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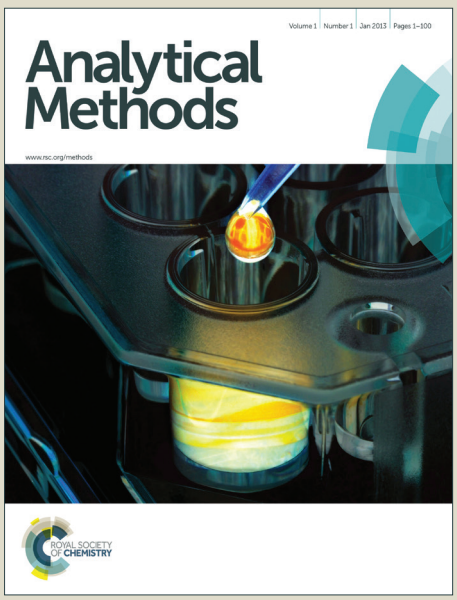

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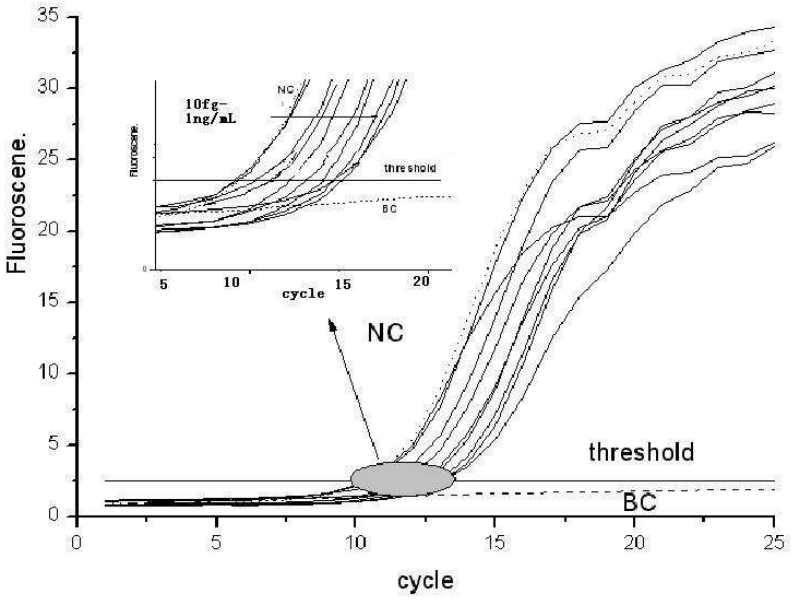
Graphical abstract

Fig. 2 Amplification curves of dilution series of Aroclor 1248 of direct competition rt-IPCR, In Fig. 2, the fluorescence signal of the curve (10 fg mL^{-1}) reaching the threshold was at around cycle 12.3, and there was a fall in the Ct value from 10 to 10^6 fg mL^{-1}. This result implied that the time expended to reach the threshold for the high concentration of PCB molecules was much longer than for the low concentration PCBs.



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Determination of multi-residue PCBs in air by real-time fluorescent quantitative immuno-PCR assay

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Abstract: Based on prepared group-specific antibodies against polychlorinated biphenyls (PCBs), a direct competitive real-time fluorescent quantitative immuno-polymerase chain reaction (rt-IPCR) assay was developed. The purpose of the assay was the determination of multi-residue PCBs in indoor air samples. In the assay, male New Zealand white rabbits were immunized with an immune antigen mixture composed of PCB12-O-BSA, PCB37-O-BSA, and PCB77-O-BSA. The specific polyclonal antibodies (pAbs) to multi-residue PCBs were obtained and used to develop a direct competitive rt-IPCR assay. The specificities of the pAbs were examined by the indirect competitive enzyme-linked immunosorbent assay (id-ELISA). The assays were found to be highly specific for PCB congeners as well as Aroclors 1248 and 1242. The effect of optimal reagent concentrations on reducing background fluorescence was also investigated. Using the optimized assay, a standard curve for Aroclor 1248 was prepared. The linear range for the determination of PCBs was10 to 10^6 fg mL^{-1} with a correlation coefficient of 0.98 and a detection limit of 10.25 fg mL^{-1}. The entire procedure was then evaluated using spiked air samples. The rt-IPCR results for the air samples were confirmed by gas chromatography/mass spectrometry and ELISA. Recovery was lower or higher with agitation but would still be acceptable or use in an on-site field test to provide rapid, semiquantitative, and reliable test results for detection of PCBs in air samples.

Key words: Immunoassay; Real time immuno-PCR; Polychlorinated biphenyls; Multi-residue detection

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1. Introduction

Polychlorinated biphenyls (PCBs) are non-flammable and chemically inert compounds widely used in different industrial societies since the last century. PCBs have been recognized as the most important persistent organic pollutants for several years due to their bioaccumulative and toxic properties.[1,2] Although their production and use have been banned in many countries, enormous amounts of PCBs are still used without proper regulation. Since their toxic properties were recognized in 1983, these complex mixtures have been detected in nearly all environmental media (soil, water, milk, dust, and so on).[3,4,5,6] The toxicology and distribution of PCBs are currently some of the most discussed topics in environmental pollution.

The concentrations of PCBs in biological materials are low, and multi-PCB congeners are simultaneously present in an environmental sample. Consequently, assay techniques for PCB detection continue to be greatly challenging.[7,8] PCBs are traditionally identified based on instrumental techniques and immunoassays, such as gas chromatography (GC)/electron capture detection (ECD) or GC/mass spectrometry (MS). However, these methods are known to manifest underlying disadvantages, including being time consuming and difficult to perform on-site. They are also deemed unsuitable for detecting very low quantities of PCBs in the environment. In contrast, immunoassays are typically very sensitive and readily adaptable to analytes for which an appropriate antibody is available.[9] Immunoassays have been used to monitor PCBs for many years.[10,11,12,13.14] There are many ways in which enzyme-linked immunosorbent assays (ELISAs) have been performed for PCB detection.[15,16,17,18]

Immuno-polymerase chain reaction (IPCR) has recently been described as an ultrasensitive and reliable analytical method for the detection of antigens.[19,20] Using PCR as a signal amplification system, IPCR enhanced the limit of detection (LOD) of a given ELISA by 100–10,000-fold. The high sensitivity and good quantification capabilities of IPCR are due to its high linearity and compatibility with established ELISA protocols. Progress in the technology and instrumentation used for the signal detection of IPCR has resulted in the development of real-time fluorescent quantitative IPCR (rt-IPCR). We have adopted this method for the quantification of antibodies to the environmental pollutants of small molecular weight such as fluoranthene, anthracene, as well as the PCB congeners PCB77 and PCB37.[21,22,23,24] Good detection results are obtained.

Commercial PCBs such as Aroclor (US) are a mixture of isomers containing 60–80 congeners

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primarily with chlorine content of 21%, 42%, 48%, 54%, and 60% by weight. Their toxicities are very different from one another in terms of the position and degree of chlorination.[25] Therefore, the screening of the total PCBs in the environment is important to provide early warnings of any accumulation to susceptible individuals prior to analysis by GC/MS. In a previous study on the detection of total PCBs by immunoassays, the antibodies used are mainly generated from one PCB congener hapten. The immunogen immunoreaction is then carried out in animals. Therefore, the antibody generated from this immunogen could only recognize limited congeners. In the current work, new polyclonal antibodies (pAbs) showing excellent affinity and specificity for three PCB congeners (PCB12, 37, and 77) and Aroclor products were introduced to promote the affinity of the antibodies against the primary PCB congeners. We also described the development of a quantitative rt-IPCR assay and assessed its potential for detecting antibodies to multi-PCBs. Whilst the lower chlorinated Aroclors are usually predominant in China, [26] and because PCB measurements in environmental matrixes were expressed as Aroclor equivalents, the assay was optimized using Aroclors 1248 as analytical surrogate standard to detect PCBs in indoor air samples. The results were confirmed by ELISA and GC/MS.

2. Materials and Methods

2.1. Chemicals and solutions

PCB12, PCB37, and PCB77 in isooctane (100 μg mL^{-1}), as well as Aroclors 1242, 1248, 1254, and 1260 in methanol (100 μg mL^{-1}), were obtained from J&K Chemical (Shanghai, China) and used for the preparation of a calibration series.

Bovine serum albumin, goat anti-rabbit IgG horseradish peroxidase (HRP), ovalbumin, as well as Freund's complete adjuvant (cFA) and incomplete adjuvant (iFA) were purchased from the Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).. N,N-dimethylformamide, N-hydroxysuccinimide, Dicyclohexylcarbodiimide, Tween-20, and hydrogen peroxide (H_2O_2; 30%) were purchased from Shanghai Guoyao Regents Co. (Shanghai, China). All reagents and solvents were analytical reagent grade and used without further purification.

An ABI StepOne Detection System (Applied Biosystems Inc., California, USA) was used for the rt-IPCR. Hot Start Fluorescent PCR Core Reagent Kits (SYBR Green I), DNA PCR Kit, and UNIQ-10 PCR DNA Extraction Kit were purchased from Sango Biotech Co., Ltd. (Shanghai, China).

The following solutions and buffers were used: (1) 0.05 M CBS coating buffer (pH 9.6) containing 1.5 g of Na_2CO_3 and 2.93 g $NaHCO_3$ L^{-1} Milli-Q water; (2) 0.01 M phosphate-buffered saline (PBS;

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pH 7.4) containing 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 L^{-1} Milli-Q water; (3) blocking solution containing PBS with 0.6% glutin; (4) PBST washing buffer containing PBS with 0.05% (v/v) Tween 20; (5) tetramethylbenzidine (TMB) solution containing 10 mg/mL TMB in absolute ethanol; and (6) citrate buffer (pH 5.5) containing 0.1 mol/L sodium citric acid and phosphate buffer.

2.2. Preparation of multiple artificial antigens

To obtain a high-affinity antibody against multi-residue PCBs in environmental samples, the immune system must be activated by multiple antigens. We used a mixture of artificial antigens to increase immunogenicity. Haptens of PCB 12, 37, and 77 were synthesized by the Friedel-Crafts acylation reaction, and termed as PCB12-COOH, PCB37-COOH, and PCB77-COOH, respectively. The immunogen and coating antigens were prepared by conjugating (PCB12-COOH, PCB37-COOH, and PCB77-COOH) with carried protein BSA and OVA, respectively. Conjugate formation was spectrophotometrically confirmed. The above synthesis steps have been described in detail elsewhere [27]. The multiple immunogens of PCBs were obtained by equally mixing with three PCB congener immunogens (PCB12-O-BSA, PCB37-O-BSA, and PCB77-O-BSA, 1:1:1 v/v/v) in sterilized physiological saline solution and emulsified with cFA.

2.3. Preparation of polyclonal antibodies and biotinylated polyclonal antibodies to PCBs

Two male New Zealand white rabbits were immunized with multiple artificial antigens(All the immunization experiments were performed by the teacher at animals laboratory of Shanghai Jiao tong university and it is in compliance with the relevant laws and school guidelines, and also has been approved by the school committee). The initial immunization was performed by injecting 50 μg of multiple immunogens dissolved in 0.5 mL of sterilized physiological saline solution and emulsified with 0.5 mL of cFA. The injections were distributed in 1.0 mL fractions to 10 sites at the back and 5 sites at the jugular regions. Seven booster immunizations were performed by injecting 50 μg of multiple immunogens in 0.5 mL of PBS and 0.5 mL of iFA at 15 day intervals. All immunizations lasted for approximately 3 months. The titers of the antiserum were then analyzed using indirect competitive (id)-ELISA. After the seventh booster injection, blood samples were collected within approximately 8 days after the last immunization for the preparation of antisera. The antiserum from the rabbit was purified by the octanoic acid/ammonium sulfate two-step precipitation method, and then introduced into SephadexA-25 and DEAE-32 columns for further purification. The purified antiserum

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was freeze dried, aliquoted, and stored at -20 °C.[28]

Antibodies were biotinylated using biotinamido-caproate-N-hydroxysuccinimide ester as previously reported.[29] Glycerin and NaN_3 were then added, and the biotinylated antibody solution was stored at -20 °C.

2.4. Detection of the specificity of the antibodies

The specificity of the antibodies was evaluated via the id-ELISA. PCB-OVA was diluted with CBS and pipetted into the wells of microtiter plates, incubated, washed three times with 200 μL/well PBST, and blocked with 0.6% glutin in PBS. Competitive compounds were then added and the mixtures incubated. After washing, 100 μL/well diluted goat anti-rabbit IgG-HRP (1:1000) was added. The mixture was incubated at room temperature for 1 h and then washed three times with PBST. The substrate solution (100 μL) was added into each well. The enzymatic reaction was terminated by adding 50 μL of 2 M H_2SO_4. The absorbance was measured at 450 nm using an automatic detection microplate reader. The entire procedure was similar to a previously reported method.[30]

2.5. Preparation and purification of biotinylated reporter DNA

Biotinylated double-stranded DNA was prepared following a method described in literature.[23] pUC19 plastid, purchased from Sango Biotech Co., Ltd. (Shanghai, China), was used for synthesizing the reporter DNA. We obtained the reporter DNA by PCR amplification using a biotinylated forward primer, 5'- G TTG TAA AAC GAC GGC CAG T -3'and a biotinylated reverse primer, 5'-GAC AGT TAC CAA TGC TTA ATC A-3'. PCR was performed as follows: at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 20 s, extension at 72 °C for 20 s, and final extension at 72 °C for 3 min. The reporter DNA was purified and retrieved using the UNIQ-10 PCR DNA Extraction Kit. DNA was quantified by UV absorbance and qualitatively checked using an agarose gel.

2.6. Direct competitive rt-IPCR protocol

We developed an antigen-coated direct competitive rt-IPCR protocol to test the samples. The processes of the assay are simplified in Figure 1. First, 30 μL of PCB12-OVA-coated antigen solution was diluted in CBS, incubated in a polypropylene PCR tube overnight at 4 °C, and treated with 0.8% glutaraldehyde solution to improve absorbability. Each tube was washed three times with 200 μL of PBST, and blocked with 200 μL of blocking solution at 37 °C for 1 h. After washing similarly as the previous washing step, 15 μl each of biotinylated pAbs and Aroclors 1248 were added, and the mixture

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was incubated at 37 °C for 1 h. Washing was performed to remove unbound biotinylated pAbs and the biotinylated pAb bound with PCBs. Only the biotinylated pAbs bound with PCBs–OVA were retained. Avidin (30 μL) was added to link with the attached biotinylated pAbs for 30 min of incubation at room temperature. Avidin is known as a protein that can bind biotin due to its high affinity with the compound. Therefore, avidin was used as a bridge to link biotinylated reporter DNA to biotinylated pAbs. After the same washing process, biotinylated DNA was added and the mixture was incubated at 37 °C for 1 h. The tubes were washed five times each with PBST and Milli-Q water to remove unbound biotinylated DNA and other remaining dissociated compounds. The PCR reaction solution mixtures were added using a Hot Start Fluorescent PCR Core Reagent Kits (SYBR Green I) following the manufacturer's instructions. The forward and reverse primers used here had the same designed base stem sequences as the biotinylated primer except for the biotin-labeled molecule. The DNA was finally quantified by real-time PCR.

The PCR cycling parameters were as follows: an initial 4 min at 94 °C; 35 cycles of 20 s at 94 °C, 20 s at 55 °C, and 20 s at 72 °C; and held at 72 °C for 3 min. Afterwards, a melt procedure was performed. This procedure consisted of a 30 s holding at 55 °C, and an additional 1 °C every 4 s until the temperature reached 98 °C.

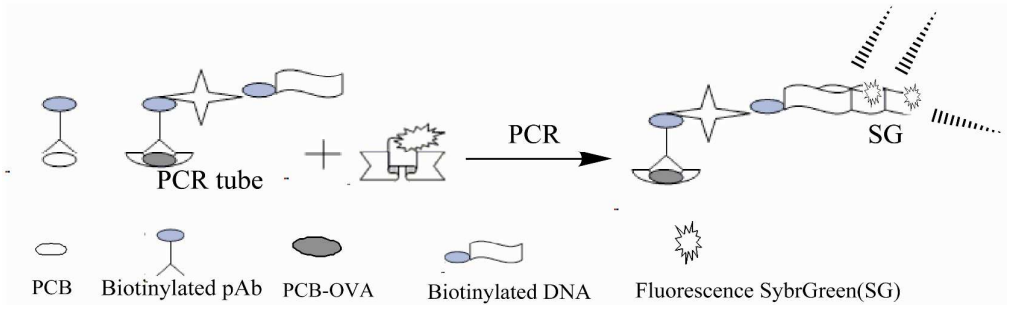

Fig. 1. Diagram of the direct competitive rt-IPCR technique.

2.7. Determination of indoor air samples

To assess the validity of the proposed method, the PCBs in indoor air samples from a typical fitment house (Shanghai, China) were determined. The passive air sampler was set at indoor stuffiness and used to collect 24 h samples, which corresponded to total air volumes between 250 and 400 m^3. The sample extraction and clean-up procedure were conducted in accordance with literature. [31] The samples were collected in bottles, diluted with PBS and dimethylsulfoxide (DMSO; adjusted to 5%), and then stored at 4 °C until use. The same air samples were subjected to ic-ELISA and GC/MS to

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compare with the rt-IPCR results. Recovery tests were performed by spiking air samples with a series of known Aroclors 1248 concentrations to determine the efficiency of the rt-IPCR assay.

3. Results and discussion

3.1. Optimized conditions in the rt-IPCR protocol

A highly sensitive detection scheme is required to monitor low levels of PCBs. The sensitivity of rt-IPCR technology is typically limited by background signal caused by the non-specific adsorption of reagents. Therefore, the solution used as a blocking reagent, different concentrations of avidin and reporter DNA, as well as coating antigen and bio-pAb concentration, were all optimized in the assays. Three sets of blocking buffers (0.6% glutin, 1% glutin, and 1% OVA) were tested, and PBST with 0.6 % glutin produced the best results. We then recorded the fluorescence intensities of different concentrations of avidin and reporter DNA in the rt-IPCR assay the highest fluorescence intensity and signal-to-background ratio were obtained at 6.5 ng mL^{-1} avidin and 0.37 ng mL^{-1} reporter DNA. Although PCB37-OVA and PCB77-OVA were both suitable as coating antigens, because the PCB12 was chosen as a representative compound of lower chlorine non-dioxin-like PCBs (NDL-PCBs) in the environment, PCB12-OVA was selected to reduce the toxicity of the experimental conditions. Based on the high specificity and amplifying efficiency of PCR, the optimal diluted proportions of the coating antigen and bio-pAb were found to be 1:3000 (original concentration = 7.5 mg mL^{-1}) and 1:2000 (original concentration = 1 mg mL^{-1}).

3.2. Cross-reactivity (CR) of the antibody for the PCB assay

To establish an IPCR system for monitoring PCBs in environmental samples, antibodies should possess high cross-reaction profiles to various PCBs. The specificities of the antibodies were evaluated via the CR of the pAb with three structurally related compounds of PCBs (PCB 12, 37, and 77), as well as four Aroclor products and other compounds with molecular structures similar to those of PCBs. CR was measured in the reaction mixtures with 5% DMSO according to the standard ic-ELISA protocol. [30] The molecular structures of the test compounds as well as their 50% inhibition (IC_{50}) and CR values are presented in Table 1.

Table 1. Specificity (cross-reactivities) of compounds structurally related to PCBs in the rt-IPCR assay

Aroclor	Molecular structures and Chlorination (%)	MDL (μg L^{-1})	IC_{50} (μg L^{-1})	Cross-reactivity（%）

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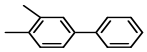
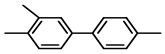
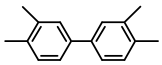
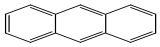
1260	60	8.3	1258.2	2.9
1254	54	0.5	60.3	61.7
1248	48	0.9	37.2	100.0
1242	12	0.6	23.9	158.5
PCB12		1.1	23.4	158.6
PCB37		0.6	16.2	229.2
PCB77		1.2	38.2	97.3
Anthracene		12.3	897.5	4.1
Naphthalene		9.3	378.2	9.8

The CR of the immunoassay toward the studied compounds was obtained using IC_{50} as the criterion. The strongest response was obtained with PCBs congeners and Aroclors as expected. Based on the CR values, the assay was evidently more responsive to formulations with low chlorination (Aroclors 1242, 1248, and 1254) than to those with high chlorination (Aroclor 1260). All these results showed the remarkable increase in the affinity of the antibodies for PCBs. All of CR showed no more than 10% CR, which demonstrated the good specificity of the pAb.

3.3. Standard curves

A standard curve was produced by plotting the average Ct values of duplicates against known Aroclor 1248 concentrations. We generated an RT-IPCR standard curve using dilutions ranging from 10 fg mL^{-1} to 1ng mL^{-1}. All samples were tested in duplicate. The linear range was used for PCB quantification in the tested samples. The sensitivity of the system was assessed after using standard samples with known isolated concentrations of a tenfold dilution series. Under the aforementioned optimal conditions, a typical dose response curve for Aroclor 1248 was obtained using the purified bio-pAb with concentrations ranging from 10 to 10^6 fg mL^{-1} (Fig. 2). The standard curves are shown in Fig. 3.

In Fig. 2, the fluorescence signal of the curve (10 fg mL^{-1}) reaching the threshold was at around cycle 12.3, and there was a fall in the Ct value from 10 to 10^6 fg mL^{-1}. This result implied that the time expended to reach the threshold for the high concentration of PCB molecules was much longer than for

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the low concentration PCBs. The biotinylated pAb were competition occupied by the PCBs from the samples and the coating antigen. There were more PCBs in the samples and fewer coating antigens that combined with the biotinylated pAb, which adsorbed on the tube surface. Similarly, the biotinylated DNA that integrated with the biotinylated pAb were fewer, so more time was needed to reach the threshold.

The fluorescent threshold was automatically set by the cycler instrument, and was defined as the mean standard deviation of fluorescence in the sample tube over baseline cycles. Along with the standard samples, a negative control containing all assay compounds except PCBs was run. A background control containing only the rt-PCR master mix without DNA template was also run. All samples were run in duplicate. In the rt-IPCR protocol, the primary step of antibody recognition of PCBs may account for a loss of precision when compared to a standard curve generated by RT-PCR of a DNA template alone. However, a semi-quantitative dose response represented out by increasing Ct with increasing the concentrations of PCBs.

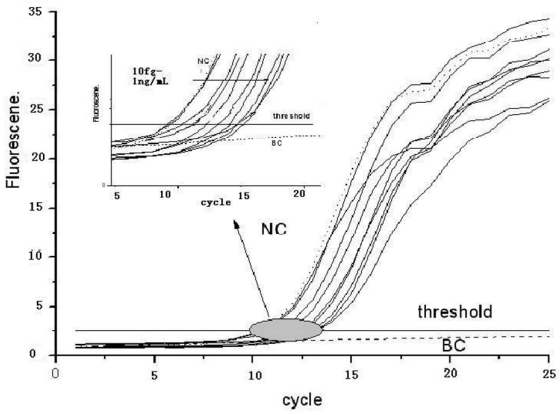

Fig. 2 Amplification curves of the dilution series of Aroclor 1248 by direct competition rt-IPCR

The detection limit of this assay calculated from nine times the standard deviation of the negative control was close to 10.25 fg L^{-1}. The standard curves displayed a correlation coefficient of 0.98 (Fig. 3), and the final concentration of Aroclor 1248 ranged from 10 to 10^6 fg mL^{-1}. Thus, the detection range of this method was 10 to 10^6 fg mL^{-1}.

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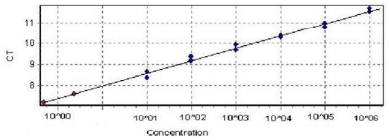

Fig. 3 Rt-IPCR standard curves. Rt-IPCR was performed on serial dilutions of known concentrations of Aroclor 1248 equaling log-fold dilutions from 10 to 10^6 fg mL^{-1} with a linear equation Y = 0.595×log(conc) + 8.556, R^2=0.98;

To show the specificity of our assay, a melt curve was generated after the amplification procedure. As shown in Fig. 4, all the DNA products amplified in the PCR tubes were only the desired products.

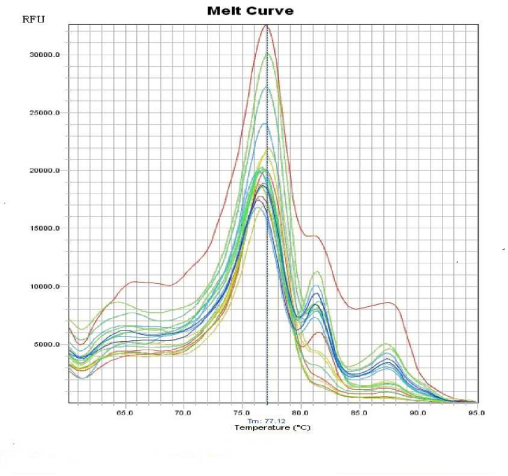

Fig. 4 Derivative of direct competition rt-IPCR melt curves of Aroclor 1248

3.4. Accuracy and Application

The indoor air samples were assessed three times using rt-IPCR with the optimized procedure. Three parallel collators from each sample were analyzed. According to the standard curves of the relationship between the Ct value and concentration, we obtained the mean concentration of each sample. The accuracy of PCB determination was evaluated by adding various amounts of Aroclor 1248 to the air samples. The recovery rates were 88.1% to 112.8%, and the relative standard deviation within a batch was below 10% (n = 6) (Table 2). ELISA and GC/MS were also performed to validate the PCB concentrations in the environmental samples detected by rt-IPCR. The results are also shown in Table 2.

Table 2. Recovery of PCBs from spiking air samples measured by the optimized rt-IPCR

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Air samples	PCBsLevels (pg/m^3)	Added (pg/m^3)	Total Found (pg/m^3)	RSD (n=6,%)	Recovery (%)	ELISA	GC/MS (pg/m^3)
Sample 1	473.1	50.0	549.8	5.5	105.1	438.6	343.2
		150.0	589.1	5.3	94.5		
		300.0	723.7	4.2	93.6		
Sample2	391.5	50.0	392.8	5.5	88.9	473.2	313.8
		100.0	461.6	4.1	93.9		
		200.0	536.6	6.6	90.7		
Sample 3	413.7	200.0	567.6	5.6	92.5	436.2	380.3
		150.0	496.5	4.6	88.1		
		300.0	805.6	8.7	112.8		

A comparison of the rt-IPCR and GC/MS results reveals that rt-IPCR had higher values. The detected concentrations were similar to those determined by ELISA. The discrepancies between the rt-IPCR and GC/MS data may be attributed to the differences in the sample preparation procedure, homogeneity of sample aliquots, variation in the data, and differences in the detection methods. The PCB mixtures in the samples may have been selectively enriched in the congeners that were bound most strongly to the PCB antibodies. In view of the errors of the experiment, the difference is acceptable. The results confirmed that this method can be satisfactorily applied as a reliable means of detecting PCBs.

4. Conclusions

A new pAb was developed for the convenient determination of PCBs in air samples to promote the application of immunoassay technology. The proposed rt-IPCR method using this antibody showed good assay stability and high specificity to one of the congeners and Aroclor products. The linear range for the determination of Aroclor 1248 was 10 to 10^6 fg mL^{-1}, and the detection limit was close to 10.25 fg mL^{-1}. The detection conditions were optimized and the sensitivity was analyzed. The method was found have high sensitivity and precision, as confirmed by ELISA and GC/MS.

Therefore, IPCR is a valid method for detecting antigens.[31] There are few reports on the application of real-time IPCR in PCB studies. Consequently, we introduced this method to detect PCBs

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and found it to be a novel way of exploring research processes, especially the routine testing of trace samples. The rt-IPCR method is not yet reproducible to the standards required for environmental monitoring, but there is little doubt that with continued refinement, it has the potential to become the most sensitive analytical method for PCB detection. The disadvantages of long incubation time and expensive equipment exist in the detection of environmental substances. Nevertheless, considering the large amount of statistical data collected during a PCB survey search, the sensitivity and robustness of rt-IPCR as an ELISA-enhancing detection tool underlines its enormous potential. The establishment of this detection method will provide an important basis for the spatial analysis, ecological security risk assessment, pollution control, and environmental management of PCBs in the air systems of the country.

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