronic waste determined by BA-ELISA and HPLC.

Samples[a,b]	Materials	BA-ELISA results (mg/kg)	HPLC results (mg/kg)
Keyboard key presses	PBT	1871.8 ± 8.8	1764.3 ± 0.3
Phone's shell	PC	22.7 ± 6.3	20.1 ± 2.9
Computer's shell	ABS	276.9 ± 1.4	273.6 ± 1.5
Circuits and circuit boards	epoxy resin	560.2 ± 6.5	554.3 ± 1.1
Plastic insulators	PVC	4650.3 ± 1.2	4432.5 ± 0.3
Capacitance[c]	PP	178 ± 10.7	166.6 ± 0.7
Printer's shell	PS	3464.1 ± 3.2	3412.3 ± 1.6

[a] The dosage limit of Tetrabromobisphenol A (TBBPA) in electronic products is < 1% (10 g/kg), according to Norway ROHs.
[b] Collected from local electronic waste recycling market in Shanghai.
[c] Product model: CBB series.

Table 5
Recoveries of TBBPA from electronic waste detected by BA-ELISA and HPLC methods.

Sample	Sample concentration (mg/kg)	Spiked level (mg/kg)	Average recovery% ± CV% (n=3)	
			BA-ELISA	HPLC
Phone's shell	22.7	10	96.7 ± 7.3	92.1 ± 5.6
		15	104.9 ± 9.1	102.7 ± 7.7
		20	117.3 ± 6.4	105.2 ± 1.3
Computer's shell	276.9	100	93.2 ± 4.2	89.6 ± 2.8
		150	113.3 ± 3.7	110.3 ± 4.4
		200	106.3 ± 5.3	99.8 ± 2.5

factor in immunoassays, and is generally used to estimate the specificity of antibodies. The CR values are calculated using Eq. (3).

3,4-Dichlorobiphenyl (PCB_{12}), 3,4,4′-trichlorobiphenyl (PCB_{37}), 3,3′,4,4′-tetrachlorobiphenyl (PCB_{77}), and bisphenol A (BPA) are selected as analogs, because they have similar structure segments with TBBPA. Although the structure of tris(2,3-dibromopropyl) isocyanurate (TBC) and hexabromocyclododecane (HBCD) is much different from that of TBBPA, HBCD and TBC are also used as BFR. So, the cross-reactivities caused by these two types of BFRs are also tested. The chemical structures of the analogs and the CR results are shown in Table 2. The obtained antibodies were specific for TBBPA. The relative antibodies exhibited high affinity and were suitable for the specific detection of TBBPA at low levels.

3.5. Repeatability of the immunoassay method

Intra-assay repeatability was estimated after performing over 10 replicates, and inter-assay repeatability was evaluated over several months. The intra-assay and inter-assay CVs were calculated to estimate the repeatability, as shown in Table 3. The intra-assay CV was < 10.51% and the inter-assay CV was < 13.23%.

3.6. Determination of TBBPA in electronic waste samples and recovery tests

The BA-ELISA method was applied to detect TBBPA in practical samples. TBBPA concentrations in electronic waste greatly varied depending on the species of plastic. The results are shown in Table 4. These practical samples were also tested on HPLC to evaluate the precision of BA-ELISA. The BA-ELISA results were slightly higher than those of the HPLC method.

The recovery of samples spiked with the target analyte was calculated to assess the analytical performance of the immunoassay. In general, a certain amount of target standard was added into samples before extraction. After heating spiked samples to melting form at low temperature, the samples were crushed into powder by a cryogenic sample crusher in liquid nitrogen. Then the spiked samples were treated as described before. All samples were tested three times using an optimized procedure to verify the repeatability. The results are shown in Table 5. The spiked samples were analyzed using BA-ELISA and HPLC for comparison. The average recovery rates and CVs of BA-ELISA were 93.2–117.3% and 3.7–9.1%, respectively. Meanwhile, HPLC showed recovery rates of 89.6–105.2% and CVs of 1.3–7.7%.

4. Conclusion

The TBBPA hapten and relative hapten–protein complexes, immunogen, and coating antigen were successfully synthesized. Specific antibodies were produced and the proposed BA-ELISA is highly sensitive and effective for the rapid detection of TBBPA in electronic waste samples. This proposed method has a low limit of detection and high sensitivity. The linear range was from 0.06 ng/mL to 5.89 ng/mL. The IC_{50} was 0.58 ng/mL, with negligible CR with structural TBBPA analogs. A series of electronic waste samples and spiked samples were tested using BA-ELISA and the HPLC method. The two methods had consistent results. The TBBPA immunoassay exhibited acceptable recovery rates and coefficients of variation. Therefore, the proposed BA-ELISA method can be used for the sensitive and selective detection of TBBPA in the environment.

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