3,3',4,4'-Tetrachlorobiphenyl (IUPAC PCB77) is one of seven indicative polychlorinated biphenyls (PCBs) in the surface sediments. The current study presents a novel polyclonal antibody for the determination of the PCB77 using indirect competitive enzyme-linked immunosorbent assay. Under optimum conditions, PCB77 was determined within the concentration range of 0.01-100 µg L\(^{-1}\), with a detection limit of 0.057 µg L\(^{-1}\). The assays were tested for their cross-reactivity profiles using 3 selected congeners and 4 Aroclor products. The assays were highly specific for coplanar PCB congeners, but less specific for Aroclor1248. The spiked recoveries from five sediment samples were 86%-114% for PCB77 from ELISA, which were satisfactory. The current study demonstrated that the developed antiserum and immunoassay procedure can be used to detect PCB77 in environmental samples. The results of the sediment analysis were confirmed by conventional GC/ECID.

**Key Words**: 3,3',4,4'-Tetrachlorobiphenyl (IUPAC PCB77), Immunoassays, Coplanar polychlorinated biphenyls, Antigen, Polyclonal antibody

**Introduction**

Polychlorinated biphenyls (PCBs) are commercially produced as complex mixtures for a variety of uses, including dielectric fluids in capacitors and transformers. Consequently, PCBs are widely distributed in the environment\(^1,2\) and are regarded as serious environmental pollutants. PCB production has been banned because of its carcinogenic properties, persistent environmental accumulation, as well as harmful effects to humans and on the ecology\(^3,5\). Although PCB products have been prohibited 30 years ago, they are still being detected in various environments because of their long-term stability\(^6,9\).

Among the 209 PCB congeners, the non-ortho chloro-substituents are called coplanar PCBs (Co-PCBs) or dioxin-like PCBs. They exhibit the highest dioxin-like activities based on their abilities to interact with the aryl hydrocarbon receptor. In terms of dioxin equivalents, PCB28, PCB 52, PCB 77, PCB118, PCB126, PCB153, and PCB169 are considered the most toxic congeners\(^10,11\). Most of researchers regard these seven PCBs congeners as indicative PCBs in the sea surface sediments. Studies on environmental PCB determinations mainly focus on these congeners\(^12-16\). Given their high toxicities relative to other congeners, assays capable of quantifying one or all of these congeners need to be established essentially.

Co-PCB concentrations in environmental samples are at much lower than level others. Therefore, great efforts are being exerted to discover assay techniques for these PCBs\(^17\). Among such detection techniques, instrumental methods are relatively accurate. Immunoassays, such as enzyme-linked immunosorbent assays (ELISAs) and those that use biosensors, are more selective and simple. The most significant advantages of ELISAs over traditional instrumental methods are their high sensitivity and specificity, simple sample preparation, and high throughput. In recent years, several competitive ELISAs with different sensitivities and specificities for the detection of Co-PCB congeners in various environmental samples have been reported. These ELISAs are based on polyclonal (pAb) and monoclonal (mAb) antibodies\(^18-21\). In detecting trace level of PCBs sample toxicity, the preparation of highly sensitive and specific antibody against Co-PCBs has significant impact. Chiu et al. and Fránek et al. have studied the determination of PCB77 by the method of ELISAs, mAbs were used by the former, and pAbs were used by the latter. Due to the diverse hapten and process of making antibody, their results show different specificities and sensitivity to PCB77. Further investigations are still needed to promote this promising approach.

Immmunochemical methods for the PCBs determination are rapidly developing, but their extensive use is hindered by the difficult production of single PCB congeners and corresponding antibodies\(^22\). In the current work, a new method was developed to produce the PCB77 congener and its artificial hapten, PCB77 butanoic acid (γ-oxo-PCB77A). A mixed anhydride reaction was used to couple the PCB77A to ovalbumin (OVA) to form an artificial coating antigen. The active ester method was used to couple the PCB77A with bovine serum albumin (BSA) to form an artificial immune antigen. Male New Zealand white rabbits were immunized with the immune antigen to obtain pAbs. Using this pAbs, a functional indirect competitive assay systems for detecting...
the PCB77 was developed.

Experimental

Reagents and Instrumentation. Dimethylsulfoxide (DMSO), Goat anti-rabbit IgG-HRP, Sephadex G-25, BSA, and OVA were purchased from the Sino-American Biotechnology Co. (Shanghai, China). Freund’s complete adjuvant was prepared in our laboratory.

Aroclor 1242, 1248, 1254, and 1260, PCB12, 15, 37, and PCB77 in isooctane (100 μg mL⁻¹) from J&K Chemical (Shanghai, China) were used to prepare a calibration series. The buffers for the immunoassay procedure were prepared by routine methods. All solutions were stored at 0-4 °C. Ultrapure water (18.25 MΩ cm⁻¹) was used in all reactions and solution preparation. All reagents and solvents were analytical reagent grade and used without further purification.

The equipment included a TU-1900 UV/Vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd), Multiskan-Mk3 plate reader (Thermo Scientific Co., Ltd), and solution preparation. All reagents and solvents were 0.1 mol L⁻¹ saline (PBS) was composed of 0.01 mol L⁻¹ sodium citric acid and phosphate buffer, pH 5.5.

Preparation of PCB77 Congener and its Antigen. The PCB congeners PCB77 was newly synthesized by the modified Gomberg-Bachmann reaction, as described in Figure 1.

3,4-Dichloroanilin (0.05 mmol) and distilled water (5 mL) were placed in a beaker and heated until almost melting. Concentrated hydrochloric acid (10 mL) was added to the mixture under vigorous stirring, and the mixture was again heated until melting. The mixture was then cooled in an ice bath, and a cold solution of NaN₂ (30%) was added dropwise. The reaction endpoint was confirmed using a starch KI indicator paper. Excess HNO₃ was eliminated by adding solid urea. Precocooned 3,4-dichlorobenzene (80 mL) was added to this ice-cold diazonium clay complex, which was slowly made basic with NaOH solution (5 mol L⁻¹), and constantly stirred at room temperature for 2 h. The reaction mixture was transferred into a 250 mL round bottom flask, and steam distillation was twice sequentially performed. The yellow crude products were extracted with n-hexane and dichloromethane (1:1 v/v), and deoxidized with 2 g of Zn and 2 mL of concentrated hydrochloric acid in anhydrous ethanol. The solvent was filtered and the white needle product appeared in crystalloid form. PCB77 was characterized by ¹H NMR, IR, and elemental analyses.

PCB77: IR (cm⁻¹) 1546, 1475, 1445, 1135, 828; ¹H NMR (δ ppm) 7.34 (dd, 2H, H-6,6' ), 7.50 (d, 2H, H-5,5' ), 7.60 (d, 2H, H-2,2'), elemental analysis calcd. for C₁₂H₄Cl₄O, C 49.3, H 2.054, N 0; found C 47.59, H 2.122, N < 0.030.

The PCB77 hapten was synthesized by the Friedel-Crafts acylation reaction using a slightly modified method. The PCB77 hapten was characterized by ¹H NMR, IR, and element analyses: IR (cm⁻¹) 3065, 2929, 1545, 1363, 878. ¹H NMR (δ ppm) 2.58 (d, 2H, CH₁₂), 7.43 (d, 1H, CH), 7.72 (d, 2H, H-2,2'), 8.0 (d, 1H, H-5), 12.1 (s, 1H, COOH). Elemental analysis calcld. for C₁₁H₁₀Cl₂O₂ C 48.9, H 2.551, N 0; found C 48.73, H 2.431, N 0.312.

Preparation of Polyclonal Antibodies. The PCB77A-BSA conjugate was used as an immunogen to immunize two female New Zealand white rabbits. The final serum was collected four months following the first immunization. The blood was collected and placed in a glass tube. The antisera was obtained by centrifugation, and the IgG fraction of the antisera was isolated by precipitation with saturated ammonium sulfate solution. After dialysis against PBS, the purified IgG fractions were lyophilized, aliquoted into vials, and stored at −20 °C until use.

ELISA Procedures. The indirect competitive ELISA (id-ELISA) procedures were performed as follow. PCB-OVA was diluted with CBS, and was pipetted (100 μL) into the wells of microtitre plates. Incubation overnight at 4 °C followed. The plates were thrice washed with 200 μL well⁻¹ PBST, and were blocked by incubation with 0.5% glutin in PBS at 37 °C for 1 h. After washing, a serially diluted analyte standard in PBS was added at 50 μL well⁻¹. Addition of purified antibodies (antisera) previously diluted with PBS (1/2000) at 50 μL well⁻¹ followed. The plates were incubated at 37 °C for 1 h and washed. Diluted goat anti-rabbit IgG-HRP (1:1000) was then added at 100 μL well⁻¹. The mixture was incubated at room temperature for 1 h and then thrice
washed. Subsequently, the substrate solution (100 μL) was added into each well. The enzymatic reaction was stopped by adding 2 M H$_2$SO$_4$ (50 μL). Absorbances were measured at 450 nm using a BIO-TEK microplate reader. Standard curves were obtained by plotting the absorbance against PCB77 concentration. Mean absorbance responses corresponded to three replicates.

**Immunossay Specificity.** To determine the specificity of our assay, three congeners and four Aroclor products were used in place of PCB77, and the same ELISA assay was performed. The standard curve for each compound was constructed (0.01–100 μg L$^{-1}$ in PBS), and their inhibition concentration (IC$_{50}$) was determined by the optimized ELISA. The cross-reactivity (CR) values were calculated according to the following equation: CR(%) = (PCB77IC$_{50}$/related compound IC$_{50}$) × 100.

**Sediment Samples and Fortification Experiment.** Five sediment samples from the inshore near Shanghai were used for analytical purposes. The sediment samples were collected and sieved to < 2 mm, and stored at 4 °C until analysis. The sample extraction and clean-up procedure was performed according to a previous study. For the immunoassay analyses, aliquots of the samples were spiked with known concentrations of the PCB77 standard solution covering the quantitative working range. The amount of PCB77 was determined based on calibration curves prepared for all sample batches.

**GC-ECD Analysis.** GC-ECD analysis was performed to evaluate the accuracy of the ic-ELISA using spBTM215-5 fused silica capillary column (30 m-0.25 mm; 0.25 μm film thickness) obtained from Agilent, a μ-ECD detector, and hydrogen as the carrier gas. The injector temperature was set to 250 °C and the detector temperature at 310 °C. The initial GC temperature was programmed to 100 °C for 2 min, then to 230 °C at 10 °C/min for 2 min. The temperature was then increased from 230 °C to 290 °C at 5 °C/min.

**Results and Discussion**

**Characterization of Immunogens.** The conjugation reactions were carried out using PCB77 hapten derivatives and carrier proteins (BSA and OVA). The reactant and product of the conjugates were scanned by UV spectroscopy. Figure 2 shows the qualitative differences between the carrier proteins and conjugates in the region of maximum absorbance of the hapten. A characteristic shoulder shifted to shorter wavelengths (PCB77 hapten, from 280 nm to 267 nm). It indicated that the coupling reaction is successful. The coupling ratios of the hapten to BSA and OVA were calculated using the molar extinction coefficient of the antigen and the BSA following formula: ratio = [ε$_{280}$ antigen-ε$_{280}$ protein]/ε$_{280}$ hapten. The molar ratios of PCB-BSA and PCB-OVA were 32:1 and 13:1, respectively.

According to the agar diffusion test, when the antigen concentration at 0.1 mg L$^{-1}$, the titers were 1:64. The ELISA titers were 1.28 × 10$^5$. These data illustrated that the PCB77-BSA conjugate was a good immunogen.

**Immunossay Optimization.** To monitor the low amounts of PCB77, a highly sensitive detection scheme was required. The scheme involved factors such as coating antigen concentration, dilution ratio of goat anti-rabbit IgG-HP, length and temperature of the antigen coated, pH, as well as ionic strength. The goat anti-rabbit IgG-HP were tested at 1:1000, 1:2000, and 1:4000 dilutions given the variations in this reagent. The results showed that a dilution of 1:1000 was the optimal working concentration. A 12 h application of the coating antigen at 4 °C was adequate. Although higher absorbance values were obtained when the coating process was longer, there was no significant gain in absorbance with increased time. Hence, overnight plate coating at 4 °C as usual was recommended in the present assay.

The influence of pH was tested by preparing PCB77 standards in pure water at pH values ranging from 5.0 to 9.0. The assay performed better in neutral or basic media, and it was inhibited below pH 6.0 (figure not shown). Different pH values can affect the activities of proteins; hence, pH 7.4 was considered optimal.

**Effect of DMSO on Dose-Response Curves.** PCBs are lipophilic compounds best extracted from environmental and food matrices using organic solvents. An effective water miscible solubilizer must also be present in the reaction mixture to solubilize PCBs for the immunochemical reaction.
Detection of PCB77 by id-ELISA in Sea Sediment Samples

The specificity of idELISA was evaluated by the CR of the pAb with three structurally related compounds of PCBs (PCB12, 15 and 37) as well as four other Aroclor products. Co-PCBs constitute a minor portion of commercial preparations, such as Aroclors and related industrial formulations. Therefore, the CR values of several Aroclor products containing unknown amounts of coplanar congeners were tested. The molecular structures of the testing compounds, IC$_{50}$, and CR values for each compound are given in Table 1. It can be seen that the antibody used in the current paper showed a high reactivity with coplanar PCB 77. From the CR values, the assay was evidently highly responsive to lower chlorinated formulations (Aroclors 1242 and 1248) than to those with higher ones (Aroclors 1254 and 1260). To the non-ortho congeners such as PCB12 and PCB37, the CR showed negative results, and the cross reaction ratios were below 8%. But to the coplanar compounds PCB15 exhibited distinct responses. However, all these congeners showed no more than 15% CR, indicating the good specificity of our method.

Calibration Curve. Under the selected optimal conditions, the calibration curve for the PCB77 antigen was constructed (Fig. 4). The linear range was 0.01 to 100 μg L$^{-1}$, and the limit of detection (LOD) was 0.057 μg L$^{-1}$. The LOD was determined as the analyte concentration giving 20% of the maximal inhibition (IC$_{20}$), and IC$_{50}$ was 5.62 μg L$^{-1}$. The IC$_{50}$ and LOD values achieved in the current study were very similar with that in using the mAb. Compared with the PCB linear range of 200 ng L$^{-1}$ to 14 μg L$^{-1}$ and the LOD of 1.3 μg L$^{-1}$, which have been reported by Fránek et al.,$^{20}$ the present method has a similar linear range level and lower LOD.

Sample Analysis. The analytical performance of ELISA is commonly assessed by the recovery of samples spiked with the target analyte. To test the accuracy and precision of the assay, the sediment samples were fortified with PCB77 at levels 1, 10, and 20 µg kg$^{-1}$ of the samples. The results of the recovery and coefficient of variation for PCB77 detection are shown in Table 2. The recovery (86%-114%) and reproducibility (relative standard deviation, RSD, < 10%) of the proposed method were satisfactory.

Conclusion

To increase the practicability of ELISA for determining PCBs in environmental samples, a new method for preparing PCB77 pAb was introduced. The molecular structures of
the hapten as well as immunogen were judged, and the titer as well as the specificity of anti-PCB77 antibody were validated. The proposed idELISA method using this antibody showed a good assay stability and high specificity to the one of most toxic coplanar congeners and Aroclor products. The optimized indirect ELISAs were calibrated in the working range of 0.01 to 100 μg L⁻¹ which appears to be sensitive enough for real sample analyses. Compared with the only previously published account of a polyclonal coplanar specific anti-PCB77 antibody, the current work developed a new synthesis scheme of the pAb, which showed a high specificity to the PCB77 and its homothetic PCBs. Some sediment samples were analyzed with satisfactory results to detect PCB77. There was good agreement between the results obtained by id-ELISA and GC/ECD, suggesting that the developed id-ELISA can be a cost-effective, fast, and reliable approach for monitoring PCB77 in sediment environments.

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