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Indirect competitive enzyme-linked immunosorbent assay of tris-(2,3-dibromopropyl) isocyanurate with monoclonal antibody

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ABSTRACT

Tris-(2,3-dibromopropyl) isocyanurate (TBC) is a heterocyclic brominated flame retardant and posses typical characteristic of Persistent Organic Pollutants (POPs). To meet the need for rapid and reliable monitoring of TBC, a monoclonal antibody was produced and an indirect competitive enzyme-linked immunosorbent assay (IC-ELISA) was developed based on the monoclonal antibody. Monoclonal antibody against TBC was generated using synthesized haptens in mice. After optimization of the immunoassay conditions, results showed that the IC_{50} and the limit of detection (LOD) were 1.59 and 0.06 μg/L, respectively. The monoclonal antibody shows high specificity and the developed IC-ELISA is with high recoveries. The precision investigation indicated that the intra-assay precision values were all below 9.2% and that the inter-assay precision values ranged from 6.7 to 11.3%. The assay of real samples gives results basically consistent with UHPLC–MS/MS. The obtained results showed that this proposed immunoassay is a potential method for rapid and reliable monitoring of TBC.

1. Introduction

The presence of anthropogenic halogenated organic compounds in the environment is an environmental issue of global concern. Substances such as dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs) have been measured in a variety of environmental media and are now regulated globally in the Stockholm Convention as persistent organic pollutants (POPs) [1]. Over the past decades, halogenated flame retardants have been widely used in large volumes as additive in household products for fire safety reasons due to their excellent capability to reduce flammability. However, increasing concerns have also been raised on the environment problems caused by them, especially for brominated flame retardants (BFRs) which are widely applied to a variety of combustible materials to reduce the risk of fire damage [2–4].

Tris(2,3-dibromopropyl) isocyanurate (TBC) is a novel additive flame retardant. With a hexabrominated heterocyclic *s*-triazine structure, TBC has high thermal stability, durability, and resistance to photodegradation [5]. It is highly effective on glass fiber reinforced plastics and is also widely used in polyolefin, polyphenyl alkenes, acrylonitrile butadiene styrene, unsaturated poly-ester, synthetic rubber and fiber [2]. In recent several years, the amount of TBC has increased because of the decline in PBDEs production and the rapid industrialization of China. However, the environmental effects of TBC are of some concern. There is evidence that TBC is semi-volatile, environmentaly persistent and has a tendency to bioaccumulate. It can be accumulated in lipid-rich organs, such as the brain, liver, intestine, etc [4]. In 2009, TBC was found, for the first time, in the natural environment around a TBC manufacturing plant in South China [2]. Moreover, studies on zebrafish embryos suggested that exposure to TBC can cause a variety of potential reproductive and endocrine disrupting toxic effects, damaging mitochondria and causing defects in gas bladder inflation [4,6]. Thus, there is reason to be concerned about their potential to cause harm to ecosystems and human health.

The current regular analysis methods of TBC are mainly based on chromatography, such as high performance liquid chromatography tandem mass spectrometry (HPLC tandem MS), which are characterized by high precision, accuracy and sensitivity [2,7,8]. However, these classical methods require complicated equipments, laborious sample pretreatment procedures, well-trained operators and are limited to laboratory scientific research [7,9,10]. Therefore, there is a growing demand for more rapid and economical methods for fast scanning of TBC.

Immunoassay as a simple, rapid, cost-effective, and high specific method is widely applied in environmental analysis, especially for the high sample through-put monitoring and on-site screening of trace level samples [7,11]. The complicated sample pretreatment procedures needed in GC–MS are not required, and a simple extraction step is generally enough [12–15]. However, few studies have been published for analysis of TBC by immunoassay due to the

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short of antibodies against TBC. The successful generation of specific and sensitive antibodies against small organic molecules, such as TBC, is greatly dependent upon the proper design of the immunizing and hapten which must be coupled to a carrier protein. In our previous work, TBC polyclonal antibodies have been prepared and applied to TBC detection by ELISA [16]. However, polyclonal antibodies may recognize similar epitopes of TBC in other antigens when they are against TBC at the same time, causing false positive results. This problem could be solved with using monoclonal antibodies which are with several advantages over polyclonal antibodies, such as the higher specificity, and the continuous production of a well-characterized product by cell culture [17–20]. In this work, a specific monoclonal antibody against TBC was therefore prepared to develop a monoclonal antibody-based ELISA method for the analysis of TBC.

Among immunoassay, immunosensors attract enduring research interests. The immobilization of antibodies on the transducer surface greatly affects antibody–antigen interactions. Various immobilization methods enable immobilization of antibodies in either an oriented or a random manner on the surface [21]. Biological interface containing antibodies covalently attached to the transducer surface using a linking layer (i.e. lipid layers, silane layers, thiol layers or polymer matrices) or using intermediate molecules (i.e. protein A and protein G) for orientation control (end-on, head-on, side-on, and lying on orientation) offers the best analytic performance [22]. In spite of this, the antibody immobilization may results in the reduction of binding capability due to steric-hindrance induced by improper orientation of the antibodies toward surface of solid substrate or partial denaturation of the antibody due to chemically harsh reaction conditions [21,23]. Hence, immunoassay without immobilization of antibodies is still an option.

In this study, antigens instead of antibodies were physically adsorbed on ninety-six-well polystyrene ELISA plate by nonspecific hydrophobic interactions between the protein-conjugated TBC and the plate surface. Analysis of TBC was realized based on the competition between the free TBC in solution and the immobilized TBC on plate for the free antibodies in solution.

2. Materials and methods

2.1. Chemicals and instruments

All reagents were of analytical grade unless specified illumination. Incomplete Freund's adjuvant, complete Freund's adjuvant, bovine serum albumin (BSA), ovalbumin (OVA), Tween-20, 3,3,5,5-tetramethylbenzidine (TMB), goat anti-mouse IgG peroxidase conjugate, polyethylene glycol 1500 (PEG), Dulbecco's Modified Eagle's Medium (DMEM), HAT medium (hypoxanthine, aminopterin, thymidine), HT medium (hypoxanthine, aminopterin) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) from Hyclone (Logan, UT, USA) was heat inactivated at 56 °C for 30 min prior to use. Mouse SP2/0 myeloma cell was bought from the Cell Bank of Chinese Science Academy (Shanghai, China).

^{1}H and ^{13}C NMR spectra were recorded on INOVA-400 instrument (Varian, USA) using tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained by Trace2000 Polaris (ThermoFinnigan, USA) or LCQ-Advantage (ThermoFinnigan, USA). Ultraviolet–visible (UV–vis) spectra were obtained by UV-2100 spectrophotometer (LabTech, USA). A cell dissociation sieve-tissue grinder kit packed with mesh 80 was purchased from Sigma-Aldrich Company. The immunoglobulin isotype was determined using the Mouse MonoAb-ID kit from Invitrogen (Carlsbad, CA, USA). CO_2 incubator (HF 151 UV) was from HealFore Development Ltd. (Shanghai, China). Protein G affinity columns were purchased from Amersham Biosciences (Sweden). The UHPLC–MS/MS analysis for TBC was carried out by using UHPLC system (Agilent 1290 Infinity, USA) equipped with a triple quadrupole mass spectrometer (Agilent 6460, USA), an automatic sample injector (Agilent G4226A, USA) and a binary pump (Agilent G4220A, USA). Polystyrene microtiter plates (96-well) were purchased from Jet Biofil Company. Absorbances were measured in a microtiter plate reader (BioTek ELx800, USA). Immunoassay competitive curves were calculated by the software of Origin 8.5.

2.1.1. Buffers and solutions

(1) Phosphate-buffered saline (PBS, pH 7.4); (2) washing buffer (PBST): a PBS solution containing 0.05% (v/v) of Tween-20; (3) coating buffer (0.05 M carbonate buffer, pH 9.6); (4) blocking buffer: PBST mixed with 1% of BSA; (5) enzyme substrate buffer (TMB+H_2O_2): 150 μL of TMB-DMSO (15 μg/L) and 2.5 μL of H_2O_2(6%, w/v) in 10 mL citrate-acetate buffer (0.1 mol/L, pH 5.5); (6) enzymatic stopping solution: 2.0 M H_2SO_4; (7) TBC standard solution: TBC was dissolved with methanol of different volumes to get the standard solutions at different concentrations.

2.2. Preparation of TBC haptens

TBC is of small molecular weight, so it requires conjugation to carrier protein in order to be immunogenic. Haptens containing reactive carboxylic acid and spacer arm of different length were designed and synthesized as shown in Fig. 1. The details of the synthesis procedures and the spectroscopic dates of these compounds please see the published work by our group [16].

2.3. Preparation of immunogens and coating antigens

The haptens-Tc1–Tc3 (shown in Fig. 1) were conjugated to ovalbumin (OVA) as immunogens and to bovine serum albumin (BSA) as coating antigens. These conjugated antigens were all prepared by *N*-hydroxysuccinimide (NHS) active ester method similarly as previous reported [24]. The hapten (20 μmol) was dissolved in anhydrous DMF (0.32 mL) in which NHS (30 μmol) and dicyclohexylcarbodiimide (DCC, 30 μmol) was added. The mixture was stirred overnight at room temperature, and then the precipitate was removed by centrifugation. The solution of the active ester was added slowly to the solution of BSA (53.6 mg) in PBS (8 mL, 0.05 M, pH 8) with vigorous stirring at 4 °C. Then this mixture was stirred gently at 4 °C for 18 h and dialyzed for 72 h with buffer changed every 12 h. At last, the conjugates were lyophilized and stored at −20 °C. UV–vis spectral data were used to confirm the structures of the conjugates and the conjugation density was determined by using the 2,4,6-trinitrobenzene sulfonic acid (TNBS) method [25]. The immunogens were prepared similarly as above.

2.4. Production of monoclonal antibody

Prior to immunization, sera from blood samples were collected from each mouse to use as a baseline control for titre comparisons with blood samples taken post immunisation.

2.4.1. Immunization

Three groups of two BALB/c female mice (8–10 weeks old) were immunized subcutaneously with the respective hapten-OVA conjugate (50 μg conjugate per mouse). The immunogen was diluted in 200 μL PBS (phosphate buffered saline), mixed with an equal volume of complete Freund's adjuvant, and stirred to prepare a water-in-oil emulsion. Three weeks after the initial injection, the

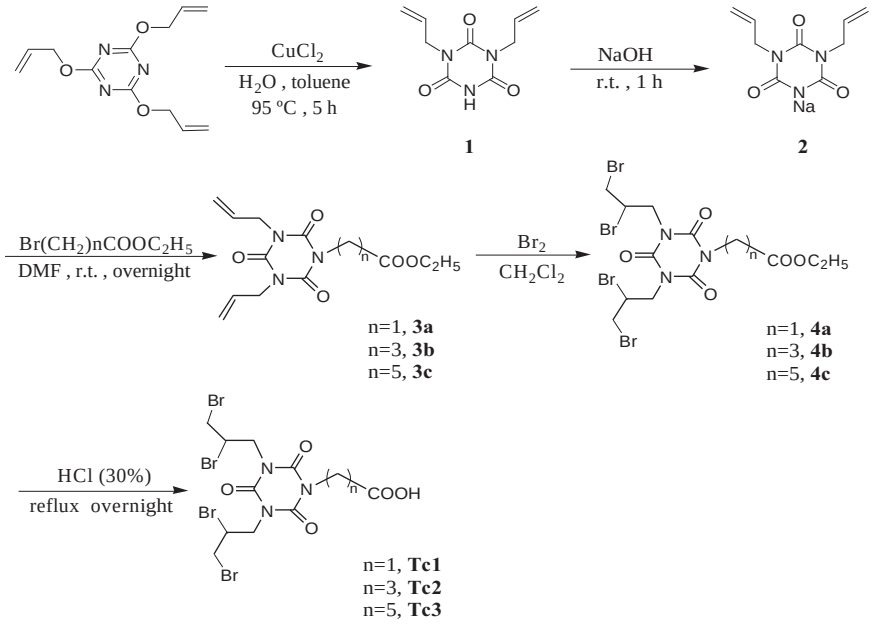


Fig. 1. Synthetic routes of haptens.

same injection with incomplete Freund's adjuvant was repeated at 2-weeks intervals thereafter. One week after the fifth immunisation, serum samples were obtained from the tail vein of each mouse, and the sera were tested for antibody titres and for analyte recognition by indirect ELISA as described below. After analysis, one mouse, immunized with hapten-Tc1-OVA, developed a serum antibody titer $>1{:}32{,}000$, the titre is the highest than the others. Therefore, this mouse was selected for hybridoma production. After a resting period of at least two weeks from the last injection with adjuvant, this mouse was received a booster intraperitoneal injection of 50 μg of protein conjugate in 200 μL of PBS. Three days later, the mouse was sacrificed and the entire spleen was aseptically removed and mashed with a glass pestle. The spleen cells were then passed through a cell dissociation sieve-tissue grinder kit packed with mesh 80 to produce a single-cell suspension for the cell fusion.

2.4.2. Cell fusion

Murine SP2/0 myeloma cells were cultured in high-glucose DMEM supplemented with 15% fatal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. Mouse spleen lymphocytes were fused with the myeloma cells at a ratio of 10:1 using 50% PEG 1500 as the fusing reagent and centrifuged. Then the fused cells were distributed in 96-well culture plates at an approximate density of 1×10^5 cells/well of DMEM supplemented with 20% fetal bovine serum (FBS) and 25 μg/mL gentamicin and incubated in a 5% CO_2 incubator at 37 °C. Twenty-four hours after plating, 50 μL of HAT selection medium (DMEM containing 20% FBS and supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine and peritoneal macrophages as feeder cells from young BALB/c mice) was added to each well. Half of the medium in the wells was replaced by fresh HAT medium every other day. Cells were grown in HAT medium for 2 weeks, then the HAT was substituted by HT medium (same as HAT but without aminopterin) when most of the nonfused cells were eliminated.

2.4.3. Hybridoma selection and cloning

Two weeks after cell fusion, when the hybridoma cells had grown about half-confluence in the well, culture supernatants were screened following a sequential double-screening process for the presence of antibodies that recognized TBC. The screening consisted of simultaneous performance of an indirect non-competitive and an indirect competitive ELISA to test the ability of antibodies to bind the hapten-BSA conjugate and to recognize TBC, respectively. The concentration of TBC in solution was 500 nM in the first screening, and 100 nM in successive experiments. The signal obtained in non-competitive conditions (absence of analyte) was compared with the competitive one when TBC was used as a competitor, and the ratio of both absorbances was used as the criterion for selecting high-affinity antibody-secreting clones. In addition, competitive ELISA were performed using serial dilutions of the culture supernatant from those wells that afforded saturated signals in the first screening experiment. The selective pressure was increased in the second assay using lower TBC concentrations, and ELISA plates were coated with antigens at 1 μg/mL to favor competition. The selected positive hybridoma cell lines which produced antibody-recognising TBC were subsequently subcloned for three times using the limiting dilution method in cloning medium. Stable antibody-producing clones were expanded until monoclones were obtained and cryopreserved in liquid nitrogen.

2.4.4. Production and characterization of monoclonal antibody

For monoclonal antibody preparation, female BALB/c mice at 8 weeks old were injected intraperitoneally with 0.5 mL of pristine. Seven days after the pretreatment, the mice were receiving an intraperitoneal injection of approximate density of 2×10^6 hybridoma cells that had been resuspended in PBS. Ascites fluid was harvested after 10–14 days of the hybridoma cell injection and was centrifuged to remove cell debris. The monoclonal antibody in the ascites fluid was purified by saturated ammonium sulfate precipitation followed by affinity chromatography on a protein G column. The concentration of antibody was measured according to the following formula: protein concentration (mg/mL) $=1.45OD_{280\ nm}-0.74OD_{260\ nm}$, where OD is the optical density [26]. The isotype of the purified antibody was determined by using a mouse monoclonal antibody isotyping kit.

2.5. ELISA procedures

ELISA was performed to screen cultured fluids of hybridomas and to titrate the optimum dilution rate (concentration) of

monoclonal antibody. Then the monoclonal antibody was used for sample analysis by IC-ELISA. All incubations were carried out at 37 °C. All assay buffers and solutions were kept at room temperature to avoid undesirable effects of temperature changes on assay precision. The procedures of ELISAs were described as follows.

2.5.1. Indirect competitive ELISA

Ninety-six-well polystyrene ELISA plate was treated with 100 μL/well of a 10 μg/mL solution of coating antigen in carbonate coating buffer and incubated overnight at 4 °C. Next day, the plate was washed three times with PBS containing 0.05% Tween-20 (PBST) and then blocked with 1% BSA in PBS (200 μL per well) by incubation for 2 h at room temperature. After washing three times with PBST solution again, 50 μL of 25% methanol in PBST was added to control and blank wells and 50 μL of TBC standard solution or sample extract was applied to the allocated wells. Then 50 μL of antibody dilution was added to the wells immediately, and the plate was incubated at 37 °C for 1 h. After washing as above, peroxidase-labeled goat anti-mouse IgG (1:4000 in PBST; 100 μL per well) was added, and the plate was incubated at 37 °C for 60 min. The plate was again washed five times as above, and then enzyme substrate buffer (100 μL per well) was added. After 10 min at room temperature, the enzymatic reaction was stopped by addition of 50 μL per well of 2 M H_2SO_4. The absorbance was immediately read at 450 nm on a Microplate Spectrophotometer.

Standard experiments were performed in quadruplicate and repeated five times. Inhibition curves were obtained by plotting average absorbance values against the logarithm of analyte concentration. For each point of the inhibition curve a minimum of five replicates was used and absorbances were the mean value of duplicate readings. The IC_{50} (the concentration of the competitor that inhibited the binding of the antibody by 50%) value, an expression of the sensitivity of immunoassay, and the limit of detection (LOD) defined as the IC_{10} value were obtained from the absorbance (Abs)-antigen concentration data following a four-parameter logistic equation: $Y=(A-D)/[1+(X/C)^B]+D$, Where A is the absorbance response at zero concentration of TBC, B is the curvature parameter that determines the steepness of the curve, C is the TBC concentration giving 50% reduction in absorbance (IC_{50}), D is the response at an "infinite" concentration of TBC, X is the TBC concentration, and Y is the corresponding absorbance.

2.5.2. Indirect non-competitive ELISA

The indirect non-competitive ELISA is identical to the indirect competitive ELISA, except that after blocking of nonspecific sites and washing, 100 μL of cell culture supernatant was added to the wells without addition of TBC.

2.6. Cross-reactivity

The specificity of the optimised ELISA was determined by using the standard solution of TBC and other related compounds. Cross-reactivity (CR) values were calculated as follows: CR%=[IC_{50} (TBC)/IC_{50} (related compound)] × 100%. The IC_{50} value of each compound was determined by IC-ELISA and each concentration level was assayed in four replicates.

2.7. Analysis of samples

2.7.1. Sample collection and pretreatment

To determine the performances of the proposed ELISA, actual samples including tap water, waster water, soil samples and human sera were analyzed. Tap water was collected from our laboratory. Waster water and soil samples were collected at the upstream of the Liuyang River which is located southwest of Liuyang, Hunan province, China. The specific sampling sites were located downstream of the waste water outlet of a TBC manufacturing plant, which has been producing TBC since 1986 with a production volume of 200 t per year. After removing the suspended solids by gravitational settling over night, water sample was filtered through filter papers and then 0.22 μm cellulose membranes to remove suspended particles. 3 × 10 mL dichloromethane (DCM) was added into 1000 mL of the filtered water sample to extract TBC. The extract was concentrated by rotary evaporation. The residue was redissolved in 30 μL of methanol and the solution was filtered by a microfiltration membrane. Then 2 μL of the solution was used for the ultra-high performance liquid chromatography–electrospray ionization tandem mass spectrometry (UHPLC–MS/MS) analysis, and 25 μL of the solution was diluted by addition of 75 μL of PBST for the analysis of IC-ELISA.

Soil and surface sediment samples were dried, ground, and homogenized to pass a 100-mesh sieve. 15 g anhydrous sodium sulfate and 10 mL dichloromethane were added to per 1 g soil sample and the mixture was subsequently extracted in an ultrasonic bath for 15 min, where dichloromethane was the extraction agent and anhydrous sodium sulfate was used to promote the salting-out of TBC. The extraction procedure was repeated three times with 10 mL dichloromethane, and then the three extraction solutions were collected and concentrated by rotary evaporation. All residues were treated according to above-mentioned methods which were used for water samples.

The human sera were collected with glass flasks (10 mL) at Hospital of Hunan University on 26 April in 2013. The treatments of these samples were according to the previously reported method with some modifications [27]. Human serum (10 μL) was mixed with distilled water and extracted with ethyl acetate. It was noticeable that a thick liquid substance always formed in the extracting step and centrifugation was helpful in separation of the organic layer. After evaporation of the organic solvents, the residue was redissolved in 25 μL of methanol and diluted by addition of 75 μL of PBST which were used for the analysis of IC-ELISA.

For the recovery study, all the samples were spiked with TBC at the concentrations of 0.5, 1.5, and 10 μg/L (for liquid samples) or μg/g (for soil samples), respectively. The spiked samples were treated according to above-mentioned methods. All analysis was carried out in triplicate.

2.7.2. UHPLC–MS/MS analysis

The UHPLC–MS/MS analysis was performed with a ZORBAXSB-C_{18} column (1.8 μm particle diameter, 2.1 mm i.d. × 50 mm length, Agilent, USA). The flow gradient with methanol (A) and water (B) was initiated at a composition of V_A:V_B=80: 20 and then increased to 100% of A with a flow rate of 0.3 mL/min in 10 min. The mass spectrometer was operated in negative electrospray ionization multiple reaction monitoring (MRM) mode with a capillary voltage of 3.3 kV. Source and desolvation temperatures were selected at 120 and 320 °C, respectively. Desolvation gas flow was 450 L/h and cone gas flow 50 L/h. Argon pressure in the collision cell was kept at 3.3×10^{-3} mbar for MS/MS measurements. For data collection, dwell time was set to 100 ms for each ion pair. Ion pairs of 727.8 > 81 and 727.8 > 79 ($[M-H]^- > Br^-$) were monitored separately for quantitative and qualitative purpose.

3. Results and discussion

3.1. Synthesis of hapten and antigen

TBC is a small and simple organic molecule which is non-immunogenic by itself and lacks a functional group for coupling to

proteins. Therefore, the synthesis of hapten and conjugation to carrier proteins are critical steps in the preparation of desirable immunoreagents. The haptens of TBC were synthesized from 2,4,6-tri(allyloxy)-1,3,5-triazine with the synthetic route shown in Fig. 1. The yield of each step was satisfied (≥ 63%) and the overall yield was also acceptable within 31–43% for five steps. The OVA and BSA conjugates were synthesized from the respective hapten. The yield of them was within 39–52%.

3.2. Production and characterization of monoclonal antibody

For the purpose of monoclonal antibody production, the mice received a total of six immunisations. After the fifth injection, the serum of each mouse from tail was tested for the presence of antibodies recognizing TBC by an indirect competitive ELISA and the best performing mouse in terms of titre detected was identified as belonging to a mouse immunised with hapten-Tc1-OVA. Therefore, this mouse was selected for subsequent hybridoma production. After cell fusion, a sequential double-screening procedure was carried out for hybridoma selection as described in Section 2.4.3. In the first screening, two simultaneous ELISAs (indirect non-competitive and indirect competitive) were performed, which helped to identify those monoclonal antibodies that bound to not only the coating conjugate but also the free analyte. The observed high number of positive clones could already be envisaged from the high titers exhibited by mouse antisera. With the aim of more efficiently selecting hybridomas for further cloning and expansion according to the affinity of the secreted antibodies, a second screening was routinely carried out following a competitive checkerboard assay. Of the 480 (5×96) wells examined, two clones (3E7, 5C6) gave strong positive signals in the IC-ELISA. Among them, the clone, 5C6, showed the highest affinity for TBC. Therefore, the supernatant of 5C6 culture was aspirated from the fusion well and subcloned for three times using the limiting dilution method. After limiting dilution and ELISA screening, clone 3D5 showing the highest affinity against TBC was selected for production of ascites fluid and was found to secrete antibody of IgG_1 isotype with κ light chain. Therefore, the monoclonal antibody obtained from 3D5 was used for the IC-ELISA.

3.3. Assay optimization

To develop highly sensitive and specific IC-ELISA, the assay conditions including dilution rate of antibody, pH, organic solvents, ionic strength, etc. were carefully optimized. The effects of these parameters were estimated by comparing IC_{50} values and A_{max} obtained under various conditions with that of a control.

Prior to the competitive experiments, the optimal concentration combination of monoclonal antibody and hapten-BSA conjugate (coating antigen) was also investigated and optimized by checkerboard assays. The optimal concentration should be that to provide the ELISA with signal response around 1.0 in the absence of analyte. As shown in Fig. 2, at 50 ng/mL of monoclonal antibody, increasing the concentration of hapten-BSA from 10 to 100 μg/mL did not significantly increase the absorbance, indicating that 10 μg/mL of hapten-BSA almost saturated the plate well surface. In addition, at the level of 10 μg/mL of hapten-BSA, dilution rate of monoclonal antibody in PBST from 50 ng/mL to 40 ng/mL resulted in the absorbance decreasing from 1.053 to 0.638. Therefore, the optimal coating concentration is 10 μg/mL and that the optimal dilution rate of monoclonal antibody should be 50 ng/mL.

Fig. 3 shows the influence of pH on the assay during the competition step in the presence of the analyte. The IC_{50} value decreases first and then increases when the pH increased from 5.5 to 9.5, a stable and relatively low IC_{50} value is observed at pH 7.5. Interestingly, there were significant changes in A_{max} for assays

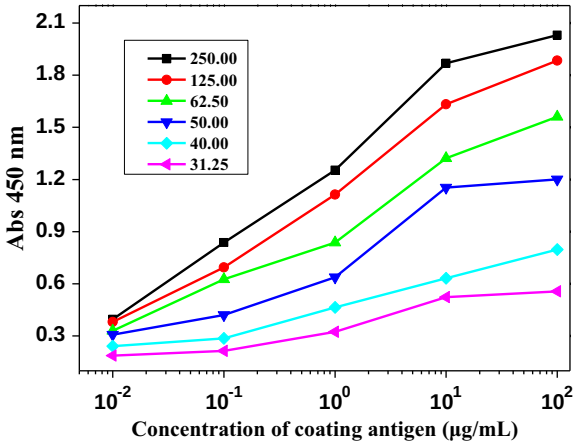


Fig. 2. ELISA checkerboard. Each point is the average of three replicates. Different lines represent the different concentrations of monoclonal antibody (ng/mL).

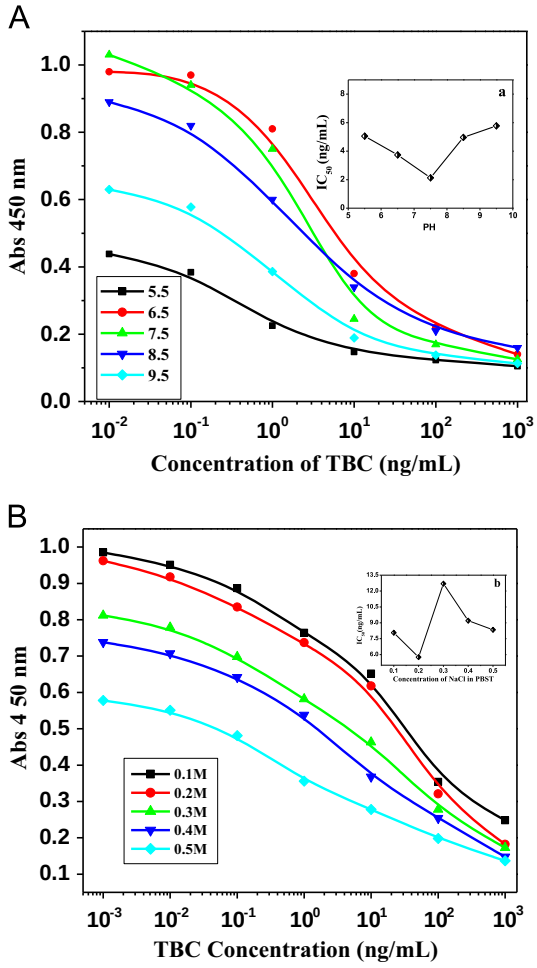


Fig. 3. Effects of assay buffer pH (A) and ionic strength (B) on the IC-ELISA. Each point represents the mean of three replicates. Insets (a) and (b) indicate the fluctuations of IC_{50} as a function of PH or ionic strength.

run in both strong acid and alkali matrix. The results indicated that the immunoreaction favored a weak alkaline solution. Therefore, a pH 7.4 PBST buffer was selected for the proposed ELISA.

To identify potential interferences from aqueous environmental samples, the ionic strength on ELISA performance was studied in medium at concentrations of NaCl ranging from 0.1 to 0.5 M (Fig. 3). A higher salt concentration (the concentration of NaCl in PBST) in

the assay system results in a lower A_{max} value and higher IC_{50} value. The reason is that increasing the concentration of NaCl can inhibit the binding of antigen with antibody. While the NaCl concentration increased from 0.1 to 0.2 M, the A_{max} values decreased from 0.985 to 0.962, a little decrease. Although the A_{max} values were very close at NaCl concentrations of 0.1 M and 0.2 M, the IC_{50} value changed a lot. With increasing the NaCl concentration from 0.1 to 0.2 M, the IC_{50} decreased from 8.07 ng/mL to 5.76 ng/mL. Therefore, PBST containing 0.2 M NaCl was used. In practice, the ionic strength of environmental samples can be adjusted by simple dilution with water or concentrated buffer.

Owing to the high lipophilicity of TBC, organic solvents should be used to improve the solubility of TBC during assays. However, the addition of organic solvents may affect the ELISA performances. The effects of chosen organic solvents including MeOH, acetone and DMSO on the assay were studied because of their water-miscible. These experiments were performed in quadruplicate and repeated three times. The effects of these organic solvents on the percent binding B/B_0 (%) were shown in Fig. 4. $B/B_0(\%) = (A - A_{min})/(A_{max} - A_{min}) \times 100$, where A is the absorbance at a given concentration of TBC, A_{max} is the absorbance at zero dose of the analyte, and A_{min} is the background absorbance. As shown in Fig. 5, the effects of these three solvents show a similar trend, little effects of these solvents on A_{max} are observed when the assay is run in concentrations of solvents up to 30%. However, the IC_{50} values showed irregular changes with increasing the solvent content, and the change trend depended on the properties of the solvent. Compared with acetone and DMSO, MeOH shows less effect on assay sensitivity (IC_{50}) and better solubility of TBC, the lowest IC_{50} (4.7 ng/mL) was obtained in 25% methanol (in PBST) solution. Based on these results, PBST containing 25% methanol as assay buffer was used. This solution did not decrease the sensitivity significantly.

In summary, the optimized ELISA conditions are: coating antigen concentration of 10 μg/mL, monoclonal antibody at a dilution of 50 ng/mL in wells, 1% BSA used for blocking the coated plate, 25% methanol in PBST (pH 7.4) assay buffer.

3.4. Antibody–antigen binding kinetics

The antibody–antigen binding kinetics is a key to affect the sensitivity. A number of techniques exist for the measurement of the antibody–antigen binding kinetics, such as surface plasmon resonance (SPR) spectroscopy, fluorescence polarization, quartz crystal microbalance (QCM) sensing, interferometry and total internal reflection ellipsometry [28,29].

Considering that external conditions (concentration, temperature, molecule diffusion, pressure, catalyst…) may influence binding rate of the antibody–antigen reaction, here we focused on the effects of antibody concentration and temperature on the binding rate based on the ELISA, with the results shown in Fig. 5. As shown in Fig. 5(A), the binding rate increases with increasing the antibody concentration. The antibody–antigen reaction reaches equilibrium within 60 min independent on the antibody concentration. Fig. 5(B) shows that the maximum binding rate is achieved at 37 °C. Also, equilibrium is reached within 60 min.

The optimal conditions should be that to provide the ELISA with absorbance around 1.0, which are: 50 ng/mL of antibody concentration, and incubation time of 60 minutes at 37 °C.

3.5. Sensitivity of the ELISA

Under the optimized conditions mentioned above, the competition curve for detection of TBC by the indirect competitive ELISA is shown in Fig. 6. From the curve, the IC_{50} value is 1.59 μg/L. A limit of detection (LOD) established at the IC_{10} is 0.06 μg/L. The monoclonal antibody shows a lower IC_{50} and LOD than the polyclonal antibody developed in our group (IC_{50}, 5.17 μg/L; LOD, 0.118 μg/L) [16].

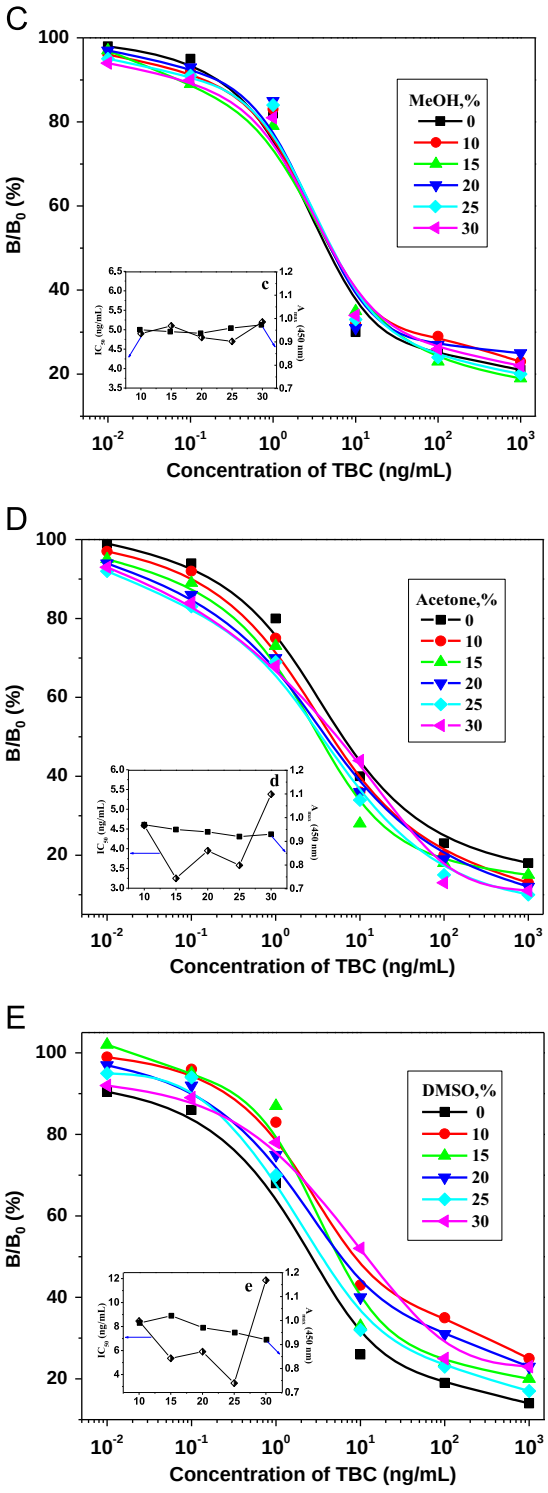


Fig. 4. Effects of different organic solvents (C)–(E) and each value represents the average of three replicates. Values refer to the final concentrations of solvents (v/v) in the competitive assay solution. Insets (c)–(e) indicate the fluctuations of A_{max} (right Y-axis) and IC_{50} (left Y-axis) [(■) A_{max};(◇) IC_{50}]as a function of solvent concentration (X-axis).

3.6. Specificity of the ELISA (cross-reactivity)

To determine the specificity of the proposed IC-ELISA, the standard solution of TBC and several other interferences were

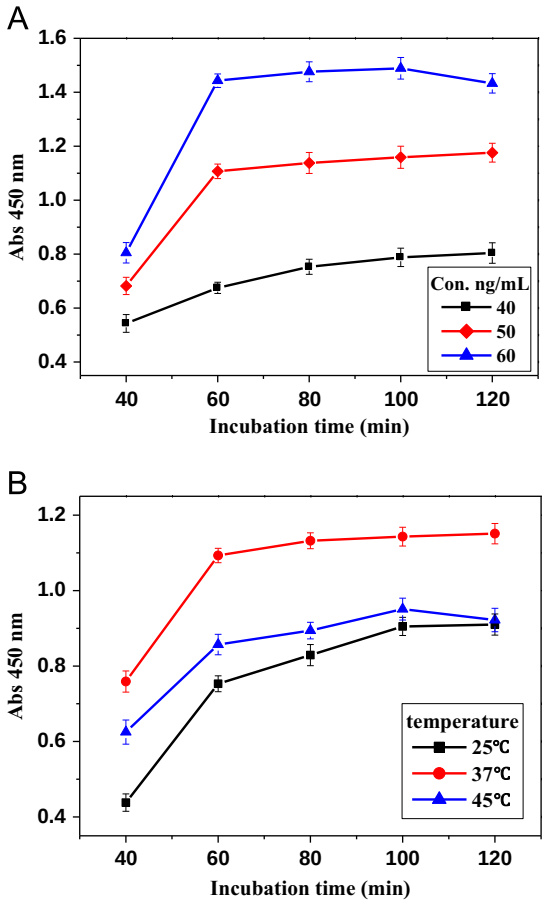


Fig. 5. Effects of concentration (A), temperature (B) to binding rate of antigen-antibody reaction based on the proposed ELISA. Each point represents the mean of three replicates.

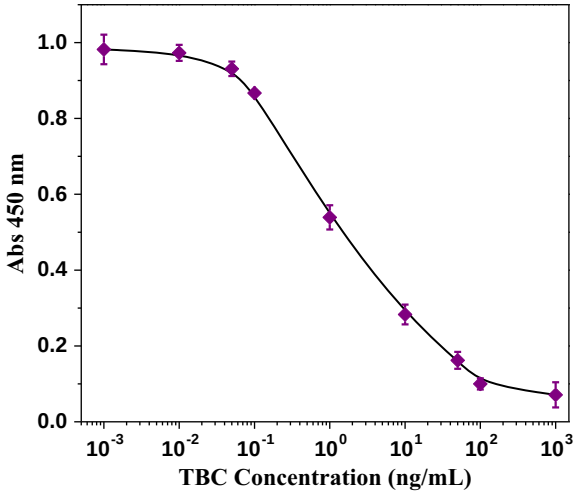


Fig. 6. ELISA inhibition curves for TBC determined on five different plates. Each symbol represents the mean absorbance from four wells.

tested for cross-reactivity. As shown in Table 1(I), all of the tested compounds show little CRs with TBC (< 2%). Among them, the highest cross-reactivity was 1.51%, obtained for 1,3-diallyl-isocyanurate with a similar structure as TBC but no bromine. Compounds with the same bromines as TBC but different structure show low CR% of < 1.24%. The CR% of other interferences with different structures and different bromines is negligible (< 0.1%). These results indicated that the developed monoclonal antibody was high specific to TBC.

3.7. Accuracy and precision of the ELISA

To test the accuracy and precision of the ELISA, actual samples including tap water collected from our laboratory, waster water and soil samples collected along Liuyang River, at the downstream of the wastewater outlet of a TBC manufacturing plant, Human sera collected from Hosipital of Hunan University were spiked with TBC at different concentrations and analyzed by the IC-ELISA. The results of recovery and coefficient variation (CVs) of the spiking experiments were listed in Table 2(I). In those samples without spiking, TBC was only detected in the waster water and soil samples. There was no detectable TBC in tap water and Human sera. It is reasonable that TBC was not detected in tap water and sera because TBC is high hydrophobic and not a widely used chemical. The trace TBC content in environment is under the limit of the detection (LOD). All the recoveries were acceptable within the range of 81–112% for different samples. The water and soil samples own the higher recovery rates, while the human sera samples are with the relatively lower recovery rates ranging from 81% to 92%. These phenomena were probably due to the binding of plasma protein with organic moleculars [30,31], resulting in the loss of analyte during the extraction step. The coefficient variations (CVs) listed in Table 2 show within-run (intra-assay) precisions of < 9.2% and between-run (inter-assay) precisions ranged from 6.7 to 11.3%, indicating that the developed ELISA has a good determining precision. The causes resulting in these variations include evaporation of solvents at the edge of the wells, non-homogeneity of the temperature or slight changes in the preparation of buffers and solutions.

3.8. Validation of the ELISA method by UHPLC–MS/MS analysis

For accuracy investigation, river water (W_1–W_4) and soil samples (S_1–S_4, G) collected at the sampling sites were analyzed by both the IC-ELISA and UHPLC–MS/MS method. The results are listed in Table 3. The TBC contents in all the samples obtained by ELISA is basically essential to those by UHPLC–MS/MS, although the ELISA always give higher results probably due to other unidentified cross-reacting compounds and/or a nonspecific interference. The garden soil (G) showed the highest TBC concentration. However, TBC in river water (W_1–W_4) collected at the upstream watershed of the Liuyang River was not detected by both the proposed method and the UHPLC–MS/MS. The reason should be that TBC in river water was diluted by rainy water because the sample was collected in a heavy rainy day. The high concentrations of TBC in soils hint the rapid distribution of TBC between the water and sedimentary interface. A majority of TBC is strongly attached to the sediment. The results also show that the TBC concentration in soil follows an increasing trend with the sediment depth.

3.9. Comparison of monoclonal antibody with polyclonal antibody

Scheme 1 To date, polyclonal- and monoclonal-antibody-based IC-ELISA have been developed in our laboratory for the analysis of TBC. The antiserum titer, sensitivity and specificity of the monoclonal antibody were on average higher than that of the polyclonal antibody. As shown in Fig. 5, the sensitivity of monoclonal antibody was higher than that of the polyclonal antibody on the basis of the IC_{50} values by IC-ELISA, the IC_{50} values were 1.59 and 5.17 μg/L for monoclonal antibody and polyclonal antibody, respectively. The results of the cross-reactivity examinations suggest that monoclonal antibody can recognize slight differences

Table 1
(I) Cross-reactivity (CR%) of monoclonal antibody of TBC and its related compounds[a].

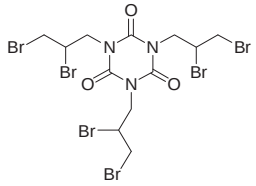
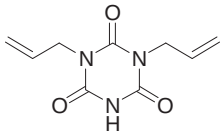
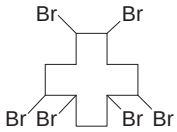
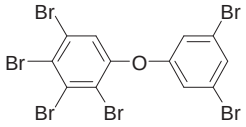
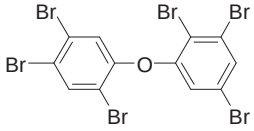
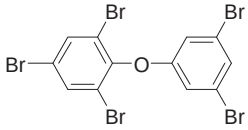
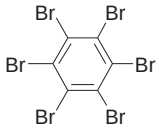
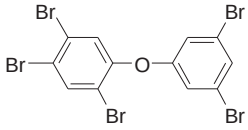
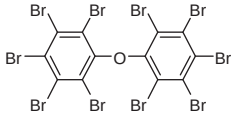
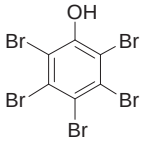
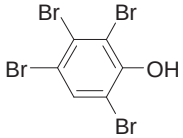
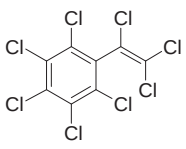
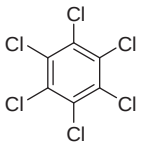
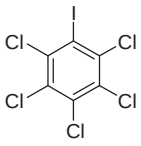
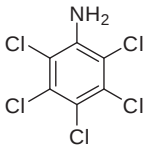
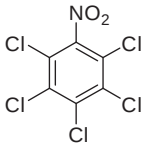
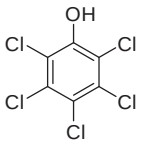
Compounds	Structures	IC_{50} (ng/mL)	CR (%)
Tris(2,3-dibromopropyl)isocyanurate		1.59	100
1,3-Diallyl-isocyanurate		105	1.51
Hexabromocyclododecane		128	1.24
2,3,3′,4,5,5′-Hexabromodiphenylether		194	0.82
2,2′,3′,4,5,5′-Hexabromodiphenylether		230	0.69
2,3′,4,5′,6-Pentabromodiphenylether		370	0.43
Hexabromobenzene		430	0.37
2,3′,4,5,5′-Pentabromodiphenylether		530	0.30
Decabromodiphenyl ether		636	0.25
Pentabromophenol		> 1,600	< 0.1
2,3,4,6-Tetrabromophenol		> 2,000	< 0.1
Octachlorostyrene		> 3,200	< 0. 05

Table 1 (*continued*)

Compounds	Structures	IC_{50} (ng/mL)	CR (%)
Hexachlorobenzene		> 16,000	< 0.01
Pentachloroiodobenzene		> 16,000	< 0.01
Pentachlorobenzenamine		> 16,000	< 0. 01
Pentachloronitrobenzene		> 16,000	< 0.01
Pentachlorophenol		> 16,000	< 0.01

(II). Cross-Reactivity (CR%) of polyclonal antibody of TBC and its related Compounds[a]

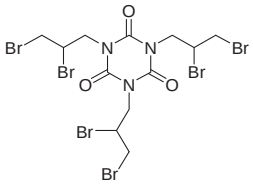
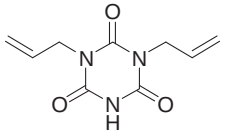
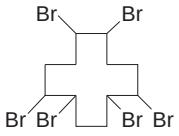
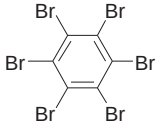
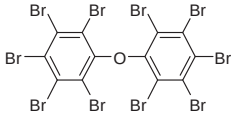
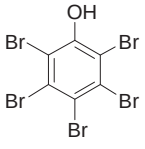
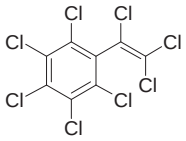
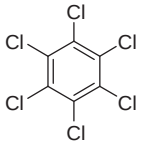
Compounds	Structures	IC_{50} (ng/mL)	CR (%)
Tris(2,3-dibromopropyl)isocyanurate		5.17	100
1,3-Diallyl-isocyanurate		259	2.00
Hexabromocyclododecane		352	1.47
Hexabromobenzene		1130	0.45
Decabromodiphenyl ether		1430	0.36
Pentabromophenol		> 2000	< 0.3

Table 1 (*continued*)

Compounds	Structures	IC_{50} (ng/mL)	CR (%)
Octachlorostyrene		> 2000	< 0.3
Hexachlorobenzene		> 2000	< 0. 02

[a] The CR% according to the expression [IC_{50} (TBC)/IC_{50}(related compound)] × 100%.

Table 2
(I) Recoveries of TBC in Tap water, waster water, Soil sample and Human sera samples measured by IC-ELISA based on monoclonal antibody.

Samples	TBC		Recovery % (n=3)	Intra-assay variation, [a]% (n=3)	Inter-assay variation, [b]% (n=3)
	Fortified	Detected			
	0	nd[c]			
Tap	0.5	0.48	96	6.3	8.1
	1	0.97	97	8.5	9.3
Water	5	5.4	108	9.2	10.1
	10	11.2	112	7.6	8.9
	0	1.26			
Waster	0.5	1.78	104	6.6	7.8
	1	2.33	107	7.5	8.4
Water	5	6.36	102	7.1	8.3
	10	11.86	106	5.9	6.7
	0	0.84			
Soil	0.5	1.33	98	7.3	8.4
	1	1.87	103	8.1	9.3
Sample	5	5.59	95	7.6	8.7
	10	9.74	89	7.8	9.6
	0	nd[c]			
Human	0.5	0.42	84	8.9	10.5
	1	0.92	92	9.1	11.3
Serum[d]	5	4.05	81	7.8	10.2
	10	8.7	87	7.3	9.6

[a] Intra-assay variation was determined by analyzing one extraction sample three times on a single day.
[b] Inter-assay variation was determined by analyzing one extraction sample on three different days.
[c] Not detected.
[d] The unit of liquid samples was μg/L, and the unit of soil samples was μg/g dry weigh based.

Table 2
(II) Recoveries of TBC in Tap water, River water, Soil sample and Human sera samples measured by IC-ELISA based on polyclonal antibody.

[a]Samples	TBC		Recovery % (n=3)
	Fortified	Detected	
	0.2	0.22 ± 0.01	110 ± 5
Tap	0.5	0.45 ± 0.05	94 ± 10
	1	0.95 ± 0.05	95 ± 5
Water	5	5.2 ± 0.1	104 ± 0.2
	10	9.0 ± 0.8	90 ± 8
	0.2	0.17 ± 0.01	85 ± 5
River	0.5	0.46 ± 0.2	92 ± 5
	1	0.94 ± 0.03	94 ± 3
Water	5	4.7 ± 0.3	94 ± 6
	10	8.8 ± 1.0	88 ± 10
	0.2	0.18 ± 0.01	90 ± 5
Soil	0.5	0.48 ± 0.3	96 ± 6
	1	1.02 ± 0.5	102 ± 5
Sample	5	4.98 ± 0.2	93 ± 4
	10	9.89 ± 0.9	98 ± 9
	0.2	0.14 ± 0.01	70 ± 5
Human	0.5	0.39 ± 0.01	78 ± 10
	1	0.91 ± 0.1	91 ± 10
Serum	5	4.0 ± 0.3	80 ± 6
	10	7.1 ± 1.5	71 ± 15

[a] The unit of liquid samples was μg/L, and the unit of soil samples was μg/g dry weigh based.

Table 3
The assayed results from the IC-ELISA together with the HPLC tandem MS results for comparison[a].

Samples[a]	W_1–W_4	S_1	S_2	S_3	S_4	G
ELISA	nd[b]	0.198	0.439	0.526	0.535	0.944
UHPLC/MS	nd[b]	0.155	0.363	0.445	0.457	0.848
RE[c](%)	–	27.74	20.94	18.20	17.07	11.32

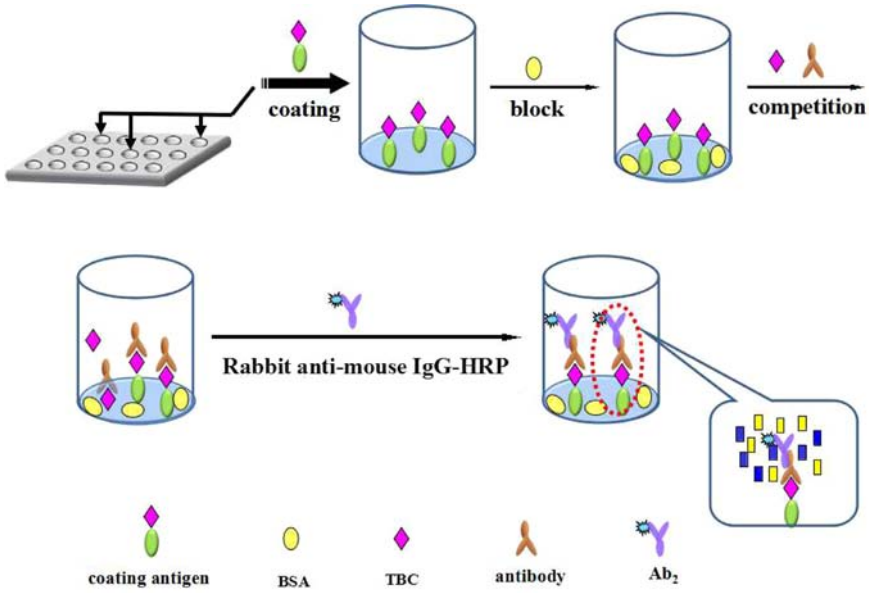
[a] The unit of water samples was μg/L, and the unit of soil samples was μg/g dry weigh based.
[b] Not detected.
[c] Relative error (with the results of UHPLC/MS as a standard).

of characteristic structures in TBC and its related compounds. These advantages of monoclonal antibody over their polyclonal counterparts is result from the scheme used to screen antibodies secreted by hybridomas, which initially selected antibodies specifically reacting with TBC of hapten-BSA and then selected from these antibodies reacting with TBC. Moreover, Polyclonal antibody tests recognize several epitopes of TBC whereas monoclonal antibody tests, by definition, bind to only one specific part of TBC [32].

In short, the performance of the monoclonal antibody test was superior to the polyclonal antibody test in our study. This is of special interest and monoclonal antibody-based ELISA is recommended for further application because of the advantages of monoclonal antibody: (i) monoclonal antibody could be obtained permanently and homogeneously, (ii) monoclonal antibody can not only provide a high degree of sensitivity and specificity, but also decrease the false positive results and would be practical for routine analysis as well compared to polyclonal sera though they are more laborious to produce and have a limited stability in changes in pH or salt concentration. If experimental conditions are kept constant, results from monoclonal antibody will be highly reproducible between experiments, which make the detection method more stable.

4. Conclusions

Monoclonal antibody of high sensitivity and specificity toward TBC was first successfully developed. They were integrated in an



Scheme 1. Schematic Representation of Indirect competitive Enzyme-linked Immunosorbent Assay Procedure.

indirect competitive ELISA which was validated for the measurement of TBC in actual samples. The monoclonal antibody showed no substantial cross-reactivities with related compounds. Both the accuracy and precision of the measurements were within the criteria of acceptance. More importantly, sensitivity and specificity of the monoclonal antibody were on average higher than that of the polyclonal antibody. Therefore, this ELISA based monoclonal antibody may serve as a reliable, simple and cost effective tool and as a promising alternative for chromatographic methods. Additionally, the monoclonal antibody may be valuable for other applications such as time-resolved fluoroimmunoassay, immunocyto- and the subcellular localization and tissue distribution of these compounds.

Acknowledgments

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