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Determination of 3,4-dichlorinated biphenyl in soil samples by real-time immuno-PCR assay

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Abstract

A real-time fluorescent quantitative immuno-polymerase chain reaction (RT-IPCR) assay was developed for the detection of non-dioxin-like polychlorinated biphenyl (PCB) congener in soil samples. Based on the construction of 3,4-dichlorinated biphenyl (IUPAC PCB12) hapten and its immunogen, the specific polyclonal antibodies (pAbs) to PCB12 was obtained and used to develop a direct competitive RT-IPCR assay. Using the optimized assay, a standard curve for PCB12 was prepared. The linear range for the determination of PCB12 was from 10.0 to 1.0×10^6 fg/mL with a correlation coefficient of 0.98 and a detection limit of 1.53 fg/mL. The RT-IPCR assays were tested for their cross-reactivity profiles using four selected congeners and four Aroclor products. The results for the soil samples correlated with the concentrations of PCBs obtained by gas chromatography/mass spectrometry. This highly specific, sensitive, and robust assay can be applied to on-site tests of PCBs and serve as a model for other pollutant immunoassays.

Key words: 3,4-dichlorinated biphenyl; antigen; polyclonal antibody; real-time immuno-PCR
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Introduction

Polychlorinated biphenyls (PCBs) continue to be an environmental concern because of their persistence, toxicity, bioaccumulative properties, and widespread distribution. Numerous reports have shown that PCBs are toxic to humans and animals (Bonefeld-Jorgensen et al., 2001), and possess carcinogenic and various toxic properties. Although their industrial use was restricted in the mid-1970s, PCBs are still produced as by-products in a wide variety of chemical processes that utilize chlorine and hydrocarbon sources, such as those employed in manufacturing chlorinated benzenes, vinyl chloride, and pigments. Moreover, the illegal dumping of PCB-containing waste with release via volatilization and uncontrolled burning may be potential sources of high PCB levels in some regions (Sakai et al., 1999; PascalI et al., 1998).

PCBs are composed of 209 congeners in theory that may enter the environment through primary and secondary sources (Durand et al., 2008). Different PCB congeners have different biological properties. Non-ortho chloro-substituents called coplanar PCBs or dioxin-like PCBs (DL-PCBs) exhibit the highest dioxin-like activities because of their ability to interact with the aryl hydrocarbon receptor (AhR). PCB77, PCB118, PCB126, PCB153, and PCB169 are considered to be the most toxic congeners in terms of dioxin equivalents (Anderson, 1991; Na et al., 2008). The determination of PCBs in environmental research has mainly focused on these congeners (Chiu et al., 2001; Shimomura et al., 2001; Tomoaki., 2006). However, in environmental and biological samples, PCBs are present as mixtures and non-dioxin-like PCBs (NDL-PCBs), accounting for the majority of the total mass. Moreover, their toxicity remains insufficiently understood. Several studies have demonstrated that NDL-PCBs have produce side effects to health via multiple toxicity pathways that do not involve the Ah receptor. These side effects include neurological, neuroendocrine, and carcinogenic effects (Hong et al., 1993).

Identification of PCBs in environmental samples presents a challenge to the bioanalytical investigator since they are present in the environment at low levels (ng/g to pg/g). Thus, immunoassays are relevant. Compared with instrumental analytical methods, such as gas chromatography/electron capture detector (GC/ECD), gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS), immunoassays are more advantageous because of their high sensitivity and selectivity, minimal requirements in terms of sample preparation, high sample throughput, and lower cost. Immunoassays have been used to monitor PCBs for many years (Carlo et al., 1996; Chuang et al., 1998; Kim et al., 2000; Danisa, 2004).

Research on immuno-methods for the detection of DL-PCBs has developed quickly in recent years. However,

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in spite of the availability of various commercial ELISA test kits to detect DL-PCBs and total PCBs (Lambert et al., 1997), limited research on the detection of NDL-PCBs congeners by immunoassays has been reported. Furthermore, the results of conventional heterogeneous immunoassays with DL-PCBs detection limits from 0.01 to 1 μg/L highlight the need for increased sensitivity, as well as other improvements, for such assays to be able approach the detection limits of GC/MS (1 pg/g sample) (Raverdino et al., 1996; Fránek et al., 2001). The immuno-polymerase chain reaction (IPCR) method has been shown to be 100–10,000 times more sensitive than conventional ELISAs (Sano et al., 1992). Improvements in the technology and instrumentation used for signal detection of IPCR have resulted in the development of a real-time IPCR (RT-IPCR) technique (Niemeyer et al., 1997, 1999). This RT-IPCR technology has been further developed by our research group for the detection of fluoranthene, anthracene, and PCB37, yielding good assay results in determinations from water and soil samples (Ye et al., 2010; Zhuang et al., 2009; Chen et al., 2009). Thus, this highly sensitive technology could be widely used for the detection of other PCBs congeners.

In the present study, PCB12 was chosen as a representative compound of lower chlorine NDL-PCBs in the environment because of its low toxicity and the similarity of its molecular structure to hapten, which designed in our lab to that of trichlorobiphenyl, which has been used primarily as impregnant in power capacitors in China (Zhao et al., 2005). In this work, a new method was developed to produce PCB congeners (PCB12), its artificial antigens, and its polyclonal antibodies. Polypropylene PCR-tubes were used as hapten-OVA conjugate binding surfaces for immuno-competition. Avidin (AV) was used to link biotinylated antibodies to biotinylated reporter DNA. A novel, direct, competitive real-time immuno-PCR assay was further developed for the determination of PCB12 in soil samples.

1 Materials and methods

1.1 Reagents

PCB12, PCB37, PCB77, and PCB15 in hexane (100 μg/mL), obtained from J&K Chemical (Shanghai, China), were used for the preparation of a calibration series. Aroclor 1242, 1248, 1254 and 1260 obtained from Dr. Ehrenstorfer (Germany), were used for cross-reaction.

Bovine serum albumin (BSA, MW 67000) and ovalbumin (OVA, MW 45000) were purchased from Sino-American Biotechnology Company (Shanghai, China). *N*,*N*-dimethylformamide, *N*-hydroxysuccinimide (NHS), and dicyclohexylcarbodiimide (DCC) were purchased from Shanghai Guoyao Regents Co. (Shanghai, China). Phosphate buffer solution (0.010 mol/L PBS, pH 7.4), carbonate buffer solution (0.050 mol/L CBS, pH 9.6) and PBST washing buffer containing PBS with 0.05% (*V*/*V*) Tween 20 were used. Hot Start Fluorescent PCR Core Reagent Kits (SYBR Green I), DNA PCR kits, and UNIQ-10 PCR DNA extraction kits were purchased from Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. (Shanghai, China). All chemicals used for the preparation of buffers and reagents were of analytical grade and purchased from Shanghai Guoyao Regents Co., unless otherwise specified.

A Rotor-Gene 3000 Detection System (Corbett Life Science Corbett Robotics Inc., USA) was used for RT-IPCR.

1.2 Preparation of PCB12 congener, hapten derivative and immunogen

The PCB congener 3,4-bichlorobiphenyl (PCB12) was freshly synthesized by the modified Gomberg-Bachmann reaction as described in Fig. 1.

First, 0.050 mmol 3,4-dichloroanlilin and 5.0 mL distilled water were placed in a 100-mL beaker and heated until the compound had melted. Then, 10.0 mL concentrated hydrochloric acid was added into the mixture, which was vigorously stirred and heated until melting. The mixture was cooled in an ice bath and a cold solution of $NaNO_2$ (30%) was added dropwise. The reaction end point was monitored using starch KI indicator paper. Finally, the excess of HNO_2 was eliminated by adding solid urea. Approximately 60.0 mL precooling benzene was added to this ice-cold diazonium clay complex, which was then basified with 5.0 mol/L NaOH solution and kept with stirring at room temperature for 2 hr. The reaction mixture was transferred into a 250-mL round-bottomed flask, and steam distillation was performed twice sequentially. The yellow crude products were extracted with *n*-hexane and deoxidized with 2.0 g Zn and 2.0 mL concentrated hydrochloric acid in anhydrous ethanol. The solvent was filtered and the white needle-like products

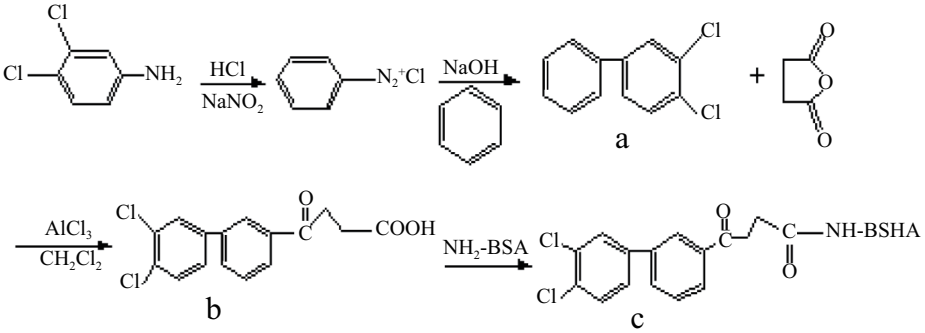


Fig. 1 Synthesis route of PCB12 immunogen.

appeared crystalloid in nature. PCB12 was characterized by ^{1}H NMR, IR and element analysis.

For PCB12: IR (cm^{-1}) 1583, 1485, 1470, 1067, 848; ^{1}H NMR (δ ppm) 7.40 (s, 1H, Ar-H),7.48 (t, 3H, Ar-H), 7.56 (t, 3H, Ar-H), and 7.68 (s, 1H, Ar-H). Elemental analysis results for $C_{12}H_8Cl_2$ are calculated: C 64.5, H 3.587, N 0; found: C 62.3, H 3.684, N < 0.030.

The hapten of the PCB12 (Fig. lb) was obtained by Friedel-Crafts acylation reaction using a slightly modified method (Pastor-Navarro et al., 2007). The resulting product, immunogen (Fig. 1c), was obtained via a slightly modified NHS ester method (Zhang et al., 2006). The conjugate formation was confirmed by their spectral characteristics using an ultraviolet spectrophotometer.

1.3 Immunization and antibody preparation

The immunization of two male New Zealand white rabbits was performed by intradermal administration of immunogen (Fig. 1c) after emulsification in complete Freund's adjuvant. The immune response consisted of an initial primary response followed by a secondary immune response. One immunization dose for rabbits contained 50 μg of the immunogen distributed in fractions of 100 μL to five sites at the back and five sites in the lumbar regions. After 4 weeks, the animals were boosted with an additional 50 μg immunogen emulsified with Freund's incomplete adjuvant (1:1, *V*/*V*). Boosts were given every 2 weeks and blood samples were drawn 7 days after each boost to check the titer of antibodies. After the sixth booster injection, blood samples for the preparation of antiserum were collected within 8 days after the last immunization.

The antibody from the rabbit was purified by octanoic acid/ammonium sulfate two-step precipitation and then introduced into SephadexA-25 and DEAE-32 columns for further purification. The purified antiserum was freeze-dried, aliquoted, and stored at –20°C.

1.4 Preparation and purification of biotinylated reporter DNA

The conjugation of biotinylated reporter DNA was prepared following the method of Joerger et al. (1995). Biotinylated double-stranded DNA, called reporter DNA, was generated by the PCR amplification of pUC19 vector DNA. The preparation process was similar to that introduced by Chen and Zhuang (2009).

1.5 Preparation of biotinylated polyclonal antibodies

The antibodies were biotinylated using biotinamidocaproate-*N*-hydroxysuccinimide ester as reported by Lind and Kubista (2005). The details of the preparation program were similar to those in the study of Chen and Zhuang (2009).

1.6 RT-IPCR procedures

The processes of the direct competitive RT-IPCR are shown in Fig. 2. The procedures were performed as follows: PCB-OVA (20 μg/mL) was diluted with CBS, pipetted (30 μL) into polypropylene PCR tubes treated with 0.8% glutaraldehyde solution to improve absorbability, and incubated overnight at 4°C. The tubes were washed three times with 100 μL/well PBST to remove unbound and other dissociative compounds. The residual adsorption sites were blocked by a 200-μL blocking buffer (PBS with 1% OVA) at 37°C for 30 min. After washing, 15 μL of biotinylated pAbs and 15 μL of PCB12 solution (concentration from 10 fg/mL to 1 ng/mL) were added and incubated at 37°C for 1 hr. The sample was washed to remove unbound biotinylated pAbs and biotinylated pAbs bound with PCBs, until only the biotinylated pAbs bound with PCBs-OVA remained. The addition of 30 μL avidin to bind the attached biotinylated pAbs was followed by incubation at room temperature for 30 min. After washing, the biotinylated DNA was added and the mixture was incubated at room temperature for 30 min. The tubes were washed five times with PBST and five times with Milli-Q water to remove the unbound biotinylated DNA. The above-mentioned processes were completed in preparation for the real-time PCR assay procedure.

DNA was finally quantified by real-time PCR. RT-PCR was performed directly in PCR tubes using a Rotor-gene 3000 real-time rotary analyzer (Corbett Life Science Corbett Robotics Inc., Australia). Fluorescence was detected

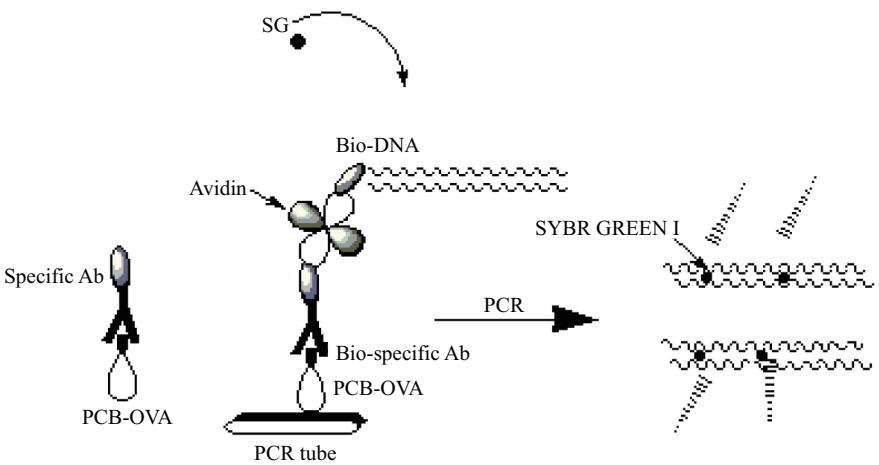


Fig. 2 Real-time immuno-PCR (RT-IPCR) strategy. Coating antigens PCB-OVA, adsorbed to the PCR tubes, is used to competing with the PCBs in combining biotinylated pAbs. Avidin is used as a bridge between the biotinylated pAbs and the biotinylated reporter DNA. The reporter DNA is amplified by PCR using a fluorescent dye SYBR GREEN I for real-time analysis.

during PCR amplification by a real-time rotary analyzer.

The PCR cycling parameters were an initial 4 min at 94°C, followed by 35 cycles of 20 sec at 94°C, 20 sec at 55°C, and 20 sec at 72°C, then holding at 72°C for 3 min. After cycles were completed, a melt procedure was performed consisting of a 30 sec step held at 55°C. The temperature was raised 1°C every 4 sec until the reached 98°C.

2 Results and discussion

2.1 Characterization of immunogens

The conjugation reactions were carried out using a PCB12 hapten derivative and carrier proteins (BSA). The reactant and product of the conjugates were scanned using UV spectroscopy. Figure 3 shows the qualitative differences between carrier proteins and conjugates in the region of maximum absorbance of hapten. The ratio of the hapten to BSA was calculated with the following: ratio = $(\varepsilon_{280\text{-antigen}} - \varepsilon_{280\text{-protein}})/\varepsilon_{280\text{-hapten}}$. The molar ratio of PCB/BSA was 34:1.

2.2 Antiserum titers

The indirect competitive enzyme-linked immunosorbent assay was used for the titration of the sera titer. Titer of the antiserum, defined as the reciprocal of the dilution that resulted in an absorbance value twice that of the blank (serum from the same rabbit before immunization), increased according to immunization times. The titers of the antisera were found to be 1:102,400. These results indicate that specific antibodies in the rabbit antiserum were produced against the immunogens.

2.3 Immunoassay optimization

The non-specific adsorption can be reduced by blocking the surface with blocking agents after the adsorption of coating antigen. Three sets of blocking buffers were tested: milk powder, herring sperm DNA, and OVA. PBST with 1% OVA yielded the best results.

Due to the extreme sensitivity of the PCR amplification step, the requirements for IPCR were much more stringent than those for conventional ELISA. The determination of the optimal reagent concentration to reduce the background fluorescence was essential. In our optimized RT-IPCR system, the appropriate concentrations of avidin and biotinylated reporter DNA were 6.5 and 1.5 ng/mL, respectively.

To improve protein adsorption, the PCR tubes were coated with 0.8% glutaraldehyde for 4 to 5 hr at 37°C prior to the reaction and then washed three times with fresh water. The fluorescence signal was compared to the tube without dealing with glutaraldehyde (data not shown).

To validate the non-specific amplification leading to the false positive results, the melt curves (not shown) were performed to estimate the non-differential amplification. The results showed that the amplifications resulted from the template DNA at 94°C.

2.4 Standard curves and sensitivity

The threshold cycle Ct value represents the PCR cycle at which the fluorescent intensity rises above the threshold; therefore, it could be used to quantify the input target concentration (Sandhya et al., 2008). The Ct was determined by setting a fluorescence threshold in the exponential phase of the amplification curves and then reading the fractional cycle number at which the amplification curve crossed the threshold. Along with the standard samples, a background control containing all assay components, except PCB12, was run. Simultaneously, another negative control (containing the RT-PCR master mix; however, no DNA was reported) was run. Fluorescence was detected during PCR amplification by a real-time rotary analyzer; amplification curves (Fig. 4) of the dilution series of PCB12 were also obtained. RT-IPCR standard curves were constructed from a dilution series of the PCB12 standard in PBS ranging from 10.0 to 1.0×10^6 fg/mL. The fluorescence threshold was automatically set by the Rotor-gene 3000 real-time rotary analyzer and defined as the mean standard deviation of fluorescence in the sample above the baseline cycles. All samples were run in duplicate. The standard curve (Fig. 5) displayed a good correlation coefficient of 0.987, thus guaranteeing the correctness of quantification. In this case, it is advantageous for analysis of several samples of defined concentration.

The standard curve generated by RT-IPCR was used to determine the concentrations of the detected PCB12. The method detection limit calculated from ten times the

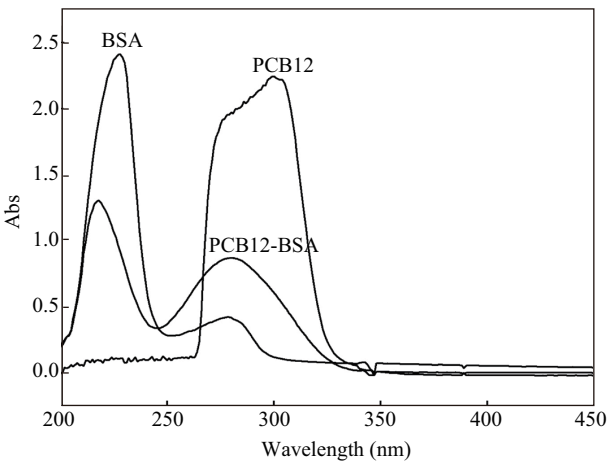


Fig. 3 UV spectra of the PCB12 hapten, conjugant and BSA.

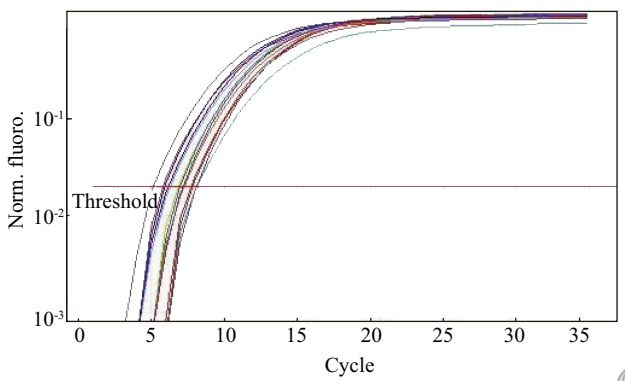


Fig. 4 Amplification curves of dilution series of PCB12 of direct competition RT-IPCR.

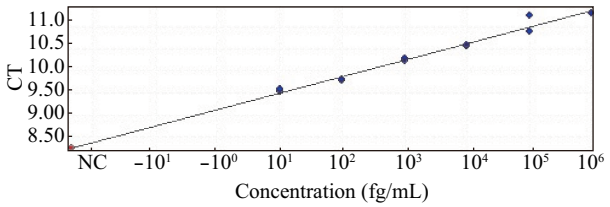


Fig. 5 RT-IPCR standard curves. RT-IPCR was performed on serial dilutions of known concentrations of PCB12 equaling log-fold dilutions from 10.0 to 1.0×10^6 fg/mL (10 fg/mL to 1 ng/mL). Correlation coefficient 0.987; $y = 0.470x + 5.416$.

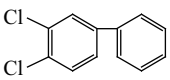
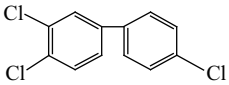
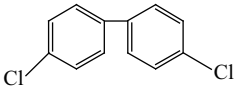
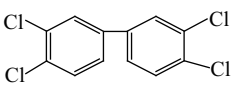
standard deviation of the blank was close to 1.53 fg/mL, indicating that the proposed assay is the most sensitive test system for detecting PCB12 congener to date.

2.5 Immunoassay specificity

To define the specificity of our method, we used four selected congeners and four Aroclor products in place of PCB12 and then performed the same IPCR assay. Measurement of cross-reactivity was performed in the reaction mixtures with 6% DMSO according to the standard IPCR protocol. The congeners were primarily chosen on the basis of their structural similarity to PCB12. The molecular structures of the tested compounds, as well as cross-reactivity values for each compound, are given in Table 1.

The cross-reactivity of the immunoassay towards the compounds applied was obtained using the 50% inhibition value (IC_{50}) as the criterion for evaluating the cross-reaction, as reported by Mauriz et al. (2007). It can be seen that the antibody showed the strongest response to PCB12 as expected; however, a significant response was also found for the coplanar congeners PCB37 (14.85%) and the Aroclor1242 (29.68%). This response may be due to similarities in the stereochemical structures of the designed PCB12 hapten and the PCB37 congener. Thus, the antibody against PCB12 appears to have significant cross-reactivity to bichlorinated and trichlorinated biphenyls. Detection of as many of these compounds as possible in the environment is desirable.

Table 1 Cross-reactivity of PCB12 structurally related compounds

Tested substance	Molecule structure	Cross-reactivity (%)
PCB12		100.0
PCB37		14.85
PCB15		8.3
PCB77		7.8
Aroclor1242	Chlorination (42%)	29.68
Aroclor1248	Chlorination (48%)	17.31
Aroclor1254	Chlorination (54%)	10.45
Aroclor1260	Chlorination (60%)	9.87

2.6 PCBs measured by RT-IPCR in soil samples

To assess the validity of the proposed method, we tested NDL-PCBs on soils from a garden and from a Songjiang suburb paddy field (Shanghai, China). The soil samples were passed through a 100-mesh sieve, carefully mixed, stored in clean glass bottles, and then kept in a refrigerator at $\leqslant$ 4°C. Microwave-assisted extractions of PCB compounds from spiked soil samples were carried out using 30 mL of acetone and hexane (V/V, 1:1) at 25°C for 25 min. The mixture was centrifuged, treated three times with oil of vitriol, and then the organic phase extraction divided into two parts: one for the RT-IPCR analysis, the other for the GC/MS analysis.

Standard PCB12 of three different concentrations was added into the soil samples, which were then run three times each. The recovery rates were 95.7% to 102.6%, and the relative standard deviation within a batch was below 9.5% (n = 9) (Table 2). These findings show that the method is reliable and rational. The recovery and reproducibility of the proposed method were also satisfactory.

To validate the concentration of the environmental samples detected by RT-IPCR, GC/MS (Shimadzu QP 2010 plus GC/MS system, Japan), with the separation performed on a 0.25-mm i.d. DB5 MS 30 m column) was performed twice and the mean values of the PCB12 concentration were obtained, as shown in Table 2. Comparing the data obtained from both techniques, a high correlation can be found between the immunoassay and GC/MS results. Although some deflection in the detected values was found, such inconsistencies may be attributed to differences in the sample preparation procedure and differences in the mechanism of the detection methods. In view of the error obtained in the experiment, the difference was considered acceptable, thereby validating our method.

3 Conclusions

A novel direct competitive RT-IPCR was developed for the convenient determination of PCB12 in soil samples to promote the application of immunoassay technology. The resulting method appeared to be able to provide a quick estimate of environmental samples contaminated by PCBs. We used the highly sensitive IPCR assay for detecting non-dioxin-like PCB congeners and selected PCB12 as the representative compound of lower chlorine NDL-PCBs in the environment; such PCBs are the main components of PCB mixtures detected in Chinese soil. Under optimized conditions, the linear range for the determination of PCB12 was 10.0 to 1.0×10^6 fg/mL with correlation coefficient of 0.987 and a detection limit of 1.53 fg/mL. Comparing the RT-IPCR results with those from GC/MS, a traditional analytical method for PCBs, the proposed method was proven to be more advantageous in terms of its sensitivity and reliability. As well, since multiple samples could be detected in one PCR cycle, the proposed method reduced sampling costs. Meanwhile, designing a synthesis strategy for PCBs and their haptens may uncover new avenues toward the creation of immunogens and antibodies for

Table 2 Recovery of PCB12 from spiking soil samples measured by the optimized RT-IPCR

Soil sample	PCB12 levels (ng/g)	Added PCB12 (ng/g)	Total found (ng/g)	RSD (n = 9, %)	Recovery (%)	GC/MS (ng/g)
Paddy field	5.45	1	6.62	5.56	102.6	
		5	11.21	6.45	107.2	4.73
		10	15.23	6.69	98.6	
Garden	3.56	5	8.78	8.53	102.6	
		10	12.98	5.74	95.7	3.02
		20	22.78	6.56	96.7	

the development of other NDL-PCB immunoassays. The RT-IPCR method was successfully applied in determining PCB37 and PCB12 levels in soil. It may also be used as a model for the high-throughput analysis of other small molecular substances, such as drugs, pesticides, and hormones. New studies to improve the methodology and techniques for comparative analysis in relation to the reference methods are in progress.

Acknowledgments

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