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Research Article

A Chemical Synthesis of Benzo[*a*]pyrene Hapten and Its Application in a Streptavidin-Horseradish Peroxidase-Based Enzyme-Linked Immunological Analysis

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A specific BaP hapten was synthesized and BaP antigens (immunogen, coating antigen) were prepared using diverse methods. Based on these works, a streptavidin-horseradish peroxidase-based enzyme-linked immunological analysis was developed and firstly used to detect BaP. Several physiochemical factors that may influence the assay performance were optimized in the assay. Under optimal conditions, a detection limit of 0.0094 ng mL^{-1} was obtained and good linearity was achieved within a range of 0.03–35.40 ng mL^{-1}. Satisfactory recovery of spiked samples (91.12–109.23%) was obtained and the coefficient of variation was acceptable. Finally, the detection results of BaP in environmental and food samples were consistent with those obtained using high-performance liquid chromatography. The proposed immunoassay is reliable and has great potential for detecting trace amounts of BaP.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic pollutants that contain 2–8 aromatic rings in linear, angular, or cluster arrangements [1]. Due to their toxicity and carcinogenicity, PAHs have attracted the attention of environmental chemists and regulatory agencies for several decades [2, 3]. Among the PAHs, benzo[*a*]pyrene (BaP) has been widely studied. In recent years, much attention has been devoted to the existence of BaP in the environment. BaP is considered the most dangerous of PAHs because of its chemical and physical characteristics and ease of accumulation in the food chain [4, 5]. BaP is usually found in exhaust gas produced by the incomplete combustion of fuels, in all smoke resulting from the combustion of organic material and in charbroiled food [6–8]. BaP can enter into water bodies mainly via atmospheric fallout, municipal/industrial effluents, urban runoff, and oil spillage [9, 10]; it is emitted to the atmosphere from different sources, including industrial processes, combustion of fossils and fuels, and irrational application of pesticide [11].

BaP is a great potential hazard to human health. It is a type of carcinogenic compound, which can cause lung, gastric, bladder, and gastrointestinal cancers [12, 13]. Recent studies of BaP have also demonstrated its involvement in the development of cardiovascular diseases [14]. BaP is mutagenic and teratogenic; it can even pass through the placenta to endanger the embryogenesis [15]. Dietary intake of BaP is a major source of human exposure [16]. Related studies have shown that people in the general population without substantial environmental and occupational exposure had a higher exposure to BaP by food ingestion [17]. Probable sources of BaP in food are environmental contamination from soil, water, and the thermal treatment used for the preparation of food, such as roasting.

Up to now, many different methods have been described for the determination of BaP residues. The main methods are gas chromatography and mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC); however, these chromatographic methods usually require long periods of detection processes that are time-consuming and the follow-up maintenance is expensive. Compared

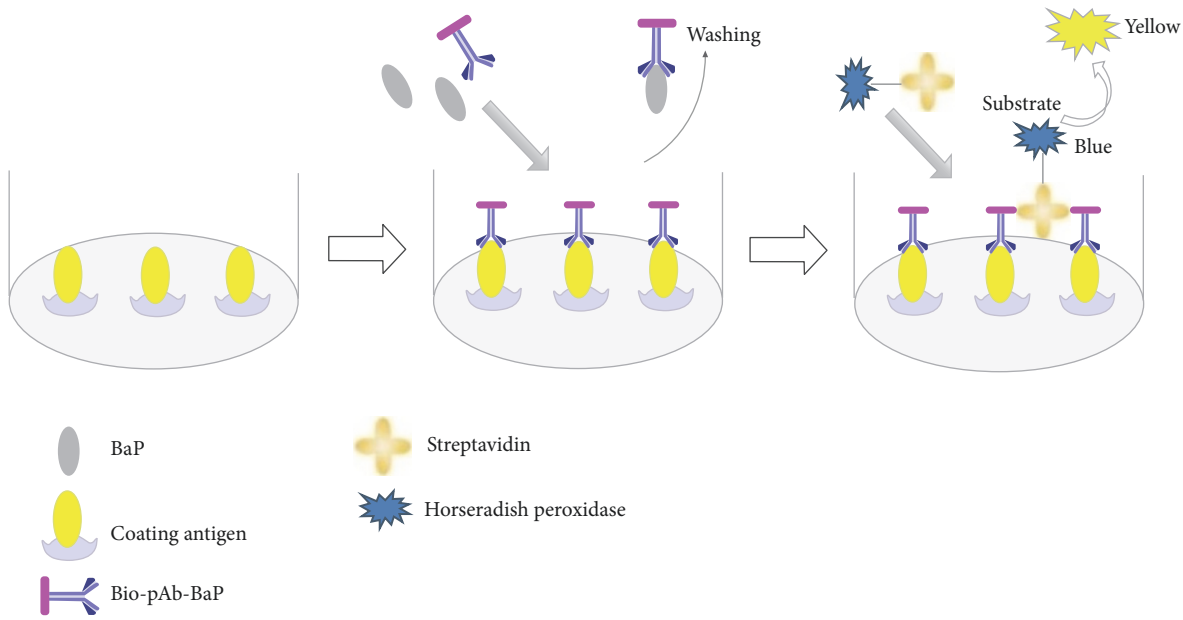


Figure 1: Principle of the SA-ELISA method. BaP coating antigens which are covered on the surface of the tubes competed with BaP analytes to combine with the bio-pAb-BaP. The horseradish peroxidase-conjugated streptavidin (SA-HRP) is fixed on the wall through the reaction with bio-pAb-BaP. As the increase of BaP concentration, less bio-pAb-BaP can be attached to the solid carrier. In this case, the amount of SA-HRP is less, and then quantitative analysis could be performed after sufficient colour development.

with chromatographic techniques, immunoassays are cost-effective, with adequate sensitivity and high selectivity [18]. Therefore, immunochemical techniques have been increasingly considered as complementary methods for residue analysis. Among these immunoassays, enzyme-linked immunosorbent assay (ELISA) is well suited for detecting trace amounts of pollutants. Some improved immunoassays were researched based on the conventional ELISA, such as fluorescence-enzyme immunoassay (FL-ELISA) and chemiluminescence immunoassay (CL-ELISA) [19, 20]. Accompanying the improved immunoassays above, the streptavidin-enzyme based ELISA can reduce nonspecific reactions with reagents due to the higher specificity and affinity between streptavidin and biotin, and it has great potential for detecting trace amounts of BaP. However, as we know, no information on detecting BaP by streptavidin-enzyme based ELISA has been reported.

In this research, a specific BaP hapten was synthesized. Diverse BaP antigens and biotinylated polyclonal BaP antibody (bio-pAb-BaP) were prepared. Based on these works, a streptavidin-horseradish peroxidase-based enzyme-linked immunoassay was developed (SA-ELISA) and firstly used for the sensitive detection of BaP. Some environmental and food samples were collected in Shanghai, China. Several physiochemical factors that may influence the assay performance were optimized, and the accuracy and reliability of the immunoassay were validated. Figure 1 shows the detection process of SA-ELISA method.

2. Materials and Methods

2.1. Buffers and Solutions. Phosphate-buffer saline (PBS: NaCl [137 mmol L^{-1}],KH_2PO_4 [2 mmol L^{-1}], Na_2HPO_4 [10 mmol L^{-1}] and KCl [2.7 mmol L^{-1}], pH 7.40), PBST (PBS with 0.05% Tween 20, pH 7.40), and carbonate buffer (CBS: Na_2CO_3 [15 mmol L^{-1}] and $NaHCO_3$ [34.9 mmol L^{-1}], pH 9.60) were used in the assay.

2.2. Materials and Chemicals. The BaP standard, other types of PAHs, chemicals for BaP hapten and antigen synthesis, 25% glutaraldehyde solution, polyethylene glycol 20,000 (PEG 20,000), N,N-dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) were purchased from J&K Chemical (Beijing, China). 3,3′,5,5′-Tetramethyl benzidine (TMB), hydrogen peroxide (H_2O_2), keyhole limpet hemocyanin (KLH), poly-L-Lysine (PLL), polyvinyl alcohol (PVA), skimmed milk powder, H_2SO_4, HCl, gelatin, Tween 20, Coomassie Brilliant Blue G250, and inorganic salts were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Bovine serum albumin (BSA), ovalbumin (OVA), dialysis bags, biotinylated N-hydroxysuccinimide ester (BNHS), and SA-HRP were all purchased from Sangon Co. Ltd. (Shanghai, China). BaP hapten was purified through column chromatography using silica gel (40 μm average particle size) obtained from Shanghai Sanpont Co. Ltd. (Shanghai, China).

2.3. Synthesis of BaP Hapten. BaP molecule does not contain functional groups that can connect with proteins. Therefore, a specific BaP hapten was synthesized (Figure 2). Then, the BaP antigens and bio-pAb-BaP were prepared. For the preparation of BaP hapten, antigens, and pAb-BaP, we followed the methods of Ma and Zhuang 2017 [21].

BaP (200 mg), n-iodosuccinimid (NIS, 210 mg), and acidic alumina (5 g) were dissolved in toluene (15 mL); the mixture was allowed to react with stirring for 4 days

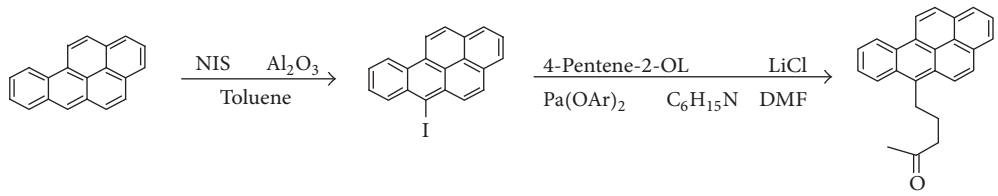


Figure 2: Synthesis of BaP hapten.

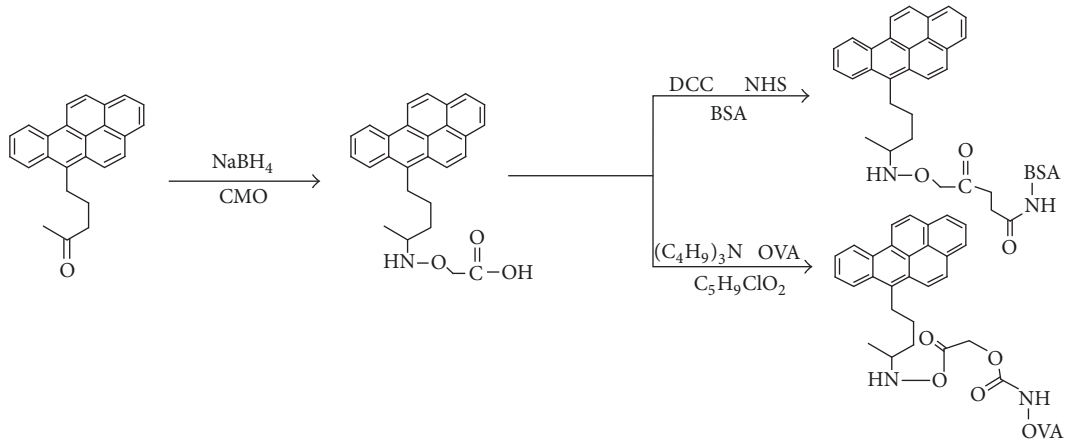


Figure 3: Synthesis of BaP immunogen and coating antigen.

at 20°C. The residual acidic alumina was washed with dichloromethane (25 mL × 4). Afterwards, the organic phase was collected and extracted by saturated sodium sulphite, saturated salt water, and double-distilled water (ddH_2O) successively. After being dried with $MgSO_4$, the mixture was concentrated down to near dryness; then, the yellow powder (6-I-BaP, 225 mg, yield: 75.3%) was obtained by recrystallization with dichloromethane and petroleum ether.

Next, 6-I-BaP (135 mg), palladium acetate (5 mg), and LiCl (10 mg) were dissolved in DMF (10 mL). Then, 4-pentene-2-alcohol (35 μL) and triethylamine (100 μL) were added. The mixture was allowed to react for 15 h at 120°C. After a completed reaction, toluene (70 mL) was added. The reaction mixture was extracted by saturated salt water and ddH_2O, respectively. After being dried with $MgSO_4$, the volatile was removed under reduced pressure. The crude product was purified through chromatography column filled with silica gel (leaching solution: tert-butyl methyl ether/petroleum ether = 1/2 (v/v)), and then the yellow powder BaP hapten (BaP = O 102 mg, yield: 87.1%) was obtained.

2.4. Immunogen and Coating Antigen. As a contact portion between carrier protein and BaP hapten, the linking arm can become an antigenic determinant to affect the specificity for conjugating with antibodies [22]. In order to reduce specific binding caused by the linking arm, BSA-BaP (immunogen) and OVA-BaP (coating antigen) were prepared by the activated ester method and mixed anhydride method, respectively (Figure 3).

BaP hapten (8 mg) and carboxymethyl amine hydrochloride (16 mg) were dissolved in pyridine (5 mL); the mixture was allowed to react in absence of light. After a completed reaction, sodium borohydride (36 mg) dissolved in ddH_2O (300 μL) was added to the reaction mixture and allowed to react for 5 h at ambient temperature. After the organic phase was evaporated to dryness, the crude product was washed with HCl solution (pH 2.0) and ddH_2O successively. After being dried, the yellow product BaP-COOH was obtained.

BaP-COOH (0.155 g) was dissolved in DMF (1 mL). N-Hydroxysuccinimide (NHS) and N, N′-dicyclohexylcarbodiimide (DCC) were dissolved in DMF (1 mL), respectively and added to the solution above successively. The mixture was allowed to react with stirring for 8 h at room temperature. After centrifugal separation, the obtained supernate was added dropwise into 10 mL of bovine serum albumin (BSA) solution (12 mg mL^{-1}, in PBS) and then stirred for 6 h at 4°C. The suspension was dialyzed against PBS for 3 days. After another centrifugal separation, the supernate (immunogen, BSA-BaP) was stored at −20°C.

BaP-COOH (0.155 g) was dissolved in DMF (1 mL), and then n-butylamine (50 μL) and isobutyl chloroformate (62.5 μL) were added sequentially. The mixture was stirred magnetically for 3 h at 4°C. Afterwards, the mixture was added dropwise into 10 mL of egg albumin (OVA) solution (12 mg mL^{-1}, in PBS) and allowed to react with stirring for 5 h at 4°C. The suspension was dialyzed against PBS for 3 days. After centrifugal separation, the supernate (coating antigen, OVA-BaP) was stored at −20°C.

2.5. Preparation of Bio-pAb-BaP. In this research, polyclonal anti-BaP antibody (pAb-BaP) was prepared by taking healthy rabbits as immune objects. Two white rabbits

were immunized with BSA-BaP through subcutaneous and intramuscular injections. The immunization lasted for 4 months. pAb-BaP was separated from rabbit serum through ammonium sulphate precipitation method, dialyzed against PBS for 5 days, and stored at −20°C.

The prepared pAb-BaP was diluted with CBS buffer at a concentration of 2.0 mg mL^{-1}. Then, the solution was mixed with 1.0 mg mL^{-1} BNHS (in DMSO) in the mass ratio of 1 : 10. The mixture was stirred for 4 h, and dialyzed against PBS for 3 days. The obtained bio-pAb-BaP was stored at −20°C.

The specificity of pAb-BaP was evaluated by testing cross-reactivity (CR) using other types of PAHs. The CR values were calculated according to the formula CR (%) = (IC_{50} of BaP)/(IC_{50} of analogues) × 100%. The chemical structures of BaP analogues and CR results are shown in Table 1. In all cases, there was a low CR (below 7%) between BaP and other structurally similar compounds, indicating that the prepared pAb-BaP was suitable for detecting trace amount of BaP.

2.6. Preparation of BaP Samples. $PM_{2.5}$ (particles in the air, the aerodynamic diameter of which is less than or equal to 2.5 μm) and agricultural soil samples were collected in Shanghai, China; the locations of sampling sites are shown in Figure 4. Vegetables and cereals were purchased from a local food market; roast lamb and duck were obtained from a barbecue shop. The treatment of environmental samples was similar to the description of the standard of People's Republic of China HJ 784-2016. First, $PM_{2.5}$ sampling membranes were broken down into pieces; agricultural soil samples were ground into powder after being freeze-dried. Then, each $PM_{2.5}$ sampling membrane (in pieces) and agricultural soil (10 g) were extracted for 16 h by soxhlet extraction with acetone/hexane (1 : 1, v/v, 50 mL) and centrifuged at 4500 rpm for 10 min. The organic layer was dried with Na_2SO_4 and then concentrated to nearly 2 mL.

The food samples were treated as described in the standard of People's Republic of China GB/T 5009. 27-2003. The vegetable samples (100 g) were grinded into pieces; the cereal samples (50 g) and barbecue food (50 g) were crushed. Next, all the samples were extracted by ultrasonic processing with hexane/acetone (1 : 1, v/v, 100 mL × 2) for 0.5 h. For the treatment of vegetables, the extract was washed with cyclohexane (100 mL × 2) and ddH_2O (100 mL × 2) successively. After being dried with Na_2SO_4, the cyclohexane layer was condensed to about 2 mL. In the case of the barbecue food and cereals, the extract mixed with CH_3OH-H_2O (9 : 1, v/v, 150 mL) was heated at 80°C for 6 h. Then, the mixture was extracted by cyclohexane (100 mL × 2); the cyclohexane layer was washed by CH_3OH-H_2O (1 : 1, v/v, 100 mL × 3) and ddH_2O (200 mL × 2) successively. Next, the collected organic layer was condensed to about 40 mL and then washed with H_2SO_4 (60%, 100 mL) and ddH_2O (25 mL × 4) successively. The organic phase was dried by Na_2SO_4 and then condensed to about 2 mL.

All the extract was purified with a florisil column which was treated with cyclohexane/dichloromethane (1 : 1, v/v, 20 mL). Benzene (10 mL) was used as the leaching solution, and then the eluent was collected and condensed to near dryness. Afterwards, the residue was redissolved in acetonitrile (0.5 mL). The treated BaP samples above were detected by SA-ELISA after being diluted by 1000 times with PBS containing 5% DMSO as well as the HPLC method.

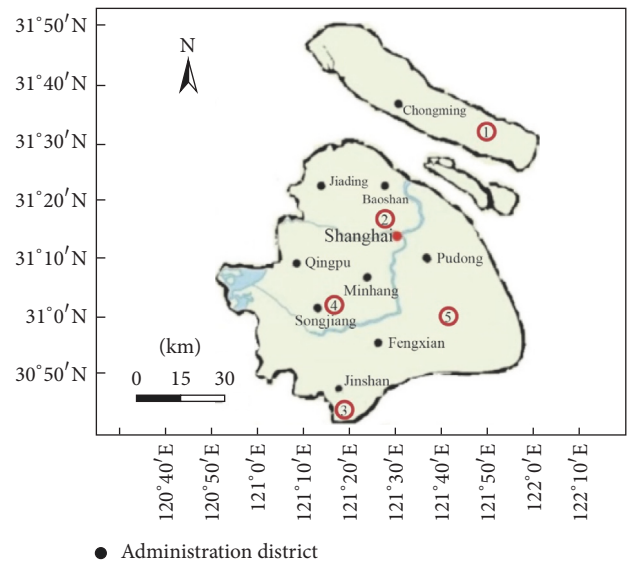


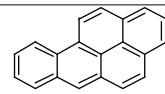
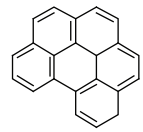
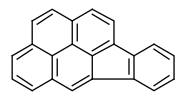
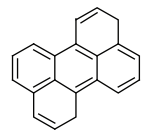
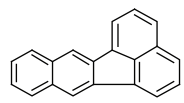
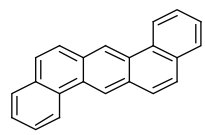
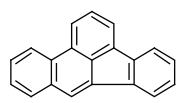
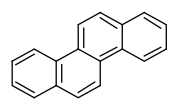
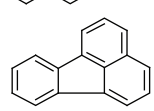
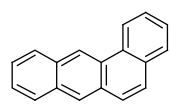
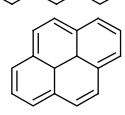
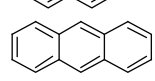
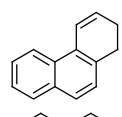
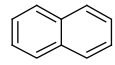
FIGURE 4: Locations of sampling sites in Shanghai, China. $PM_{2.5}$ samples were collected at sampling sites (1), (2), and (3). (1) Chongming Island; (2) central area of Hongkou; (3) Jinshan District. Agricultural soils were collected at sampling sites (4) and (5) (local vegetable bases).

2.7. Establishment of the Immunoassay. The SA-ELISA determinations were performed in a 96-well microtitre plate. First, microplates were covered with 100 μL/tube of coating antigen at 4°C overnight. After washing, the unbound active sites were blocked with 200 μL/tube of blocking reagent. Then, the mixture was incubated for 1 h at 37°C. Next, 50 μL/tube of BaP standard and 50 μL/tube of bio-pAb-BaP dilution were added after additional washing and incubated for 1 h at 37°C. After another washing step, 100 μL/tube of SA-HRP dilution was added, and the mixture was incubated for 1 h at 37°C. After a final washing step, 100 μL/tube of TMB + H_2O_2 substrate solution was added. The enzymatic reaction was stopped by adding 50 μL terminating solution (2 mol L^{-1} H_2SO_4) after sufficient colour development. The absorbance of each well was immediately recorded in dual-wavelength mode (absorbance in 450 nm as test and 630 nm as reference).

Under optimal conditions, the standard curve was constructed by plotting average inhibition (%) values of six replicates against the logarithm of BaP concentrations. Limit of detection (LOD) was evaluated in terms of IC_{10} (concentration at which a compound inhibits a particular phenomenon by 10%). Inhibition was calculated as follows (A_{max} is the absorbance in absence of BaP; A_{min} is the absorbance of blank sample; and A_s is the absorbance of BaP standard solutions):

$$\text{Inhibition (\%)} = \frac{(A_{max} - A_{min}) - (A_s - A_{min})}{A_{max} - A_{min}} \times 100\%. \quad (1)$$

Table 1: Cross-reactivity of pAb-BaP with structural analogues.

Analogues	Structure	IC_{50} (ng mL^{-1})	Cross-reactivity (%)
Benzo(a)pyrene		0.67	100
Benzo[ghi]perylene (BghiP)		30.45	2.2
Indeno[1,2,3-cd]pyrene		23.10	2.9
Perylene		10.31	6.5
Benzo[k]fluoranthene		24.81	2.7
Dibenz[a,h]anthracene		9.71	6.9
Benzo[b]fluoranthene		23.93	2.8
Chrysene		19.14	3.5
Fluoranthene		111.67	0.6
Benz[a]anthracene		18.11	3.7
Pyrene		17.63	3.8
Anthracene		335.12	0.2
Phenanthrene		223.33	0.3
Naphthalene		>3350	<0.02

3. Results and Discussion

3.1. Characterization of BaP Hapten and Antigens. The molecular weight of BaP hapten is 336.374. As shown in Figures 5, 6(a), and 6(b), [M^+] ion peak was 337.16, Mass spectrometry was similar to the characterization of BaP = O. ^{1}HNMR (400 MHz $CDCl_3$): δ = 2.18 (t, 2H, 4′-H), 2.22 (s, 3H, 1′-H), 2.68 (t, 2H, 3′-H), 3.78 (t, 2H, 5′-H), 7.83–7.85 (m, 2H, 8-H, 9-H), 7.95 (d, 1H, 4-H), 7.97 (t, 1H, 2-H), 8.07 (d, 1H, 1-H), 8.21 (d, 1H, 12-H), 8.28 (d, 1H, 7-H), 8.34 (d, 1H, 5-H), 8.61–8.64 (m, 1H, 3-H), 9.07 (d, 1H, 11-H), 9.14–9.16 (m, 1H, 10-H) ppm. ^{13}CNMR (100 MHz $CDCl_3$): δ = 25.0 (C-4′), 27.4 (C-5′), 30.1 (C-1′), 43.1 (C-3′), 122.1 (C-11), 123.6 (C-10), 123.8 (C-12c), 124.4 (C-5), 124.6 (C-1), 125.0 (C-7), 125.6

Table 2: The optimization of coating antigen.

Coating antigen	A_{0max}	IC_{50} ($\mu g\ L^{-1}$)	A_{0max}/IC_{50}	Abs (negative group)
OVA-BaP	0.85	1.19	0.71	0.071
KLH-BaP	0.87	1.21	0.72	0.102
PLL-BaP	0.91	1.13	0.81	0.121

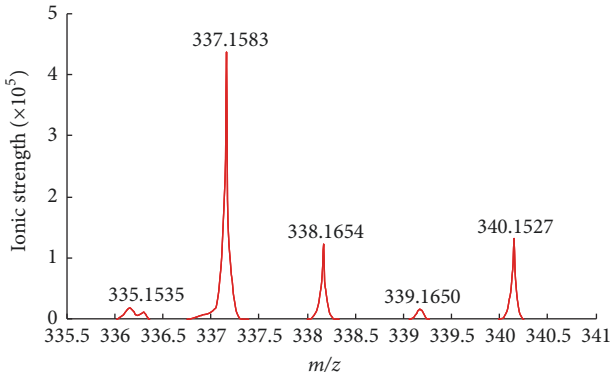


Figure 5: Mass spectrum of BaP hapten.

Table 3: The optimization of coating condition for OVA-BaP.

Coating condition	A_{0max}	IC_{50} ($\mu g\ L^{-1}$)	A_{0max}/IC_{50}
4°C overnight	1.07	1.73	0.62
25°C			
1 h	0.76	2.39	0.32
2 h	0.81	2.21	0.37
37°C			
1 h	0.92	2.26	0.41
2 h	1.05	1.78	0.59
43°C			
1 h	0.72	2.51	0.29
2 h	0.63	3.37	0.19

(C-3, C-9), 126.0 (C-2), 126.3 (C-8), 126.5 (C-10b), 126.9 (C-4, C-12b), 127.3 (C-5a), 128.1 (C-12), 128.3 (C-10a), 129.9 (C-6a), 131.2 (C-3a), 131.5 (C-12a), 132.6 (C-6), 208.8 (C-2′) ppm. According to the characteristic results above, the BaP hapten was synthesized successfully.

The prepared BaP-COOH-protein conjugates BSA-BaP and OVA-BaP were identified by the ultraviolet-visible (UV-vis) spectrophotometer, respectively. As shown in Figure 7, there were relationships and differences among the characteristic peaks of BaP-COOH, proteins, and conjugates. Moreover, new absorption peaks appeared at 361 nm (for BSA-BaP) and 373 nm (for OVA-BaP) in the UV-vis spectrum of BSA-BaP and OVA-BaP. The results revealed that the conjugates were successfully prepared. Measured by Coomassie Brilliant Blue assay, concentrations of BSA-BaP and OVA-BaP were 5.02 and 2.83 mg mL^{-1}, respectively.

3.2. Optimization of Reaction Conditions. Coating antigens prepared in different carrier proteins have different structures and molecular weights, which can lead to different abilities to react with the prepared antibody specifically. We selected KLH and PLL as carrier proteins and prepared another two types of coating antigens using the mixed anhydride method described above. Different coating antigens (OVA-BaP, KLH-BaP, and PLL-BaP) were compared. As shown in Table 2, there were no obvious differences between the IC_{50} values as well as A_{0max}/IC_{50}. Considering the background interferences, OVA-BaP, which obtained the minimum absorbance value (Abs) of the negative control group, was more appropriate for the immunoassay.

The interaction between proteins may affect the adsorption of proteins on the surface of polystyrene carrier at high temperature. Moreover, a long time of incubation has influences on the coating effect and the activity of coating antigens. Therefore, the appropriate incubation condition has a significant effect on the coating step. Several incubation conditions (4°C overnight, 25°C for 1 h and 2 h, 37°C for 1 h and 2 h, and 43°C for 1 h and 2 h) during the coating step were investigated. As shown in Table 3, IC_{50} value is the lowest and A_{0max}/IC_{50} is maximum in the condition of incubation at 4°C overnight. Therefore, the coating step was completed at 4°C overnight.

The optimal concentrations of bio-pAb-BaP and coating antigen were used to improve the sensitivity of the immunoassay. The number of immune complexes reduces rapidly if concentrations of the reagents are not appropriate. A chessboard assay was used to optimize the working concentrations of coating antigen and bio-pAb-BaP. The optimal reaction conditions are the lowest bio-pAb-BaP and coating antigen concentrations that resulted in the maximum absorbance (A_{0max}) of approximately 1.0. As shown in Table 4, the optimal coating antigen concentration was 5.66 $\mu g\ mL^{-1}$ and bio-pAb-BaP was at a dilution to 1 : 2000 (0.493 $\mu g\ mL^{-1}$).

Blocking is to eliminate the unoccupied sites on microplates; otherwise the unoccupied sites may interact with the components, such as bio-pAb-BaP and SA-HRP during the subsequent steps. The optimum blocking reagent should achieve the minimal background interference. Several blocking reagents (in PBS) were investigated. As shown in Figure 8(a), 1% gelatin, which achieved the minimal background interference (0.079), was used in the immunoassay.

Different incubation temperatures during the blocking step were compared. The optimum incubation temperature should cause the least background interferences, that is, the lowest absorbance value. As shown in Figure 8(b), the absorbance values obtained at 37°C and 43°C were obviously lower than others. Considering the effect on the activity of the coating antigen, 37°C was selected as incubation temperature during the blocking step.

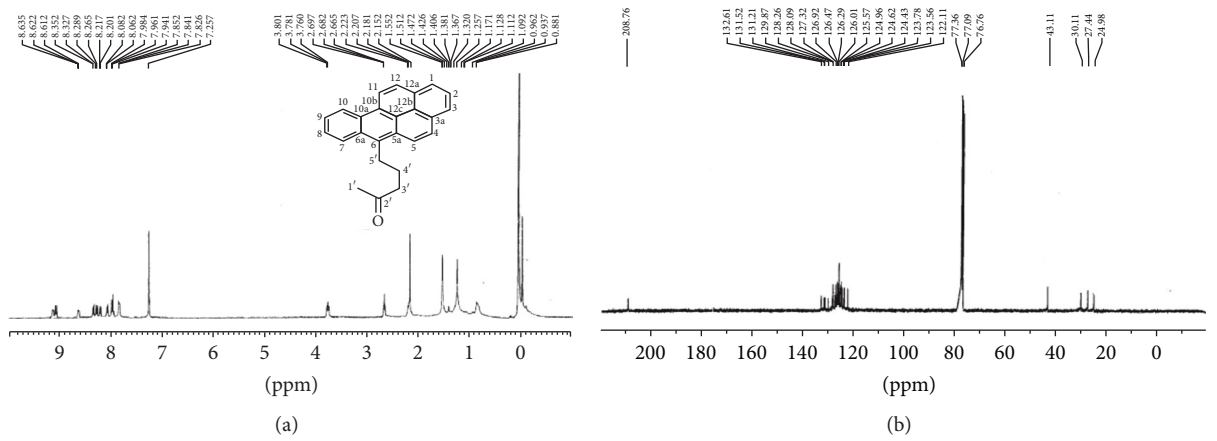


Figure 6: (a) The ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectrum of BaP hapten. (b) The ^{13}C-NMR spectrum of BaP hapten.

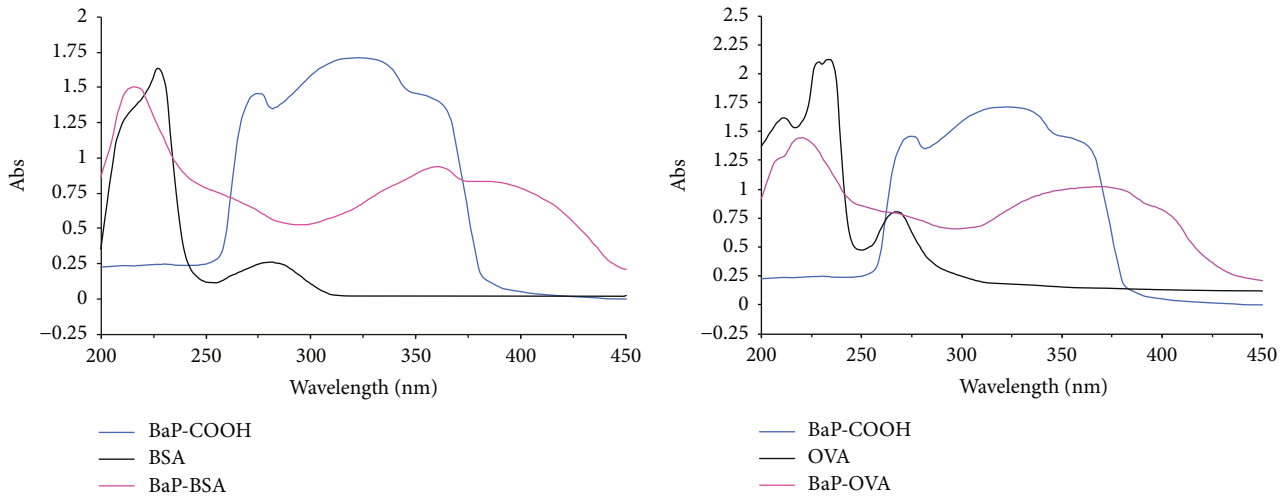


Figure 7: UV-vis spectrum of BaP-COOH, proteins, and conjugates. The proteins and conjugates were dissolved in PBS; the BaP-COOH was dissolved in DMSO.

As BaP is a type of hydrophobic organic compound, organic solvent needed to be added in the preparation of the BaP solution. Different concentrations of organic solvent may affect the adsorption capacity of the antibody. The effect of DMSO in different concentrations on the assay was investigated. The result is shown in Figure 8(c). PBS containing 5% DMSO, which induced the lowest IC_{50} value (0.22), was selected to improve the analyte solubility in the follow-up test.

Dilutions of SA-HRP (500, 1000, 1500, 2000, and 3000) were also investigated. The nonspecific reaction will increase at high SA-HRP concentrations; however the sensitivity of the immunoassay may be influenced at low SA-HRP concentrations. As shown in Figure 8(d), A_{0max}/IC_{50} was the maximum and the IC_{50} value was the lowest when the SA-HRP was diluted with PBST to 1 : 1000 (IC_{50} = 0.49 μg mL^{-1}, A_{0max} = 1.07). Hence, the SA-HRP was at a dilution of 1 : 1000.

In the immune reaction of antigen and antibody stage, different incubation times (15, 30, 45, 60, 75, and 90 min) were investigated. As shown in Figure 8(e), the A_{0max} value increased with the increase of incubation time. The IC_{50} value (0.63) was lowest at 60 min and A_{0max}/IC_{50} was the maximum. So, an incubation time of 60 min was selected for the reaction system.

In the reaction system, the hydrogen bonding force of antigen and antibody may be influenced by pH value; different ionic strength also has an effect on the specificity of the immune reaction. As shown in Figures 8(f) and 8(g), A_{0max} decreased with the increase of salt concentrations and the lowest IC_{50} (1.81) was induced at an ionic strength of 0.1 mol L^{-1}. A_{0max} values decreased with the increase of pH and the appropriate IC_{50} (1.50) was induced at pH 7.40. So, the salt concentration was 0.1 mol L^{-1} and the pH of buffer was 7.40 in the immunoassay.

3.3. Standard Curve and Linear Working Range for BaP. A series of concentrations of BaP standard solutions (0.001 ng mL^{-1}, 0.005 ng mL^{-1}, 0.01 ng mL^{-1}, 0.05 ng mL^{-1}, 0.1 ng mL^{-1}, 0.5 ng mL^{-1}, 1 ng mL^{-1}, 5 ng mL^{-1}, 10 ng mL^{-1}, 50 ng mL^{-1}, 100 ng mL^{-1}, 500 ng mL^{-1}, and 1000 ng mL^{-1})

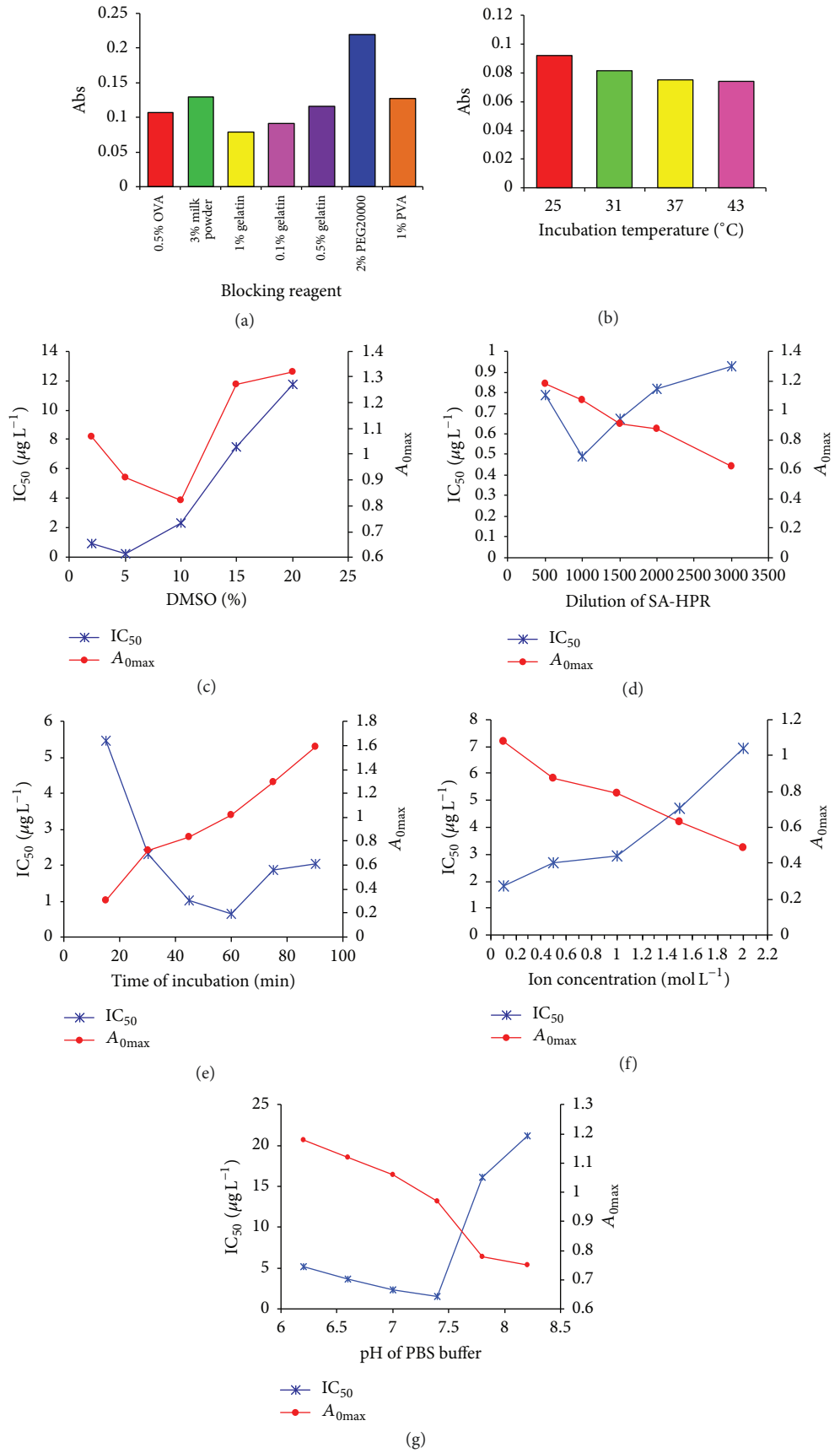


Figure 8: The optimal conditions of the immunoassay. (a) Blocking reagent. (b) Incubation temperature during blocking step. (c) DMSO concentration in PBS buffer. (d) SA-HRP concentration. (e) Incubation time. (f) Ion concentration of PBS. (g) pH of PBS.

Table 4: Optimal concentrations of OVA-BaP and bio-pAb-BaP.

Dilutions of bio-pAb-BaP[b]	OVA-BaP concentration (μg mL^{-1})[a]					
	28.3	14.15	5.66	2.83	1.42	0.71
100	2.855	2.166	2.011	1.972	2.244	2.612
500	1.997	1.657	1.721	1.851	1.879	2.446
1000	1.275	1.419	1.396	1.367	1.208	1.468
2000	0.865	1.036	1.039	0.910	0.820	0.907
4000	0.393	0.750	0.902	0.490	0.392	0.636
Blank	0.136	0.129	0.167	0.159	0.137	0.162

[a]The initial concentration of the prepared OVA-BaP was 2.83 mg mL^{-1}; the OVA-BaP was diluted with PBS buffer. [b]The initial concentration of the prepared bio-pAb-BaP was 0.985 mg mL^{-1}; the bio-pAb-BaP was diluted with CBS buffer.

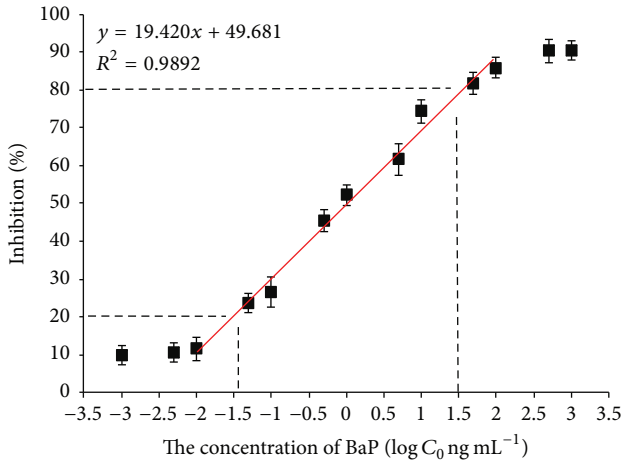


Figure 9: Standard curve of SA-ELISA.

were prepared by PBS with 5% DMSO, and the inhibition was determined under optimal conditions. As shown in Figure 9, linear regression equation is as follows: $y = 19.420 \log C_0 + 49.681$; $R^2 = 0.9892$. Linear working range, which was determined as concentration range that causes 20–80% colour inhibition, was 0.03–35.40 ng mL^{-1}. IC_{50}, which is an important criterion for evaluating sensitivity of the immunoassay, was 1.04 ng mL^{-1}. The limit of detection (IC_{10}) was 0.0094 ng mL^{-1}. In comparison with the sensitive indirect competitive enzyme-linked immunosorbent assay for detecting BaP [22], in which antibody-enzyme conjugates were used (LOD is 0.024 ng mL^{-1}), LOD of the SA-ELISA is obviously lower.

3.4. Repeatability of the Immunoassay. The stability of the proposed immunoassay was estimated by the coefficient of variation (CV). Different concentrations of BaP standard samples (0.05 ng mL^{-1}, 0.1 ng mL^{-1}, 0.5 ng mL^{-1}, 1 ng mL^{-1}, 5 ng mL^{-1}, 10 ng mL^{-1}, and 50 ng mL^{-1}) were run in the SA-ELISA method (Table 5). The intra-assay repeatability was evaluated after performing six replicates, and the interassay repeatability was evaluated over several weeks. The CV of intra-assay was <10% and the CV of interassay was <12%, indicating that repeatability and stability of the immunoassay were well.

3.5. Determination of BaP in Practical Samples. The proposed SA-ELISA method was used to detect BaP in several environmental and food samples. The results are shown in Table 6. As the combustion of fossil fuels and industrial production process contribute greatly to BaP emissions, BaP concentration of $PM_{2.5}$ samples collected at site 3 which is near a small industrial district was the highest. BaP concentration at site 2 was also significantly high, which was mainly caused by BaP emission of automobile exhaust and human daily lives. In addition, BaP concentration in the agricultural soil sample at site 4 was generally higher than that of site 5. This may be mainly caused by the influences of a small gas distribution plant near site 4.

These practical samples were also tested by HPLC to validate the immunoassay. As shown in Table 6, compared with HPLC analysis, the results detected by SA-ELISA were slightly higher. This may be caused by nonspecific adsorption of reagents used in the immunoassay and the cross-reactivity of pAb-BaP with other PAHs existed in the samples, which failed to be analyzed by HPLC method and could contribute to SA-ELISA-derived concentrations. However, the results of the immunoassay still have good consistency with those of HPLC. Statistical study showed that the correlation coefficient was 0.9887 (linear regression equation: $y = 0.9555x - 0.3447$), and p value from the paired sample t-test in comparison with the two methods was 0.0025. That means the difference was statistically significant at 0.05 levels. As the immunoassay was performed in a 96-well microtitre plate in a high throughput detecting manner, it took about 6 h to complete the analysis of 17 treated BaP samples, while HPLC spent about 76.5 h for the analysis (analysis for each sample needs about 15 min and the column needs to be washed after each detection (30 min); six reptiles were tested for each practical sample); it can be concluded that this immunoassay is rapid and effective.

3.6. Recovery Tests. The recovery of several practical samples spiked with BaP analyte was calculated to evaluate accuracy and precision of the immunoassay. Three practical samples (A_2, P_3, and spinach) were spiked with BaP standard in different concentrations ranging from 1–50 ng g^{-1} before extraction. The spiked samples were treated as described above and then tested by the SA-ELISA method. As shown in Table 7, the recovery of SA-ELISA was 91.12–109.23% and the

Table 5: The variability of intra-assay and interassay for BaP.

Object	BaP concentration ($ng\,mL^{-1}$)						
	0.05	0.1	0.5	1	5	10	50
Intra-assay CV (%)	9.69	7.71	6.27	5.99	4.81	4.06	3.52
Interassay CV (%)	11.91	10.96	10.61	9.69	9.02	7.65	6.79

Table 6: Determination of BaP concentration by SA-ELISA and HPLC.

Samples (n = 17)	BaP concentration ($ng\,g^{-1}$, mean ± SD); n = 6	
	SA-ELISA	HPLC
$PM_{2.5}$ particles (n = 3)		
P_1	5.91 ± 0.106	5.67 ± 0.046
P_2	12.11 ± 0.129	11.96 ± 0.053
P_3	31.03 ± 0.201	30.09 ± 0.101
Agricultural soil (n = 2)		
A_1	7.99 ± 0.142	6.02 ± 0.061
A_2	12.98 ± 0.119	10.11 ± 0.049
Vegetables (n = 5)		
Ipomoea aquatica	0.75 ± 0.061	0.69 ± 0.018
Brassica alboglabra Bailey	1.01 ± 0.087	0.95 ± 0.035
Radish	0.95 ± 0.079	0.87 ± 0.034
Spinach	1.62 ± 0.077	1.52 ± 0.020
Chinese cabbage	1.79 ± 0.095	1.51 ± 0.031
Cereals (n = 3)		
Rice	2.81 ± 0.052	2.27 ± 0.015
Millet	3.92 ± 0.107	3.12 ± 0.038
Corn	2.91 ± 0.091	2.51 ± 0.026
Barbecue food (n = 4)		
Roast meat	6.21 ± 0.122	6.01 ± 0.051
Roast fish	7.97 ± 0.136	6.21 ± 0.048
Roast bean curd	5.97 ± 0.127	5.72 ± 0.053
Roasted duck	6.62 ± 0.112	6.45 ± 0.040

CV was 4.63–8.79%. It can be concluded the immunoassay presents good accuracy.

3.7. Comparison with the Correlated Immunoassay. In the highly sensitive BA-IPCR method for detecting BaP (linear range is $5\,pg\,L^{-1}$–$10\,ng\,L^{-1}$; LOD is $2.85\,pg\,L^{-1}$) [21], the analysis is based on the PCR technique and the fluorescence labeled in DNA is used as detection signal. The BA-IPCR assay requires an additional step to pretreat PCR tubes to improve the adsorption capacity while there is no need for this operation in the SA-ELISA method. Moreover, after the similar coating and blocking processes, it only needs to add bio-pAb-BaP for immune reaction and to add SA-HRP for chromogenic reaction prior to the final instrumental detection in the SA-ELISA method; however, the BA-IPCR needs step-by-step use of bio-pAb-BaP, streptavidin, biotinylated DNA (bio-DNA), and then PCR mixture, which is much more complicated. With the 96-well microtitre plate, the analysis of nearly 100 BaP samples can be achieved within 6 h while the BA-IPCR spends about 12 h (including the pretreatment of PCR tubes). Therefore, the proposed SA-ELISA is more rapid and convenient. Although the BA-IPCR assay is more sensitive, the actual linear working range is limited due to its trace levels, which may lead to a certain limitation for the detection of some practical samples. In addition, the price of bio-DNA used for PCR analysis is very expensive; this increases the economic cost greatly. Therefore, we consider the SA-ELISA method has a better applicability for detecting BaP samples.

4. Conclusions

In this study, a specific BaP hapten was synthesized, and diverse BaP antigens and relative polyclonal antibody were prepared; then, a streptavidin-horseradish peroxidase-based enzyme-linked immunosorbent assay was developed and used for the sensitive and high throughput monitoring of BaP in environmental and food samples. Under optimal conditions, this proposed method had a low limit of $0.0094\,ng\,mL^{-1}$. Linear working range was from $0.03\,ng\,mL^{-1}$ to $35.40\,ng\,mL^{-1}$. The recovery of spiked samples ranged from 91.12% to 109.23%. IC_{50} value of the assay

Table 7: Determination of BaP in spiked samples.

Samples	BaP concentration ($ng\,g^{-1}$)	Spiked level ($ng\,g^{-1}$)	Average recovery (%) ± CV (%) ($n = 6$)
Spinach	1.62	1	105.61 ± 5.49
		2	102.35 ± 7.61
		5	91.12 ± 4.63
A_2	12.98	5	97.03 ± 6.22
		10	103.11 ± 7.09
		20	95.77 ± 5.26
P_3	31.03	10	99.09 ± 8.79
		20	109.23 ± 7.61
		50	92.71 ± 8.26

was $1.04\,ng\,mL^{-1}$. The SA-ELISA could selectively detect BaP with negligible CR values below 7% against several structural BaP analogues. Meanwhile, the results of BaP detection for several practical samples were consistent with those obtained by HPLC, indicating the proposed method was accurate and reliable. Moreover, due to the application of a 96-well microtitre plate, this immunoassay had potential for the batch-testing detection of BaP samples. Therefore, the established SA-ELISA is a useful option for the batch detection of BaP in environmental and food samples with specificity and sensitivity.

Ethical Approval

The studies with animals were performed following all institutional and national guidelines for the care and use of laboratory animals.

Conflicts of Interest

The authors have no conflicts of interest regarding the publication of this paper.

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