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Development of a sensitive ELISA for the analysis of the organophosphorous insecticide fenthion in fruit samples

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Abstract

Two fenthion haptens, 4-(4-(dimethoxyphosphorothioyloxy)-2-methylphenylamino)-4-oxobutanoic acid (H1) and 6-(methoxy(4-(methylthio)phenoxy)phosphorothioylamino)hexanoic acid (H2), were synthesized. H1 was conjugated with bovine serum albumin (BSA) and H2 with ovalbumin (OVA) by the active ester method. Then H2-OVA conjugate was used as coating antigen, while H2-BSA conjugate was used to produce polyclonal antibodies. After optimization, an effective competitive indirect enzyme-linked immunosorbent assay (ELISA) for determination of fenthion was established with the new combination of antibody/antigen, I_{50} of which was 0.01 ng/ml, and there was only cross reactivity (CR) with fenitrothion (4.5%), and CRs with other tested pesticides were all below 0.1%. The recoveries obtained by standard fenthion addition to the different fruit samples such as grape, peach, pear and tomato were all from 79.8% to 106.0%. Therefore, the optimized ELISA may become a new convenient and satisfied analytical tool for monitoring fenthion residues in agricultural samples.

Keywords: Organophosphorus pesticide; Fenthion; Hapten; Antibody; ELISA

1. Introduction

Organophosphorous insecticides (OPs) are a group of highly toxic compounds which are extensively used as agricultural and domestic pesticides (Costa, 1988). OP toxicants generally elicit their effects by inhibition of acetylcholinesterase, which leads to the accumulation of the neurotransmitter acetylcholine (Ach) in synapses, over stimulates the post synaptic cholinergic receptors with consequent signs of neurotoxicity (Ecobichon, 1996; Gallo & Lawryk, 1991). So non-target organisms, such as human, fish, bee and so on, are also threatened by the insecticides.

Fenthion, *O*,*O*-dimethyl *O*-[3-methyl-4-(methyl-thio)-phenyl]phosphorothioate is one of OP insecticides. It is also moderately toxic to mammals (Brun, Garcés-García, Puchades, & Maquieira, 2004). Due to its extensive application to crops and cattle, the occurrence of fenthion residues in food and the environment has been widely reported (Tsatsakis et al., 2003). Thus, many countries classified fenthion as a restricted use pesticide and gave a strict standard of maximum residue limited (MRLs) for the chemical in farm produce, and China government is not an exception.

Several methods have been described for the determination of fenthion at trace levels, including high performance liquid chromatography (Cabras, Plumitallo, & Spanedda, 1991), flow injection (Hernandez, Carabias, Becerro, & Jiménez, 1988), colorimetric and bioassay techniques (Devi, Mohandas, & Visalakshy, 1986), cholinesterase-based biosensors (Lee, Kim, Cho, & Lee, 2002), and gas

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chromatography (Arrebola, Martínez Vidal, Gonzaález-Rodríguez, Garrido-Frenich, & Sánchez Morito, 2003). However, more specific, sensitive, rapid and economical analytical methods for the detection of fenthion residues are needed. In particular, an immunoassay method would be a useful analytical tool (Brun et al., 2004). Immunoassays for fenthion have been reported (Brun et al., 2004; Cho, Kim, Hammock, Lee, & Lee, 2003; Kim et al., 2003a, Kim, Cho, Lee, & Lee, 2003b) recently. In our previous work, five haptens with different spacer-arm attachment sites on the structure of the organophosphorus insecticide fenthion were synthesized and 15 combinations of immunizing/coating hapten were selected for studies of assay sensitivity and specificity for fenthion. Finally, we found the most sensitive combination which was different from other people'. With this novel combination of immunizing/coating hapten, we developed a sensitive ELISA for detection of fenthion residues in several fruit samples.

2. Materials and methods

2.1. Chemicals and instruments

Chemical reagents for hapten synthesis and pesticide standards used for cross-reactivity studies were supplied by Jiangsu Pesticide Research Institute (Nanjing, China). Analytical grade solvents were from Sinopharm Group Chemical Reagent Co., Ltd (Shanghai, China). Tween 20, *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC), bovine serum albumin (BSA), ovalbumin (OVA), and complete and incomplete Freund's adjuvant were purchased from Sigma (St. Louis, USA). Tetramethylbenzidine (TMB) and peroxidase-labeled goat anti-rabbit immunoglobulins (GAR-HRP) were obtained from Huamei Biotechnology Co. (Luoyang, China). All other reagents used were analytical grade. Thin-layer chromatography (TLC) was performed on 0.25 mm, precoated silica gel 60 F254 on aluminum sheets (Merck, Darmstat, Germany). Column chromatographic purifications were carried out with silica gel (60–230 mesh), from Qingdao Haiyang Chemical Co., Ltd (Qingdao, China).

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with a Bruker ARX pectrometer (300 MHz, Rheinstetten, Germany). Chemical shift values are given in parts per million (ppm) downfield from internal standard deuterium chloroform. Coupling constants are expressed in Hz and the abbreviations s, d, t, m and ar, represent singlet, doublet, triplet, multiplet and aromatic, respectively. UV–Vis spectra were recorded on a Bechman 640 spectrophotometer. Polystyrene 96-well microtiter plates were from Costar (Corning, Massachusetts, USA). A microplate washer from Prolong New Technology Co. (Beijing, China) was used to wash ELISA plates. Absorbance (A) was measured using a microtiter plate reader (Thermo Electron Co., United States); this device was controlled by a personal computer containing the standard software package EasySoftware.

2.2. Synthesis of haptens

2.2.1. Synthesis of immunizing hapten (H1)

To a solution of compound **1** (2.1 g, 7 mmol) in ethyl ether (40 mL) were added 9:1 acetic acid:HCl (21 mL) and zinc dust (4.1 g, 60 mmol). The reaction mixture was stirred for 30 min at room temperature, and then refluxed for 30 min. The mixture was decanted from the reaction flask and the zinc was washed with ether. The combined organic phase was then washed with water and dried over K_2CO_3. The solvent was evaporated and the residue was subjected to column chromatography (dichloromethane, R_f: 0.55) to give the product as a yellow-red syrup (1.8 g, 79%).

^{1}H NMR ($CDCl_3$) δ: 2.15 (s, 3H), 3.82 (s, 3H, CH_3-OP), 3.87 (s, 3H, CH_3-OP), 6.61 (d, $J = 8.5$ Hz, 1H, Ar), 6.84 (s, 1H, Ar), 6.86 (d, $J = 8.5$ Hz, 1H, Ar); ^{13}C NMR ($CDCl_3$) δ: 123.0, 119.4, 115.7, 77.9, 77.5, 77.1, 55.5, 18.0; MS (EI) m/z (%): 247 (M^+, 80), 138 (100).

To a solution of compound **2** (1.7 g, 6 mmol) in dichloromethane (90 mL) was added succinic anhydride (0.7 g, 6 mmol). After the mixture was stirred at room temperature for 18 h, the solution was concentrated. The recrystallization of the residue from ethyl ether gave H1, 4-(4-(dimethoxyphosphorothioyloxy)-2-methylphenylamino)-4-oxobutanoic acid, as a brown solid (1.9 g, 90%).

^{1}H NMR ($CDCl_3$) δ: 2.23 (s, 3H), 2.70 (t, $J = 3.1$ Hz, 2H), 2.80 (t, $J = 3.1$ Hz, 2H), 3.84 (s, 3H, CH_3-OP), 3.88 (s, 3H, CH_3-OP), 7.02 (d, $J = 9.2$ Hz, 1H, Ar), 7.38 (s, 1H, Ar), 7.68 (d, $J = 9.2$ Hz, 1H, Ar), 8.25 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ: 178.1, 171.2, 148.2, 132.9, 125.6, 123.1, 119.2, 119.1, 55.6, 31.7, 29.9, 18.3; MS (ESI) m/z (%): 346 ($M-H^+$, 44).

2.2.2. Synthesis of coating hapten (H2)

Compound **6** (H2), 6-(methoxy(4-(methylthio)phenoxy)phosphorothioylamino)hexanoic acid, was prepared as Kim et al.described (2003a). The product was a colorless liquid and its ^{1}H NMR was same as the reference (Kim et al., 2003a). MS (ESI) m/z (%): 376 ($M-H^+$, 21), 753 ($2M-H^+$, 100).

2.3. Preparation of hapten–protein conjugates

For immunization purposes, H1 was covalently attached through its carboxylic acid moiety to the lysine groups of BSA by the active ester method (Langone & van Vunakis, 1982). Additionally, H2 was coupled to OVA to obtain coating antigens with the same method as the above. Then, the immunogen (H1-BSA) and the coating antigen (H2-OVA) were purified by dialysis (Zhang et al., 2006) against phosphate buffer (PB: 0.02 mol /L, pH 6.8). The conjugates were stored at −20 °C until use. Finally, UV–Vis spectral data supported the structures of the final conjugates. The hapten density (the number of the hapten molecules per molecule of protein) of conjugates was estimated directly by mole absorbance ε.

Hapten density $= (\varepsilon_{conjugation} - \varepsilon_{protein})/\varepsilon_{hapten}$

2.4. Production of polyclonal antibodies

The immunogen, H1-BSA (2 mg in 0.5 ml PBS), was suspended in 0.5 ml Freund's completed juvant and injected intramuscularly into two female New Zealand white rabbits (01 and 02). Animals were boosted at 21-day intervals with the same immunogen suspended in 0.5 ml Freund's incomplete adjuvant. Seven days after each boost, blood was obtained by bleeding the ear vein of the rabbit. When no titer enhancement was observed, whole blood was collected, allowed to coagulate overnight at 4 °C. Then, serum was separated by centrifugation. Finally, the antisera (pAb01 and pAb02) were added equal volume glycerin and stored at −20 °C.

2.5. Screening of antisera by checkerboard titration

The polyclonal antibodies raised against the immunogen (H1-BSA) were screened against the coating antigen (H2-OVA) by checkerboard titration. The antiserum with higher titer values was selected in this study for competitive ELISA as described by Zhang et al. (2006). Then, to have a rough estimate of appropriate dilutions of antiserum and coating antigen, several dilutions of serum were titrated against varying amounts of the coating antigen. The procedure for the checkerboard assays was the same as that for the competitive assays (see Section 2.6) except that only solvent instead of pesticide solution was added into the well at the competition step.

2.6. Competitive indirect ELISA (CI-ELISA)

Microplates were coated overnight at 4 °C with 50 μl per well of the appropriate coating antigen concentration in 0.05 M carbonate–bicarbonate buffer (pH 9.6). After washing with PBST (PBS with Tween 20: 8 g/l NaCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, 0.2 g/l KCl, and 0.05% Tween-20, v/v), the surface of the wells was blocked with 100 μL of a 1% gelatin in PBS (or 1% OVA, 3% skimmed milk powder) for 1 h at 37 °C. After another washing step, 25 μl per well of antiserum diluted in PBS and 25 μl per well of analyte solution were added, and incubated for 1 h. Following a washing step, goat anti-rabbit HRP conjugate (1:2000 in PBST, 50 μl per well) was added and incubated for 1 h at 37 °C. The plates were washed again, and 50 μl per well of TMB solution (3.3 μl of 30% H_2O_2, 400 μl of 0.6% TMB in DMSO per 25 ml of acetate buffer, pH 5.5) was added. The color development was stopped after 10–15 min with 2 M H_2SO_4 (25 μl per well). The absorbance was measured at 450 nm. Sigmoidal curves were fitted to a logistic equation (Raab, 1983) from which I_{50} values (concentration at which binding of the antibody to the coating antigen is inhibited by 50%) were determined.

2.7. Optimization of a CI-ELISA

The screening of the variables to set up competitive ELISA procedures was performed in conjugate- and antibody-coated formats, following the protocols described by Tijssen (1987).

Assay optimization was performed using fenthion as the competitor analyte. A set of experimental parameters (ionic strength, pH, organic solvent and blocking agents) was studied sequentially to improve the sensitivity of the immunoassay. The main criteria used to evaluate immunoassay performance were I_{50} and R^2 of their linear equation. The effect of pH was evaluated using different PBS solutions, ranging from pH 5.0 to 9.0. To estimate the influence of salt concentration, PBS at 0, 20, 40, 80, 160, 320 and 640 mM was tested. Also, the effect of organic sovent (5%, 10% and 20% of methanol; 10% and 20% of DMF and acetone; v/v) on immunoassay performance was studied. Finally, the influence of blocking reagent (1% OVA, 1% gelatin and 3% skimmed milk powder) was investigated.

2.8. Evaluation of the optimized CI-ELISA

2.8.1. Cross-reactivity studies

The specificity of the ELISA procedures was determined against several organophosphorus insecticides, and calculated as follows: CR (%) $= [I_{50}$ (fenthion)$/I_{50}$ (interferent)$] \times 100$. Here, CR (%) of fenthion was defined as 100%.

2.8.2. Sensitivity and precision

According to the results from Section 2.7, the optimized ELISA conditions were determined, and then under the best parameters, competitive inhibition ELISA in serial concentrations of fenthion standard (0.00001–10,000 ng/ml) was repeated five times at different times in order to work out the mathematical simulation equation and linear detection range, and evaluate the precision (intra-assay variability and inter-assay variability) and sensitivity.

2.8.3. Accuracy (analysis of spiked samples)

The accuracy was evaluated by spiked samples experiment. To study spike recovery, fruits of grape, peach, pear and tomato were spiked with different concentrations of fenthion and analyzed in a blind fashion by the ELISA protocol. These fruit samples were purchased from a local supermarket. For the spike-and-recovery test, four final concentrations (0, 2, 20, 50 ng/ml) of fenthion for each of the above samples were prepared. Fruit samples first were finely chopped, and then 2 ml methanol was added to 1 g each of the samples and shaked for 2 h. After stability of several minutes, the supernatant was finally analyzed by the ELISA without any other purification procedure (the samples were diluted with PBS-methanol buffer for 20 times).

3. Results and discussion

3.1. Hapten synthesis and conjugation

The synthesis of haptens is the key step in a procedure of research on rapid immunoassay for pesticide residue. Some haptens of fenthion (Brun et al., 2004; Kim et al., 2003a, 2003b) have been reported. Their final immunizing haptens were all with the spacer arm to the thiophosphate moiety. Here, we designed and synthesized a new combination of immunizing/coating hapten on the basis of our screening work recently (Zhang et al., 2007). The hapten (H1) with the spacer arm to fenthion aromatic ring was treated as immunizing hapten, while the hapten (H2) with the spacer arm to the thiophosphate moiety was used as coating hapten. This combination was different from those reported and was very effectively proved by the following results.

3.2. Screening of antisera by checkerboard titration

The antisera of terminal bleeds from two rabbits were screened against H2-OVA coating antigen using a checkerboard titration method with the coated antigen format (Kim et al., 2003b). The heterologous assay, in which the different haptens were used in coating antigen and immunogen, showed that titer value of pAb01 was 1/64,000 and titer value of pAb02 was 1/128,000. So the antiserum of pAb02 with higher affinity was selected for further development.

3.3. Optimization of a CI-ELISA

To identify potential interferences from environmental samples, the effects of pH and ionic strength on ELISA performance were evaluated in this study. In system pAb02/H2-OVA, a lower salt concentration ($\leqslant$0.08 M) in the assay system resulted in lower I_{50}, whereas a higher salt concentration ($\geqslant$0.32 M) in the assay system resulted in lower R^2 value of its linear equation owing to lower optical densities (OD). According to data from Table 1, 0.16 M of salt concentration was selected in CI-ELISA system. In the herterologous system, when analyte was dissolved in buffer at different pH values ranging from 7.4 to 9.0, no significant effect upon the I_{50} was detected, but acid matrix resulted in lower sensitivity, indicating that the assay could effectively detect fenthion at pH values ranging from 7.4 to 9.0. Ionic strength strongly influenced ELISA performance. For solvent optimization, we tested methanol as it is commonly used as the eluant in SPE, DMF, and acetone because they are common cosolvent used in immunoassay to improve analyte solubility. As observed in Table 2, the lowest I_{50} was found at 5% methanol (0.032 ng/ml). As for the effect of blocking reagents, OVA, gelatin and skimmed milk powder were tested, because they are usually used in ELISA system to eliminate non-specific binding. Finally, 1% gelatin resulted in the lowest I_{50} (0.017 ng/ml) was selected.

Through studies of several factors, the main parameters of ELISA procedure were determined: concentration of coating antigen H2-OVA was 1.2 μg/ml, dilution of pAb02 was 1:8000, the blocking reagent was 1% gelatin, the co-solvent was 5% methanol, pH was 7.4–9, and ionic strength was 0.16 M.

3.4. Evaluation of the optimized CI-ELISA

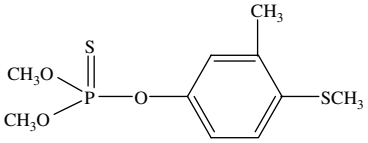
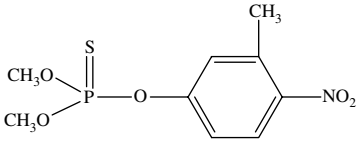
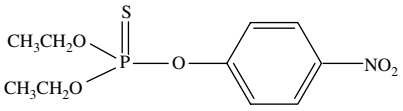
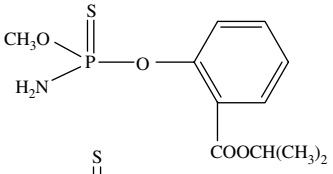
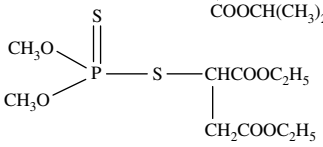
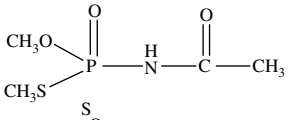
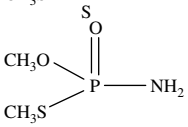
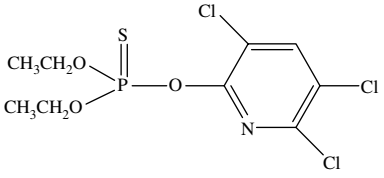
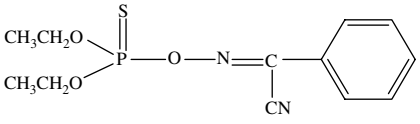
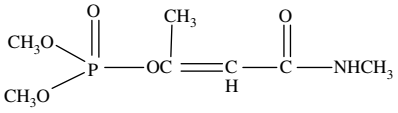
3.4.1. Cross-reactivity

To determine the specificity of the optimized CI-ELISA, several organophosphorus pesticides were tested for cross-reactivity. Table 2 shows the cross-reactivity that was found by the assay. The interference observed was negligible. The highest interference was obtained for fenitrothion, which showed across-reactivity of 4.5% (lower than 14% reported by Kim et al., 2003b; and near about the result of 3.1% reported by Brun et al., 2004). The low cross-reactivity of the antibody for this pesticide is understandable, because it has the same thiophosphate structure as fenthion and its aromatic structure is very similar to fenthion. Therefore, it can be concluded that the CI-ELISA developed for fenthion is highly specific (see Figs. 1 and 2).

Table 1
Effect of ionic strengths, organic solvents, pH values and blocking agents on the ELISA sensitivity (I_{50})

Factors		I_{50} (ng/mL)	R^2 of linear equation	Factors		I_{50} (ng/mL)	R^2 of linear equation
Ionic strengths (mol/L)	0	>1000	–	Organic sovent	5% Methanol	0.032	0.99
	0.02	>1000	–		10% Methanol	0.823	0.98
	0.04	244.375	0.96		20% Methanol	13.009	0.91
	0.08	1.132	0.89		10% DMF	21.783	0.94
	0.16	0.076	0.99		20% DMF	109.227	0.99
	0.32	0.012	0.79		10% Acetone	63.086	0.87
	0.64	0.001	0.58		20% Acetone	217.496	0.91
pH	5.0	76.830	0.86	Blocking agents	1% Gelatin	0.017	0.97
	6.0	16.971	0.92		1% OVA	0.926	0.99
	7.4	0.069	0.98		3% Skimmed milk powder	1.162	0.74
	8.0	0.068	0.94				
	9.0	0.079	0.91				

Table 2
Cross reaction of antibody FpAb2 with fenthion and its analogues

Analogues	Structures	I_{50} (ng/mL)	Cross reactivity (%)
Fenthion		0.01	100.0
Fenitrothion		0.22	4.5
Parathion		49	<0.01
Isocarbophos		>40,000	<0.01
Malathion		>100,000	<0.01
Acephate		>100,000	<0.01
Methamidophos		>100,000	<0.01
Chlorpyrifos		>100,000	<0.01
Phoxim		>100,000	<0.01
Monocrotophos		>100,000	<0.01

3.4.2. Sensitivity and precision

Under the optimized conditions mentioned above, the CI-ELISA procedures were conducted in quintuplicate with a set of standard concentration of fenthion at different times (within three days). Then a competitive curve representing the average was obtained (Fig. 3a). We used logistic (B/B_0) as the lateral coordinates (y), logarithm of concentration of fenthion (ng/ml) as the longitudinal coordinates (x). After conversion of Fig. 3a, we could observe that in

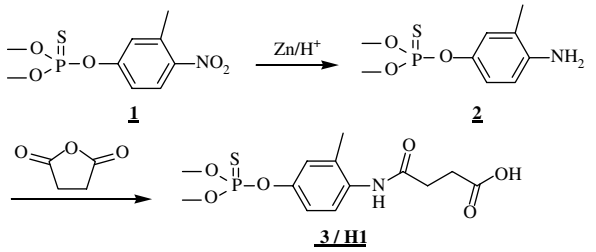


Fig. 1. Synthesis route for immunizing hapten (H1).

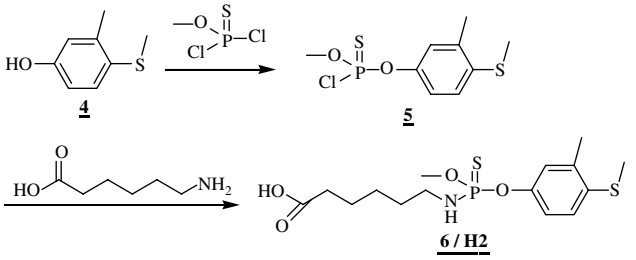


Fig. 2. Synthesis route for coating hapten (H2).

the range of 0.00001–10 ng/mL, the graph between "y" and "x" was linear (Fig. 3b), and the regression equation was obtained ($y = -0.7575x - 1.4928$, $R^2 = 0.984$). In this optimized CI-ELISA, I_{50} value was 0.01 ng/ml and the limit detection (I_{20}) was 0.0002 ng/ml.

The variability of intra-assay and inter-assay of the ELISA curve for fenthion was used to show the precision of this protocol. The intra-assay variability was given by the average of six replicated wells in one microplate. The inter-assay variability was given by the average of five replicated microplates at different times. After calculation described by Liu, Xu, and Wang (1998), the intra-assay average variation coefficient was 2.7%, and the inter-assay average variation coefficient was 5.2%, which showed that it was feasible to determine fenthion using the CI-ELISA.

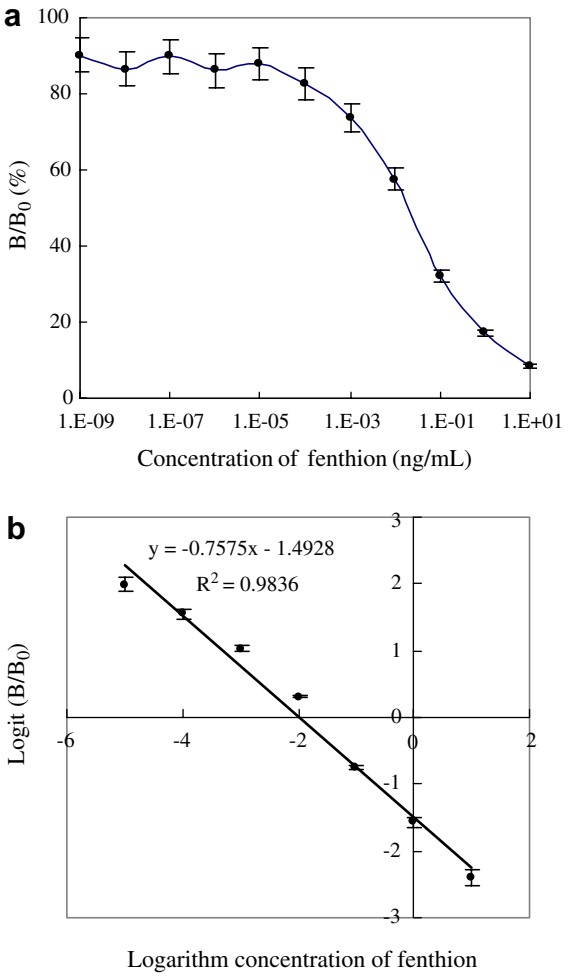


Fig. 3. Competitive indirect ELISA curve for fenthion. "a" is binding curve of the CI-ELISA, and "B/B_0" is binding ratio of antibody/coating antigen in wells; "b" is the detection line conversed from "a", and "Logit (B/B_0)" equals to logistic value of "B/B_0".

Table 3
Recovery test of fenthion in grape, peach and river water

Samples	Spiked (ng/mL)	Theoretical (ng/mL)	Found (ng/mL)	Average recovery ± SD (%)
Grape fruit	0	0	0.000 ± 0.002	–
	2	0.050	0.042 ± 0.003	84.0 ± 6.0
	20	0.500	0.465 ± 0.034	93.0 ± 6.8
	50	1.250	1.024 ± 0.098	81.9 ± 7.8
Peach fruit	0	0	0.001 ± 0.001	–
	2	0.050	0.052 ± 0.003	104.0 ± 6.0
	20	0.500	0.472 ± 0.022	94.4 ± 4.4
	50	1.250	1. 194 ± 0.131	95.5 ± 10.5
Pear fruit	0	0	0.000 ± 0.001	–
	2	0.050	0.046 ± 0.006	92.0 ± 10.2
	20	0.500	0.444 ± 0.029	88.8 ± 7.0
	50	1.250	0.998 ± 0.062	79.8 ± 6.2
Tomato fruit	0	0	0.002 ± 0.002	–
	2	0.050	0.053 ± 0.007	106.0 ± 13.0
	20	0.500	0.472 ± 0.054	94.4 ± 9.6
	50	1.250	1.174 ± 0.128	93.9 ± 10.8

3.4.3. Accuracy (analysis of spiked samples)

The spiked recoveries were used to represent the accuracy of this CI-ELISA. Therefore, fenthion residues in samples of fruits of grape, peach, pear and tomato were detected using the optimized CI-ELISA, after the simple sample former disposal procedure described above. As can be seen from Table 3, the average recoveries of spiked grape fruit were from 81.9% to 93.0%, those of spiked peach fruits were from 94.4% to 104.0%, those of spiked pear fruits were from 79.8% to 92.0%, and those of spiked tomato fruits were from 93.9% to 106.0%. Overall, the CI-ELISA developed in this study can accurately determine fenthion residues in samples of fruits of grape, peach, pear and tomato after the simple and rapid extraction procedure.

4. Conclusions

Here, an effective ELISA for fenthion was developed using a novel combination of immunizing/coating hapten. The "efficiency" is mainly displayed on three aspects. The first one is its high sensitivity, limit detection (I_{20}) of the optimized CI-ELISA method is 0.0002 ng/mL, and its I_{50} is 0.01 ng/ml, which is an assay method for fenthion with the highest sensitivity among those reported at present. The second one is that sample former disposal is of great speediness and simplicity because of the highly enough sensitivity and it does not need to be concentrated after the pesticide in sample dissolves enough in organic solvent.

So the ELISA can be lest the complicated former disposal procedure needed by chromatography (Arrebola et al., 2003). The third one is utility. The recoveries obtained by standard fenthion addition to the different samples such as fruits of grape and peach and river water were all from 79.8% to 106.0%. Therefore the optimized ELISA may become a new convenient and satisfied analytical tool for monitoring fenthion residues in environment and agricultural samples.

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