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A sensitive immunoassay for parathion based on covalent linkage between small molecules hapten microtiter plates surface

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Abstract A sensitive competitive inhibition enzyme-linked immunosorbent assay (CIELISA) for the detection of parathion has been developed. In this assay, a small molecule hapten II (O,O-diethyl O-4-aminophenyl phosphorothioate) was covalent linked to glutaric dialdehyde treated-microtiter plates. In addition, 4-(ethoxy(4-nitrophenoxy) phosphorothioylamino) butanoic acid-ovalbumin (hapten I–OVA) conjugate served as the coating antigen for comparison with directly hapten II covalent linked plates in the CIELISA format. The developed assay demonstrated highly sensitivity (IC_{10} was 0.08 ng mL^{-1}) selectivity and stability. In samples analysis, the results of parathion detected by this assay were in accordance with which obtained by high-performance liquid chromatography.

Keywords Competive inhibition enzyme-linked immunosorbent assay · Covalent linkage · Parathion · Small molecule hapten

Abbreviations

CIELISA	Competitive inhibition enzyme-linked immunosorbent assay
CR	Cross-reactivity
GD	Glutaric dialdehyde
GM	Gas chromatography-mass
GC	Gas chromatography
HPLC	High-performance liquid chromatography
MWs	Molecular weights
MS	Mass spectrum
NMR	Nuclear magnetic resonance
NHS	N-hydroxysuccinimide
OP	Organophosphates
PBS	Phosphate-buffered saline
SM-hapten	Small molecule hapten
LOD	The limit of detection
TLC	Thin-layer chromatography
OVA	6-Aminocaproic acid, ovalbumin
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
APTES	3-Aminopropyltriethoxysilane

Introduction

The pesticides contaminants in foods and environment are great concern for the public and regulatory due to their chemical hazards to human health [1]. In China, about 1 million tons of various pesticides were used per year, and a quarter of them were organophosphates (OP) chemicals [2]. Parathion belongs to the OP pesticides and has the potential to be involved in the activities of cholinesterase and disrupt the nervous system of humans, resulting in convulsions, poor vision, tremor, dyspnea, lung oedema as well as respiratory arrest [3, 4]. More critically, parathion is associated with an increasing risk of cancer in malignant transformation of some cells through altering genomic instability [5–7]. Parathion was highly toxic (estimated median lethal dose of 11.68 to 7.21 mg kg^{-1}), producing dose-dependent inhibition of brain and plasma cholinesterase activity, hyperglycemia and elevated plasma corticosterone concentration [8, 9]. Several organizations such as the United

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States Environmental Protection Agency list parathion as a possible human carcinogen [10, 11].

Due to its high chemical stability and persistence, parathion and their oxidation products can not only remain in crops, but also disperse into the environment (water, soil and so on), eventually transmitting into the food chain through the bio-accumulation [12, 13]. The hazards of parathion contaminants to human health are incontestable [14–16]. Therefore, it is still necessary to regular monitor parathion residues in foods and environment. There have been routine methods for monitoring parathion, including gas chromatography-mass (GM) [17, 18], gas chromatography (GC) [19–21], and high-performance liquid chromatography (HPLC) [22, 23]. However, they usually involve sophisticated equipment and a large number of separate analytical procedures, resulting in more-complex, time-consuming and laborious screening procedures. In addition, new detection assays for parathion have been developed recently, such as acetylcholinesterase biosensor [24], molecule imprinted polymeric sensor [25], electrochemical sensor [26], fluorescence probing strategy [27], nanoporous gold/MWCNTs electrode sensor [28] and so on. Unfortunately, they still required complicated fabrication processes and special instruments. Alternatively, enzyme-linked immunosorbent assays (ELISAs) is recognized as a valuable tool in pesticide residual analysis and a complements conventional analytical method due to its cheap, rapid, sensitive and selective characteristics [29–31].

Many competitive inhibition enzyme-linked immunosorbent assays (CIELISAs) have been developed for monitoring OP pesticides to date [32–35]. In these CIELISAs, the small molecule hapten (SM-hapten) should be conjugated with carrier protein when immobilized on the surface of microtiter plates because SM-haptens do not have multiepitopes. However, this approach has many disadvantages for some purposes. Firstly, the formation of SM-hapten-protein conjugate is a time-consuming process and has poor reproducibility, which is unfavourable for assay standardization [36, 37]. Secondly, immobilization process of SM-hapten-protein conjugates on the PS surface depends on hydrophobic interactions between SM-hapten-protein conjugate and the surface of plates. This interaction often contains significant conformational changes which lead carrier protein easily to screen SM-hapten, thus resulting in unsuitable presentation and orientation of SM-hapten [38].

For avoiding these drawbacks, the direct attachment of SM-hapten to the surface of microtiter plates would be an interesting approach [39, 40]. The surface of plates can be pre-activated and modified with different functional groups, such as hydroxyl, amino, carbonyl and carboxyl; these functional groups could link SM-hapten through covalent linkage [41]. The polystyrene surface has been modified by a mixture of HNO_3–H_2SO_4 and 3-aminopropyltriethoxysilane (APTES) for directly linking bisphenol A, atrazine and 2,4-dichlorophenoxyacetic acid [36, 38]. However, such method has potential risk on the operators due to using strong acids. In addition, the small peptide haptens were covalent attached to the plate surface treated with ϒ-radiation activation [39]. The g-irradiated was also used to modify the plate surfaces for directly binding p-aminophenylalanine [40]. But, these methods are time-consuming and difficult to control. A facile method was later developed by treating the plate with a polymerized glutaric dialdehyde (GD) to directly immobilize macromolecules [42] and O-pinacolyl methylphosphonic acid [43].

Herein, a highly sensitive CIELISA was developed for the detection of parathion based on a covalent linkage between the SM-hapten II (O,O-diethyl O-4-aminophenyl phosphorothioate) and microtiter plate surface. In this assay, the plate surface was treated with GD network to introduce aldehyde group that can cross-link with amino group of hapten II. As a comparison, the other parathion derivative (Hapten I) OVA conjugate (4-(ethoxy(4-nitrophenoxy) phosphorothioylamino) butanoic acid-ovalbumin (hapten I-OVA) conjugate) was prepared and coated on the plate surface by hydrophobic interactions. After optimization of reaction conditions, the developed assays were further applied in food/water matrices including corn, rice, cucumber and drinking water. Furthermore, the detected amounts of parathion were also confirmed by HPLC.

Experimental

Materials

Parathion, methyl parathion, atrazine, monocrotophos, chlorpyrifos, diazinon, n-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 6-aminocaproic acid, ovalbumin (OVA) and goat anti-rabbit IgG-HRP conjugate secondary antibody were purchased from Sigma-Aldrich (USA). The rabbit polyclonal antibody against parathion was obtained from Shanghai Biotechnology Institute (Shanghai, China). Common solvents (including GD) and salts were obtained from Tianjin Regent Corporation (Tianjin, China). The reaction progress was monitored by thin-layer chromatography (TLC) using GF254 silica gel on glass plates. The water used was prepared by a Milli-Q system (Millipore, Bedford, MA). Coating buffer was 0.05 M carbonate buffer (pH 9.0). Washing buffer (PBST) was 0.01 M phosphate-buffered saline (PBS) with 0.05% Tween 20 (pH 8.5). The 96 wells ELISA microtiter plates (medium binding) were purchased from Wanger Biotech Co. (Beijing, China).

The ^{1}H nuclear magnetic resonance (NMR) spectrum of hapten II was obtained with an AVANCE DMX 500 spectrometer (Bruke, Berlin, Germany). The hapten I–OVA conjugates were determined by Reflex III MALDI-TOF-Mass Spectrum (Bruker Inc., Germany). A Multiskan MK3 ELISA reader (Thermo, USA) was applied for the determination of absorbance. 6930 centrifuge (KUBOTA Inc., Japan) and MS3 digital orbital shaker (IKA Inc., Germany) were used for the preparation of hapten I, hapten I–OVA conjugate and hapten II. HPLC system with coupled with UV–Vis UV-detector (Waters, USA) was employed for detection of drinking water samples.

Haptens syntheses

The synthetic routes for hapten I and hapten II are illustrated in Fig. 1. Two approaches were utilized in the haptens syntheses: (1) use of a spacer attached to a carboxyl group of parathion, (2) displacement of a nitro-group of parathion by amino group. Their syntheses procedures were described as follows.

4-(ethoxy (4-nitrophenoxy) phosphorothioylamino) butanoic acid (hapten I) was prepared by the following steps. Ethyl dichlorothiophosphate (2.5 g), K_2CO_3 (10 g), 4-nitrophenol (1.5 g) were mixed thoroughly in 10 mL of acetonitrile. After stirring for 1 h at 37 °C, the mixture was filtered by Celite, and the solvent was removed under reduced pressure. The obtained solid (250 mg) was dissolved in 2 mL of cooled MeOH and mixed with a mixture of KOH (125 mg) and 4-aminobutyric acid in 1.5 mL of MeOH. After stirring for 10 min, the reaction mixture was filtered and extracted with 1 M HCl-chloroform. After drying over anhydrous sodium sulphate, hapten I was obtained. ^{1}H NMR ($CDCl_3$): δ = 8.23 (d, J = 8.8, ArH, 2H), 7.37 (d, J = 8.0, ArH, 2H), 4.21 (m, CH_2OP, 2H), 3.40 (m, NH, 1H), 3.18 (m,CH2NH, 2H), 2.47 (m,CH_2COOH, 2H), 1.87 (m, $CH_2CH_2CH_2$, 2H), 1.38 (t, J = 6.8, CH3, 3H).

O,O-diethyl O-4-aminophenyl phosphorothioate (hapten II) was synthesized by the following procedures. O,O-diethyl-O(4-nitrophenyl) thiophosphate (4 g) was dissolved in ethyl ether (40 mL) and extracted with 1% cold Na_2CO_3 solution (4 × 10 mL). After removing the water layer, 20 mL of acetic acid/hydrochloric acid mixture (9:1, V/V) and 4 g of zinc powder were added, then stirred and reflowed for 45 min. After purification, an orange-red oily hapten II was obtained.

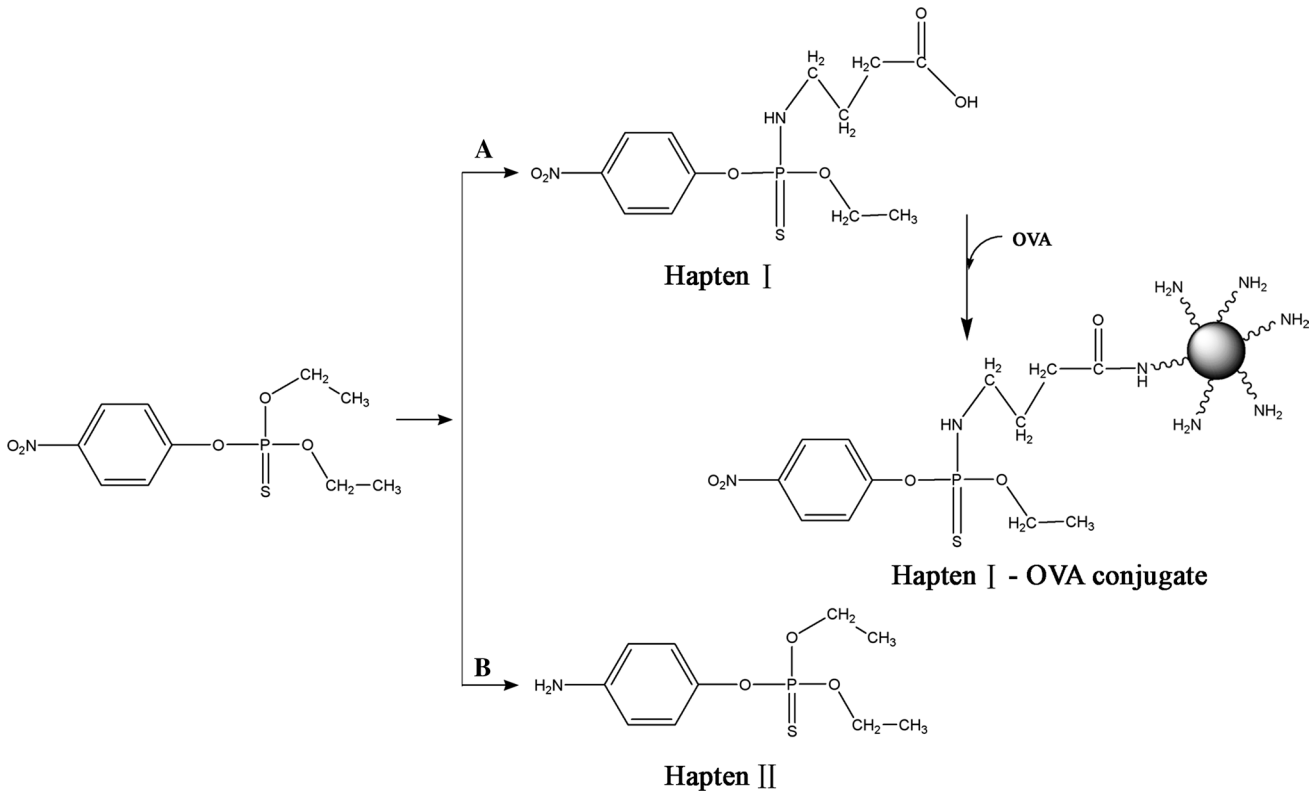

Fig. 1 Synthesis of hapten I, hapten I-OVA conjugate and hapten II. **a** 4-(ethoxy (4-nitrophenoxy) phosphorothioylamino) butanoic acid (hapten I) and hapten I-OVA conjugate. A spacer was attached to a carboxyl group of parathion to form hapten I, and the obtained hapten I was then conjugated with OVA to form hapten I-OVA conjugate. **b** O,O-diethyl O-4-aminophenyl phosphorothioate (hapten II). A nitro-group of parathion was displaced by amino group to form hapten II

Hapten I–OVA conjugate preparation

Hapten I–OVA conjugate was prepared using active ester method [44, 45]. Hapten I–OVA conjugate was characterized by mass spectrum (MS). Assuming that the molar absorptivity of hapten was the same for the conjugated and free forms [36, 41], the identification of hapten, i.e. coupling ratios (the number of hapten molecules per molecule of protein carrier) of the conjugate could be approximately estimated directly by molecular weights (MWs) shown in Eq. (1):

$$\text{Coupling ratio} = (\text{MWs}_{\text{Conjugate}} - \text{MWs}_{\text{OVA}})/\text{MWs}_{\text{Hapten}} \quad (1)$$

CIELISA procedures using the hapten I–OVA conjugate coated plates

Microtiter plates were coated with hapten I–OVA conjugate by adding 100 μL of the conjugate. After incubating at 4 °C overnight, the plates were treated with 50 μL the rabbit polyclonal antibody against parathion and 50 μL different concentration of free parathion (100, 20, 4, 0.8, 0.16, 0.032, 0.0064, 0.00128, 0.000256 and 0 μg mL^{-1}) for 1.5 h at 37 °C. The plates were thoroughly washed with PBST. 100 μL of goat anti-rabbit IgG-HRP conjugate secondary antibody at 1:3000 diluted in PBS was then added into each well of the plates. Afterwards, 100 μL tetramethylbenzidine substrate systems were added into each well and incubated for 10 min at 37 °C in dark. Absorbance at 450 nm was determined in an ELISA reader.

CIELISA procedures using the hapten II covalent linked plates

Microtiter plates were covalent linked hapten II molecules directly following the procedure of works with some modifications [41]. Standard microtiter plates were pre-treated with a dilution of GD in carbonate buffer (0.05 M, pH 9.0) at 37 °C for 2 h. After washing 3 times with PBST buffer, an optimized dilution of hapten II in PBS (0.05 M, pH 8.5) was incubated at 37 °C for 2 h. The plates were then washed with PBST buffer thoroughly. By incubating with a selected blocking reagent in PBS (pH 8.5) at 37 °C for 1 h, the remaining binding sites on the modified surface of microtiter plates were blocked. After washed thoroughly with PBST, the plates were treated with 50 μL of the rabbit polyclonal antibody against parathion and 50 μL standard concentration of free parathion (100, 20, 4, 0.8, 0.16, 0.032, 0.0064, 0.00128, 0.000256 and 0 μg mL^{-1}) for 1.5 h at 37 °C. Then, 1:3000 dilution of the goat anti-rabbit IgG-HRP conjugate secondary antibody was added into each well of the plates. The plates were incubated for 45 min at 37 °C and washed with PBST for 6 times. The next steps were followed as described above.

Analysis of data

Experimental wells were blanked against wells in which the rabbit polyclonal antibody against parathion was omitted. Data analysis was performed as described elsewhere [37–39]. The absorbance was recorded at 490 nm, and data analysis was performed by normalizing the absorbance using the absorbance based on $\%B/B_0$ value.

$$\%B/B_0 = (A - A_{\text{ex}})/(A_0 - A_{\text{ex}}) \times 100 \quad (2)$$

where A: absorbance of parathion at standard concentration, A_0: absorbance at zero parathion concentration, and A_{ex}: absorbance at excess parathion concentration.Curve fitting was performed by use of Eq. (3).

$$Y = [100/(1 + (X/C))B] \quad (3)$$

in which C is the test midpoint (Hapten value), B is the slope of the curve at the IC_{50}-value, and X is the analyte concentration.

Optimization of CIELISAs

To detect parathion in foods and environmental samples, it is essential to develop CIELISA with optimum sensitivity. Therefore, effects of different concentration of coating hapten and GD, blocking buffers and antibodies dilution were studied. The main criteria used to evaluate immunoassay performance were competitive inhibition curve and the absorbance values.

Cross-reactivity

In our study, cross-reactivity (CR) of CIELISAs was evaluated by using its analogues: methyl parathion, atrazine, monocrotophos, chlorpyrifos and diazinon. The CR values were calculated according to Eq. (4) [37, 41].

$$\text{CR}\,(\%) = \text{IC}_{50}\text{ of parathion / IC}_{50}\text{ of analogues} \times 100 \quad (4)$$

Sample extraction and spiking method for CIELISAs

Real samples including drinking water, cucumber, rice and corn were spiked and analysed by CIELISAs. Cucumber, rice and corn samples were supplied from a local supermarket in Tianjin. Three kinds of drinking water were obtained from the Haihe River, Tuanbo Lake and Dongli Lake, respectively, in Tianjin.

The cucumber sample preparation was carried out following the previous report [25]. Parathion was spiked in cucumber at three final concentrations (5, 10, 20 ng mL^{-1}). One-gram spiked cucumber was mixed with 2 mL methanol and then shaken for 24 h. After centrifuging at 6000 rpm for 15 min, the supernatant was evaporated to dryness under vacuum. The obtained residue was then dissolved in

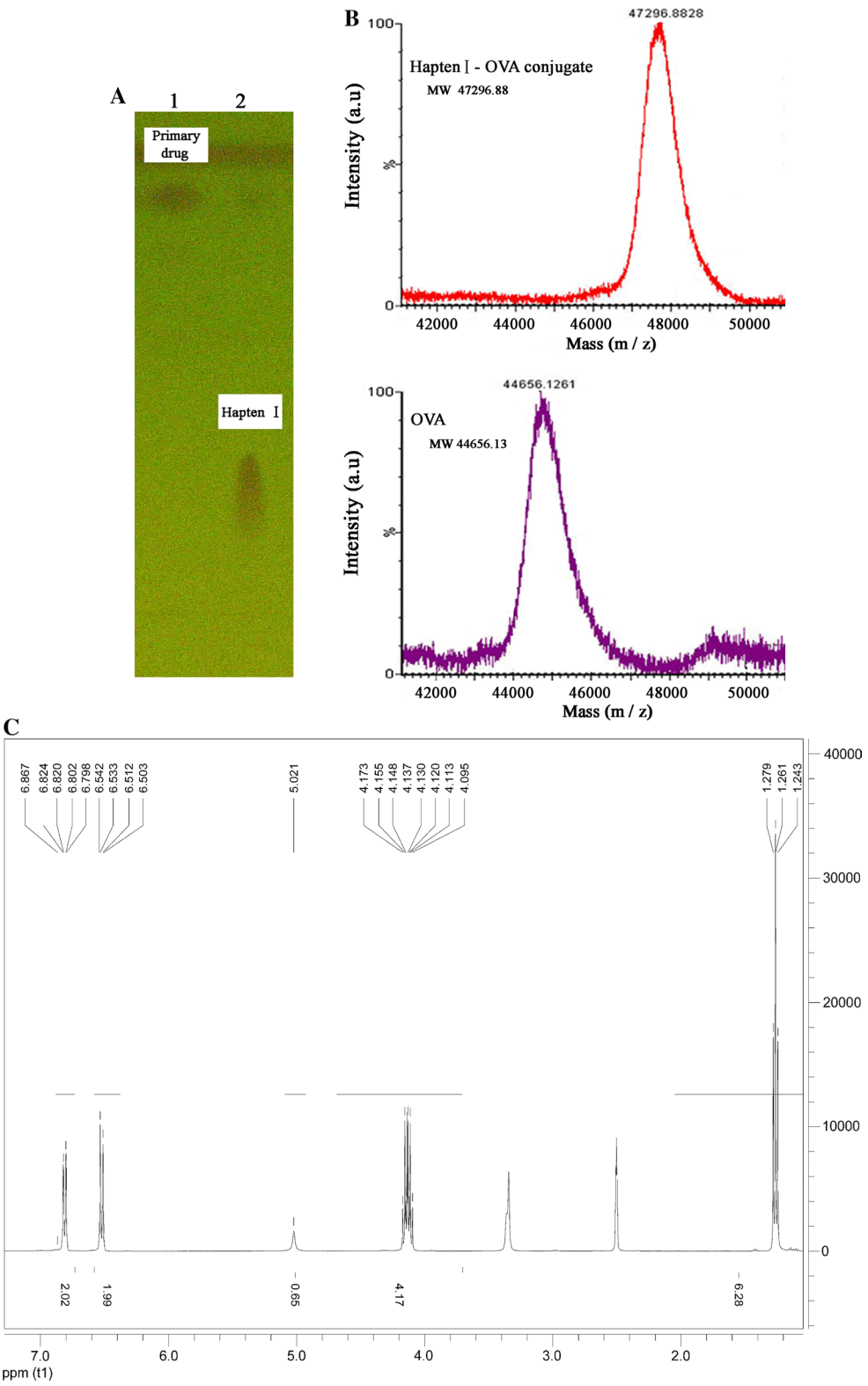

Fig. 2 Verification of hapten I, hapten I-OVA conjugate and hapten II. **a** TLC analysis of hapten I. Compared with the parathion original drug, there was new product (hapten I) generated. **b** Mass spectra of hapten I-OVA conjugate and OVA. Their MWs were 47,296.88 and 44,656.13, respectively. **c** ^{1}H HMR spectrum of hapten II. $\delta = 1.26$ (t, $J = 5.7$, CH_2CH_3, 6H), 4.17–4.95 (m, CH_2CH_3, 4H), 5.02 (s, Ph–NH, 1H), 6.503–6.867 (m, PhH, 4H)

1 mL PBS with 5% methanol and analysed by CIELISAs without any other purification procedure.

Rice and corn samples were prepared with the same procedure. Concretely, the spiked samples were mixed with 2 mL of methanol/water (75:25, V/V) and then shaken using a rotary shaker at 250 rpm for 15 min. The mixture was kept for 24 h. The aliquot of the extract was then diluted with 1 mL PBS with 5% methanol.

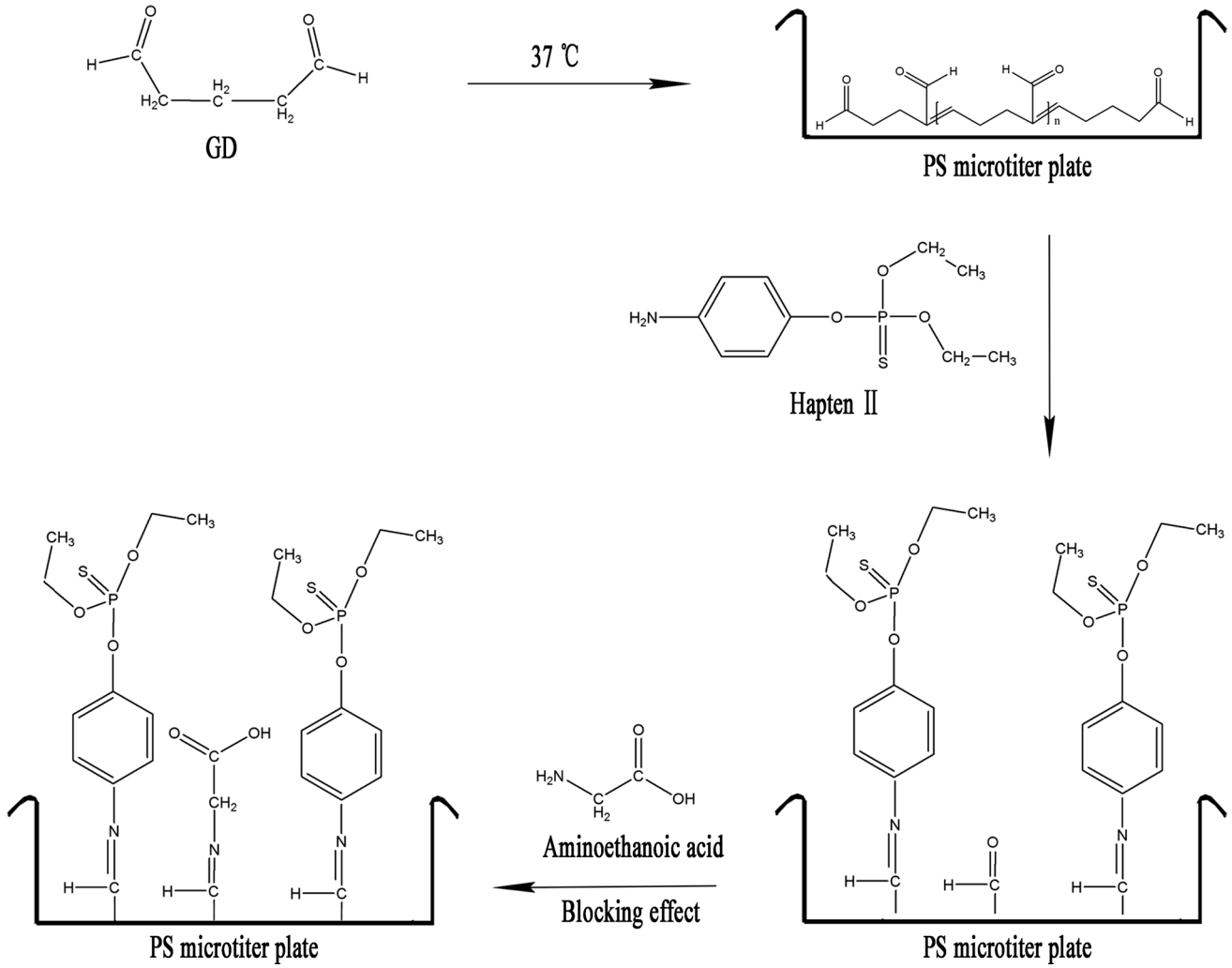

Fig. 3 Reaction mechanism of direct covalent linkage of hapten II and the PS surface of microtiter plates. The GD was used to treat the microtiter plate surface for 2 h to introduce aldehyde groups that can covalent link hapten II. And, aminoethanoic acid was used to block the excess aldehyde groups of the modified surface

Table 1 Effect of blocking reagents on absorbance values ($n = 3$)

Blocking reagents	$A^{a}_{blocking}$	A^{b}_{bg}	A^{c}_{0}	A^{d}_{ex}
BSA	0.209 ± 0.04	0.109 ± 0.03	0.407 ± 0.03	0.249 ± 0.02
6-aminocaproic acid	0.335 ± 0.02	0.234 ± 0.04	1.127 ± 0.02	0.375 ± 0.03
Aminoethanoic acid	0.241 ± 0.05	0.102 ± 0.01	1.192 ± 0.03	0.234 ± 0.01
Ethanolamine	0.294 ± 0.03	0.339 ± 0.03	1.607 ± 0.05	0.768 ± 0.04
Guanidine	0.275 ± 0.01	0.236 ± 0.02	0.829 ± 0.04	0.423 ± 0.03
Acrylamide	0.317 ± 0.04	0.375 ± 0.04	0.987 ± 0.02	0.846 ± 0.05

Using aminoethanoic acid and BSA, there were higher A_0 value and lower A_{bg} value

[a] Surface only coated with 6-aminocaproic acid, aminoethanoic acid, BSA, ethanolamine, guanidine or acrylamide, respectively

[b] Absorbance at negative group

[c] Absorbance at zero hapten concentration

[d] Absorbance at excess hapten concentration

The following steps were just the same as the cucumber preparation.

The water sample was filtered by the Millipore filtration (0.45 μm) and then was spiked with parathion standard (1, 5, 10 ng mL^{-1}) in methanol and diluted with 10% methanol-PBS for CIELISAs.

Lake water samples preparation for HPLC method

Two lake water samples (A and B) were collected from two different lakes in Tianjin. HPLC method was carried out by some reports [46, 47]. HPLC instrument was utilized for the experiments, and the obtained data were collected and processed by the empower software. The optimized chromatographic conditions were as follows:

Column: C18 (250 mm × 4.6 mm, 5 μm)
Mobile phase: acetonitrile–water (50:50, v/v)
Flow rate: 1.0 mL^{-1} at room temperature
Detection wavelength: 270 nm.

Results and discussion

Haptens syntheses

The obtained hapten I was characterized by TLC analysis. In Fig. 2a, it was learned that a new product was generated and hapten I preparation was initially confirmed. Figure 2b shows the mass spectrograms of hapten I–OVA conjugate and OVA. It was determined that MWs of hapten I–OVA conjugate and OVA were

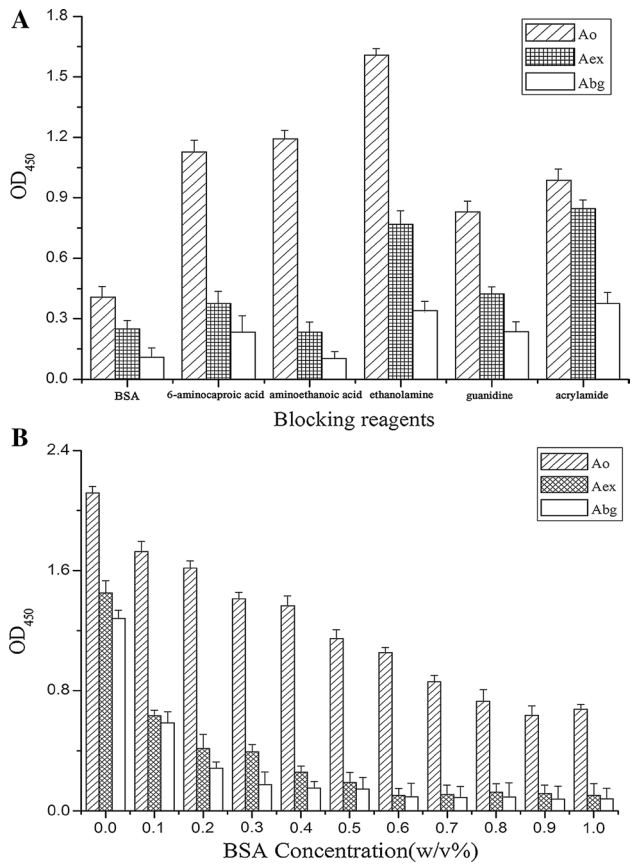

Fig. 4 Blocking effects of blocking reagents and BSA concentration of diluting PBS. A_0 value is the absorbance at zero hapten concentration; A_{ex} value means the absorbance at excess hapten concentration; A_{bg} value is the absorbance at negative group. *Each point* represents the mean ± SD (standard deviation, $n = 3$). **a** Effect of different blocking reagents on the absorbance values. A_0 value is the absorbance at zero hapten concentration. When using aminoethanoic acid, there were higher A_0-A_{ex} difference and lower value of Abg. **b** Effect of BSA with different concentration on the absorbance values. Higher A_0-A_{ex} difference and lower A_{bg} were achieved when BSA concentration beyond 0.6% (M/V)

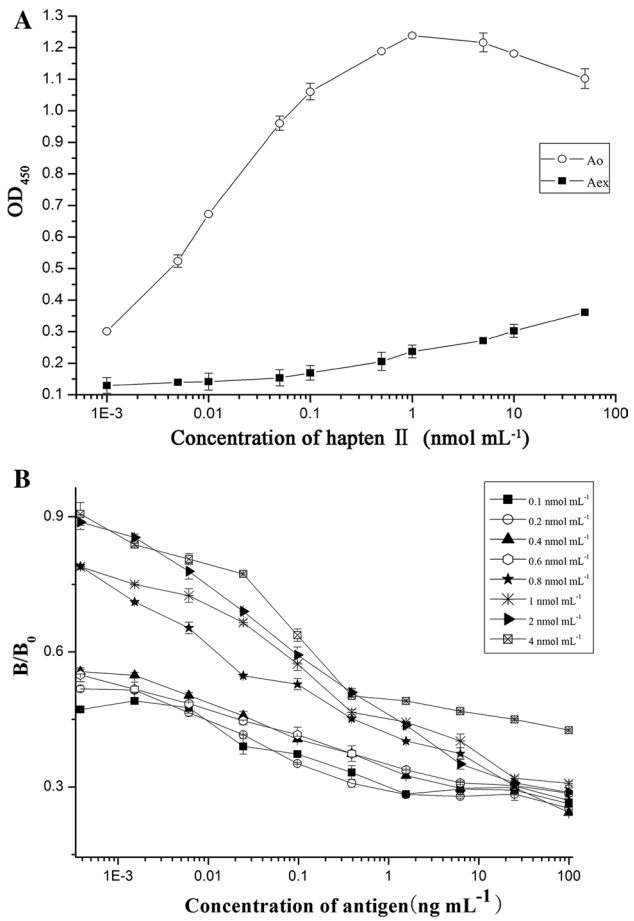

Fig. 5 Effect of hapten II concentration on genetic parameters of CIELISA. A_0 value is the absorbance at zero hapten concentration; A_{ex} value means the absorbance at excess hapten concentration. *Each point* represents the mean ± SD (standard deviation, $n = 3$). **a** Effect of the concentration of hapten II on A_0-A_{ex} difference. From 0.1 to 4 nmol mL^{-1} of hapten II concentration, higher A_0-A_{ex} difference was achieved. **b** Effect of the concentration of hapten II on competitive inhibition. There was the obvious linear correlation using 2 nmol mL^{-1} of hapten II

47,296.88 and 44,656.13, respectively, and the coupling ratio was 7:1 according to Eq. (1). The synthesized hapten II was characterized by ^{1}H NMR in Fig. 2c. ^{1}H HMR (DMSO + D_2O): $\delta = 1.26$ (t, $J = 5.7$, CH_2CH_3, 6H), 4.17–4.95 (m, CH_2CH_3, 4H), 5.02 (s, Ph–NH, 1H), 6.503–6.867 (m, PhH, 4H).

As described in Fig. 3, microtiter plates were pre-treated with GD at 37 °C to introduce aldehyde groups, which were used to cross-link with amino groups of hapten II. Aminoethanoic acid was then utilized for blocking excess aldehyde groups of the surface.

Selection of blocking effect

Sensitivity of an assay depends on the solid phase condition to a great extent. Ideal solid phase should be consistent, stable and have low background signal. We chose medium binding ELISA plates, because it can largely eliminate non-specific absorption, as described in the product manual.

In this assay, the non-specific binding of antibodies on the surface was prevented by the following steps. In the blocking step, several blocking reagents (BSA,

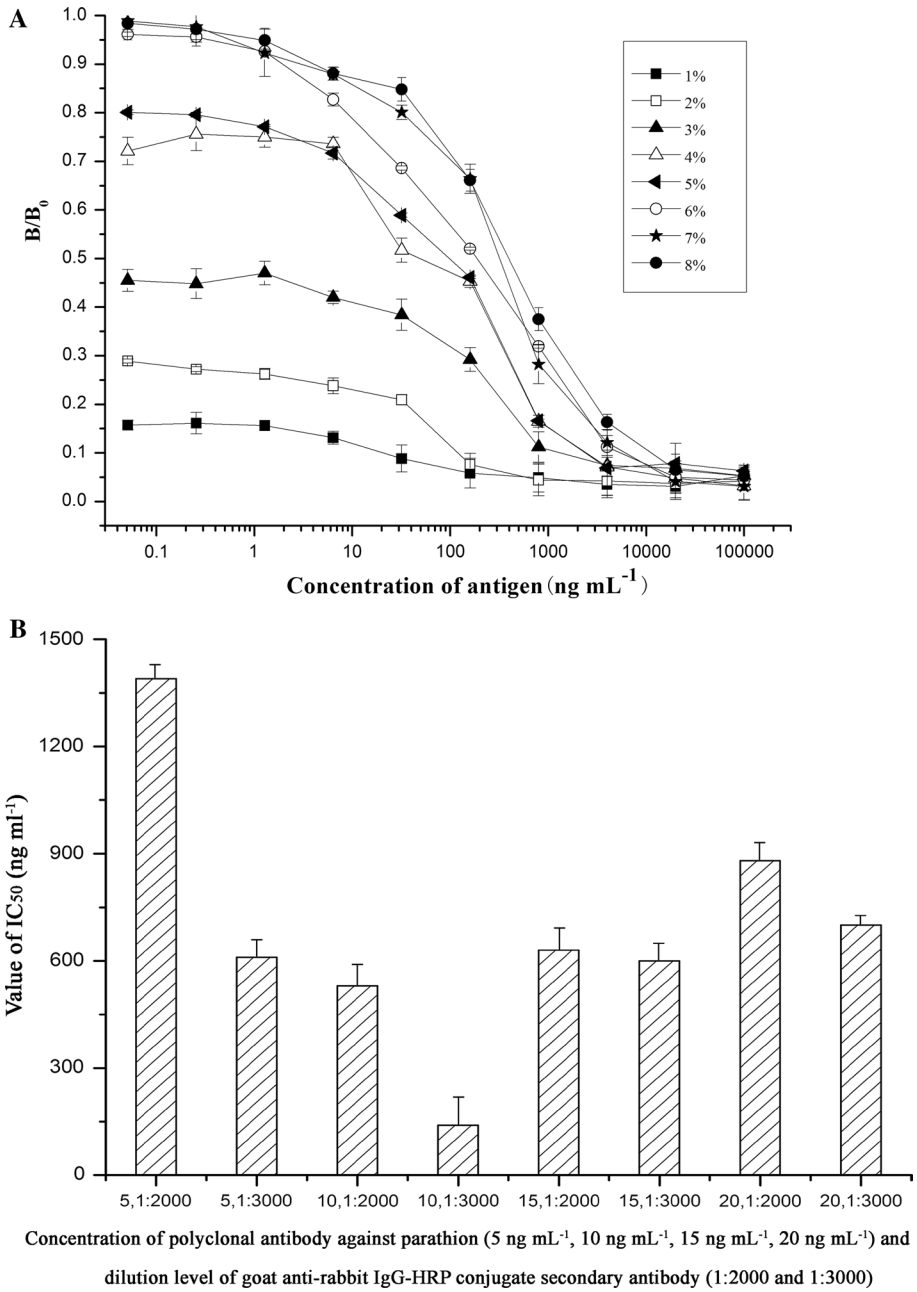

Fig. 6 Effect of the concentration of GD, antibodies on genetic parameters of CIELISA using the hapten II covalent linked plates. *Each point* represents the mean ± SD (standard deviation, $n = 3$). **a** Effect of the concentration of GD solution on competitive inhibition. When using 6% a dilution of GD, the linear correlation was achieved. **b** Concentration of polyclonal antibody against parathion (5, 10, 15, 20 ng mL^{-1}) and dilution level of goat anti-rabbit IgG-HRP conjugate secondary antibody (1:2000 and 1:3000)

6-aminocaproic acid, acrylamide, ethanolamine, guanidine and aminoethanoic acid) were utilized for blocking the excess aldehyde groups of the surface modified with a GD layer. As shown in Table 1, aminoethanoic acid and BSA prevented non-specific binding of assay components to a large extent (A_{bg}) without influencing the specific binding of the primary antibody (A_0). In Fig. 4a, there was higher A_0-A_{ex} difference and lower value of A_{bg} while using aminoethanoic acid. It indicated that aminoethanoic acid could prevent non-specific binding of antibodies to the surface and the high ratio A_0/A_{ex} was desirable for assay purpose. On the other hand, the non-specific binding could be further avoided when antibodies were diluted in PBS with BSA. As shown in Fig. 4b, A_0, A_{ex} and A_{bg} values all trended to decrease with the increase of BSA concentration. A_{ex} and A_{bg} almost run through the lowest values, and the positive value (A_0) was decreased greatly when BSA concentration beyond 0.6% (M/V). Thus, aminoethanoic acid was used for blocking the excess aldehyde groups of the modified surface (Fig. 4a), and 0.6% BSA in PBS was selected to dilute the antibodies (Fig. 4b).

Optimization of hapten II concentration

As shown in Fig. 5a, when the absorbance values A_0 and A_{ex} were close to the background value (A_{bg}), it was indicated that hapten II was not covalent linked on the surface of microplates. The A_0 signals increased with increasing of hapten II concentration, but run through a maximum value at approximately 1 nmol mL^{-1}. This phenomenon may be explained by steric effects, which arising from the fact that each atom with a molecule occupies a certain amount of space. If atoms are brought too close to each other, there is an associated cost in energy due to overlapping electron cloud, and this may influence the molecule preferred shape (conformation) and reactivity. In other words, the steric effects can affect the reaction between antibody and antigen. When the hapten II was beyond 1 nmol mL^{-1}, the mass of hapten II influenced each other to generate conformational changes, which resulted in unfavourable recognition between the rabbit polyclonal antibody against parathion and hapten II [36].

From 0.1 to 4 nmol mL^{-1} of hapten II concentration, there was higher A_0-A_{ex} difference (Fig. 5a). So we selected this range of concentration for further optimization of hapten II concentration. According to Eq (2), the linear correlation was obvious and sensitivity was highest when hapten II concentration was 2 nmol mL^{-1} in Fig. 5b. Thus, we chose 2 nmol mL^{-1} of hapten II as coating concentration in this study.

Optimization of GD concentration, the rabbit polyclonal antibody against parathion and goat anti-rabbit IgG-HRP conjugate secondary antibody concentration

The competitive inhibition assay was performed to select the optimized concentration of GD. According to the previous studies [41], a range of 1−8% GD was tested. Based on Eq. (2), the linear correlation was manifest in the use of a 6% dilution of GD in Fig. 6a. So 6% GD was chose in the subsequent experiments. Figure 6b shows a chequerboard titration of the rabbit polyclonal antibody against parathion and goat anti-rabbit IgG-HRP conjugate secondary antibody concentration by using competitive inhibition assays. From the result, it was learned that the optimal concentration of the rabbit polyclonal antibody against parathion was 10 ng mL^{-1} and the proper titer of goat anti-rabbit IgG-HRP conjugate secondary antibody was 1:3000.

Sensitivity

The typical standard curves of CIELISAs are given in Fig. 7. In the case of CIELISA using the hapten I–OVA conjugate coated plates, the IC_{50} value and the limit of detection (LOD, calculated as IC_{10}) for parathion were 375 and 1.25 ng mL^{-1}, respectively. However, in CIELISA using the hapten II covalent linked plates, the IC_{50} value and LOD were 15 and 0.08 ng mL^{-1}, respectively. In view of LOD, the sensitivity of the CIELISA using the hapten II covalent linked plates was improved 15-fold compared with that using the hapten I–OVA conjugate coated plates. The

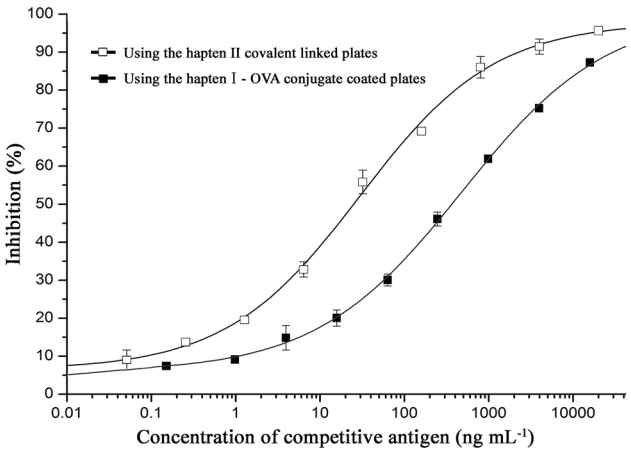

Fig. 7 Standard curves of CIELISAs using the hapten I-OVA conjugate coated plates and the hapten II covalent linked plates. Their IC_{10} values were 1.25 ng mL^{-1} and 0.08 ng mL^{-1}, respectively. *Each point* represents the mean ± SD (standard deviation, $n = 3$)

great improvement of sensitivity would be resulted from the following reasons. The reactive site of hapten I was easily shielded by carrier protein OVA in the preparation of hapten I–OVA conjugate due to its low MWs and the single reactive site of SM-hapten. In addition, the immobilization of hapten I-OVA conjugate on the microtiter plate surface mainly depended on hydrophobic interactions. It may involve significant conformational changes which lead carrier protein easily to shield SM-hapten during this immobilization. However, the directly covalent linkage of hapten II on the PS plates may release more reactive sites of SM-hapten since there was no interference of carrier protein, thus improving the reaction efficiency of SM-hapten and antibody reactions.

As a result, our CIELISA using the hapten II covalent linked plates achieved a highly sensitive detection of parathion (as low as 0.08 ng mL^{-1}), which was less than or approximately close to the detection borderline of traditional chromatograph methods [18–21, 46–48] and the reported CIELISAs [49, 50] and was below the legislated maximum residue levels [51].

Table 2 Cross-creativities of the inhibition assay with parathion, methyl parathion, atrazine, monocrotophos, chlorpyrifos and diazinon ($n = 3$)

Compounds	CR (%)
Parathion	100
Methyl parathion	18
Atrazine	<0.01
Monocrotophos	<0.01
Chlorpyrifos	<0.01
Diazinon	<0.01

CR for parathion was 100% and that was highest than the other analogues

Although IC_{50} value could not be determined accurately due to the limited solubility of the compounds at high concentrations, it was clear that inhibition was less than 50% at 1500 ng mL^{-1}

Specificity

In this study, CR data of CIELISA using the hapten II covalent linked plates are summarized in Table 2. It was learned that the assay had CR of 100% to parathion, 17% to methyl parathion and less than 0.01% to other analogues. Except for parathion, each IC_{50} value was greater than the highest concentration of compounds used (1500 ng mL^{-1}). So the CIELISA using the hapten II covalent linked plates was very specific against parathion.

Recovery and precision

Four kinds of real samples (river water, cucumber, rice and corn) were spiked with parathion and analysed by the developed CIELISAs. As shown in Table 3, the recoveries for river water ranged from 83.0 to 101.0% with CVs of 6.65–11.13% in CIELISA using the hapten II covalent linked plates, which showed better stability than that using the hapten I–OVA conjugate coated plates. The food samples included cucumber, rice, corn were also analysed by CIELISAs after being fortified with parathion at 5.0, 10, 20 ng mL^{-1}. The recoveries of CIELISA using the hapten II covalent linked plates were ranged from 86.3 to 110.2% with CVs of 5.95–13.59%. The fortifications of food samples were carried out by incubating the chopped ground rice in fortification solutions for 24 h. Therefore,

Table 3 Accuracy and precision of parathion in spiked samples (River water, cucumber, rice and corn) by the CIELISAs using the hapten II covalent linked plates and the hapten I-OVA conjugate ($n = 3$)

Sample	Spiked (ng/g)	Using the hapten II covalent linked plates			Using the hapten I–OVA conjugate coated plates		
		Measured (ng/g)	Recovery (%)	CV (%)	Measured (ng/g)	Recovery (%)	CV (%)
River water	1	0.8 ± 0.09	83.0	11.13	0.8 ± 0.01	80.3	12.28
	5	5.1 ± 0.37	101.0	7.46	5.1 ± 0.37	102.0	7.44
	10	9.5 ± 0.63	94.7	6.65	9.3 ± 0.70	93.3	7.42
Cucumber	5	4.9 ± 0.66	97.6	13.59	4.9 ± 0.62	98.2	12.61
	10	10.2 ± 0.94	102.3	9.24	9.6 ± 0.83	95.6	8.66
	20	18.6 ± 1.17	92.9	6.32	18.1 ± 1.09	90.5	6.02
Rice	5	5.4 ± 0.41	108.8	7.48	5.7 ± 0.42	112.9	7.44
	10	11.0 ± 0.67	110.2	5.95	10.5 ± 0.63	105.2	6.03
	20	20.8 ± 2.03	104.1	9.75	21.7 ± 2.12	108.7	9.77
Corn	5	4.4 ± 0.47	88.2	10.71	4.2 ± 0.42	84.3	9.98
	10	9.1 ± 0.80	91.3	8.69	8.9 ± 0.76	88.5	8.54
	20	17.3 ± 1.41	86.3	8.21	16.1 ± 1.30	80.6	8.06

Table 4 Results from analysis of parathion in lake water samples by CIELISAs with using the hapten II covalent linked plates and the hapten I-OVA conjugate coated plates, and HPLC ($n = 3$)

Lake water samples	Spiked (ng/g)	Using the hapten II covalent linked plates			Using the hapten I–OVA conjugate coated plates			HPLC		
		Measured (ng/g)	Recovery (%)	CV (%)	Measured (ng/g)	Recovery (%)	CV (%)	Measured (ng/g)	Recovery (%)	CV (%)
A	1	1.0 ± 0.11	104.2	10.57	0.9 ± 0.13	88.2	14.78	1.1 ± 0.92	106.6	8.57
	5	4.8 ± 0.58	96.6	12.01	4.7 ± 0.64	93.5	13.68	4.8 ± 0.37	96.2	8.05
	10	10.2 ± 1.04	101.7	10.27	9.3 ± 1.32	92.7	14.24	10.1 ± 0.96	102.5	9.34
B	1	1.1 ± 0.09	109.3	8.26	0.9 ± 0.08	87.3	9.28	0.9 ± 0.08	93.8	9.35
	5	4.8 ± 0.55	96.8	11.42	5.2 ± 0.63	104.1	12.09	5.5 ± 0.45	109.4	8.15
	10	9.2 ± 0.91	92.1	9.91	10.6 ± 1.13	107.5	10.51	10.6 ± 0.91	105.5	8.62

pesticides may have penetrated quite deeply into the interior of grains during the incubation period. However, the CR data suggested that pesticides can be nearly completely recovered by the extraction procedure adopted in this study.

Analysis of lake water samples

CIELISAs and HPLC were utilized to detect parathion in lake water samples to assess the applicability and validity of the developed methods. As shown in Table 4, determination of parathion measured by three methods was similar. It was learned that the CIELISA using the hapten II covalent linked plates could be used for the determination of parathion in real samples.

Compared with GC, HPLC and other methods [17–23], our proposed approach eliminated sophisticated equipment, and complicated operation processes. For low-pressure gas chromatography–tandem mass spectrometry (LP-GC/MS–MS) for parathion [52], the sample preparation procedure consisted of ten steps, and for GC–MS analysis [53], it generally needed eight steps to prepare real samples, which were time-consuming and complicated processes. For fluorescence probing sensors [22], acetylcholinesterase biosensor [24], and molecule imprinted polymeric sensor [54, 55], complex fabrication was required to prepare carbon spheres, CdTe quantum dots and molecular imprinted polymer, and complex instruments (surface plasmon resonance, electrochemical working station and fluorescence spectrophotometer) were utilized to generate a readable optical or electronic signals. Therefore, our constructed method was characterized by (a) low cost, easy construction and simple operation; and (b) stable, sensitive and selective detection.

Conclusion

In summary, we have developed a highly sensitive CIELISA in which a SM-hapten was directly covalent linked the PS surface of microtiter plates for the detection of parathion. The developed assay not only refrained from a complex process of hapten-protein conjugation, but also showed highly sensitivity, stability and selectivity. Further research should be focused on exploiting more means to directly link SM-hapten to microtiter plates and applying some signal amplification systems simultaneously to further improve the sensitivity and stability of immunoassays.

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