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Analytical Methods

Development of a McAb-based immunoassay for parathion and influence of the competitor structure

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ABSTRACT

A specific monoclonal antibody (McAb) for parathion was produced. Based on this McAb, a battery of competitors as coating antigens were used to develop homologous and heterologous indirect competitive enzyme-linked immunosorbent assays (ELISAs) for parathion. The relationship between the heterology degree of the competitor and the sensitivity of the corresponding immunoassay was investigated. Results showed that, when the specific McAb was used in the ELISA experiment, competitors should have a certain degree of homology with the immunizing hapten for immunoassays, and the best performance occurred when the competitor hapten was highest heterologous to the target analyte. With the most suitable competitor, a sensitive and selective ELISA was developed. The IC_{50} value of the ELISA was 2.94 ng/ml with a detection limit (IC_{20}) of 0.70 ng/ml. The average recoveries of parathion in spiked water, soil, cucumber and rice were 88.09%, 93.15%, 91.37% and 83.42%, respectively.

1. Introduction

Organophosphate pesticides are considered as the substances of high environmental impact due to their toxicity and persistence (Roex, Keijzers, & Gestel, 2003). They can be found in many compartments of the ecosystem and in the organisms. Parathion (O,O-diethyl O-4-nitrophenyl phosphorothioate), belongs to the organophosphorus pesticides, can deactivate the enzyme acetylcholinesterase, disrupt nerve function, and lead to paralysis and death (Buratti, Volpe, Meneguz, Vittozzi, & Testai, 2003; Fenske, Lu, Barr, & Needham, 2002). Due to its high toxicity and persistence, parathion is fully prohibited to be used in agriculture in many countries over the world. Nowadays, the residue of parathion is still one of the important items in the detection of pesticide residues in the international trade. The conventional methods for detection of parathion involving gas chromatography (GC) (Bai, Zhou, & Wang, 2006; Basheer, Alnedhary, Rao, & Lee, 2007) and high-performance liquid chromatography (HPLC) (Rotich, Zhang, & Li, 2003) are sensitive and reliable. However, these applications are relatively slow and usable only in laboratory scale. For high sample throughput and on-site monitoring of pesticide residues, it is important to develop and explore technologies that more rapid, economical and without need of complicated derivatization steps.

Enzyme-linked immunosorbent assay (ELISA) is proven to be simple, cost-effective, and do not require sophisticated instrumentation (Abad et al., 1999; Nunes, Toscano, & Barceló, 1998). Further more, the immunoassay is demonstrated as a powerful tool involved in high sample throughput and on-site screening in pesticide monitoring programs (Ferguson, Kelsey, Fan, & Bushway, 1993; Gabaldón, Maquieira, & Puchades, 1999). All of these features make the immunoassay a very promising analytical tool. Many ELISA methods have been established for parathion. A radioimmunoassay was developed by Ercegovich et al. (1981). The resulting assay did detect parathion, but the limit of detection (LOD) was very high. Ibrahim, Morsy, and Hewedi (1994) produced a monoclonal antibody (McAb) to parathion and ELISA was developed. But the immunoassay showed significant cross-reactivity with parathion-methyl (50%). Garrett, Appleford, Wyatt, Lee, and Morgan (1997) produced a recombinant anti-parathion antibody (ScFv). LOD for the McAb-based and ScFv-based ELISAs were 16 and 20 ng/ml, respectively. Recently, Zeng et al. (2007) developed a McAb-based ELISA for parathion with the sensitivity (IC_{50}) and the detection limit (IC_{10}) of 360 and 26 ng/ml, respectively.

In our previous work (Liu et al., 2007), we developed a more sensitive polyclonal antibody-based immunoassay for parathion (LOD was 0.31 ng/ml). Because McAb offered a more definite specificity than polyclonal antibodies and an unlimited production, in this work, a specific McAb was produced. Furthermore, as an increase of the detectability by introducing a certain degree of heterology in the chemical structure of the competitor was often reported (Galve, Baeza, Camps, & Marco, 2002; Holthues, Fukumura, Sound, & Baumann, 2005; Kim, Cho, Lee, & Lee, 2003; Kim, Kim, Lee, & Lee, 2007), the influence of the competitor structure on McAb-based immunoassay sensitivity and specificity was

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also investigated. Additionally, theoretical computer models were used to objectively assess the degree of heterology existing between the analyte and the haptens. Finally, a heterologous indirect ELISA (the immunizing hapten and the coating hapten differ in their molecular structures) based on this McAb was developed, and applied to detect parathion residue in water, soil, cucumber and rice samples.

2. Experimental

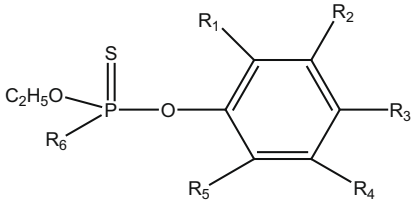
2.1. Immunoreagents, chemicals and instruments

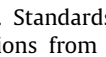
Table 1 showed the structures of parathion and the haptens (hapten **1**–**14**) used in this study. The preparation of the immunizing antigen (hapten **1**-BSA) and the coating antigen (hapten **1**–**14**-OVA) had been described in a previous paper (Liu et al., 2007). Parathion and other pesticide standards were obtained from National Standards Company (China).

Ovalbumin (OVA, MW45000), bovine serum albumin (BSA, MW67000), peroxidase-labelled goat anti-mouse immunoglobulins, culture media RPMI-1640, hypoxanthine–aminopterin–thymidine (HAT) and hypoxanthine–thymidine (HT) medium supplements, pristane, complete and incomplete Freund's adjuvants, dimethyl sulphone (DMSO), polyethylene glycol (PEG) 3350 were purchased from Sigma–Aldrich (Spain). SP2/0 mouse plasmacytoma line was from Shanghai Institute of Cell Biology (China). *O*-phenylenediamine (OPD) and Tween 20 were purchased from Shanghai Chemical Reagents Company (China). Phosphate-buffered saline (PBS, 10 mM, pH 7.4), carbonate-buffered saline (CBS, 50 mM, pH 9.5) and phosphate–citrate buffer (pH 5.6) were self-prepared. All other chemicals and organic solvents were of analytical grade or better.

The ELISA was carried out in 96-well polystyrene microplates (COSTAR, High Binding Plates, US). Plates were washed with a DEM plate washer (Beijing Tuopu Analytical Instruments Co. Ltd., China) and absorbencies were read with a 550 plate reader (Bio-Rad, America).

Table 1
Chemical structures of parathion and its haptens.



Compound	R_1	R_2	R_3	R_4	R_5	R_6
Parathion	H	H	NO_2	H	H	C_2H_5O
Hapten **1**	H	H	NO_2	H	H	$NH(CH_2)_4COOH$
Hapten **2**	H	H	NO_2	H	H	$NH(CH_2)_3COOH$
Hapten **3**	H	H	NO_2	H	H	
Hapten **4**	F	H	NO_2	H	H	$NH(CH_2)_3COOH$
Hapten **5**	NO_2	H	H	H	H	$NH(CH_2)_3COOH$
Hapten **6**	H	NO_2	H	H	H	$NH(CH_2)_3COOH$
Hapten **7**	NO_2	H	F	H	H	$NH(CH_2)_3COOH$
Hapten **8**	NO_2	H	H	F	H	$NH(CH_2)_3COOH$
Hapten **9**	H	H	NH_2	H	H	C_2H_5O
Hapten **10**	H	H	C_2H_5	H	H	$NH(CH_2)_3COOH$
Hapten **11**	H	H	$C(CH_3)_3$	H	H	$NH(CH_2)_3COOH$
Hapten **12**	Cl	H	Cl	H	H	$NH(CH_2)_3COOH$
Hapten **13**	Cl	H	F	H	H	$NH(CH_2)_3COOH$
Hapten **14**	OCH_3	H	H	H	OCH_3	$NH(CH_2)_3COOH$

2.2. McAb production and characterisation

2.2.1. Immunization

BALB/c female mice (8–10 weeks old) were immunized with hapten **1**-BSA conjugate. First dose consisting of 100 μg of the conjugate was intraperitoneally injected as an emulsion of sterilized physiological saline and complete Freund's adjuvant. Booster injections were given 2, 4 and 6 weeks after the initial dose, with the same amount of immunogen emulsified in incomplete Freund's adjuvant. One week after the last injection, mice were tail-bled and titres of antisera were determined by indirect ELISA. After a resting period of at least 3 weeks from the last injection in adjuvant, mice selected to be spleen donors for hybridoma production received a final soluble intraperitoneal injection of 100 μg of conjugate in physiological saline (without adjuvant), 3 days prior to cell fusion.

2.2.2. Cell fusion

SP2/0 murine myeloma cells (screened by 8-Azaguanine) were cultured in RPMI-1640 media supplemented with 2 mM glutamine 4.5 g/l glucose, 15 mM HEPES and 15% foetal bovine serum. Cell fusion procedures were carried out essentially as described by Nowinshi, Lostrom, Tam, Stone, and Burnette (1979). Mouse spleen lymphocytes were fused with myeloma cells at 5:1 ratio using PEG 3350 as the fusing agent. The fused cells were distributed in 96-well culture plates (approximately 4×10^5 cells/100 μl of RPMI-1640/well), and 100 μl of HAT selection medium (RPMI-1640 medium supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine) was added to each well 2 or 3 days after plating. Half of the medium of the wells was replaced by fresh HAT medium on the 6 day post-fusion and by HT medium (HAT medium without aminopterin) on the 10 day post-fusion.

2.2.3. Hybridoma selection and cloning

Twelve to fourteen days after cell fusion, culture supernatants were screened with homologous coating conjugate for the presence of antibodies that recognised parathion. Selected hybridomas were subcloned by limiting dilution. Stable antibody-producing clones were expanded and stored in liquid nitrogen.

2.2.4. McAb characterisation

Selected antibody-producing clones were cultured in 150-ml flasks, and the supernatant was collected. Class and subclass determination was performed using Pierce ImmunoPure Monoclonal Antibody Isotyping Kit (HRP/ABTS). With the supernatant of cell culture medium with proper dilution and the coating antigen hapten **1**-OVA, established competitive indirect ELISA for parathion.

2.3. Competitive indirect ELISA

For competition assays, the antibody and coating antigen concentrations were optimised with checkerboard titration. All incubations were carried out at 37 °C. Standards were prepared in 10% methanol–PBS by serial dilutions from a stock solution in methanol, using borosilicate glass tubes. The ELISA was run as described as a preceding paper (Liu et al., 2007). Briefly, Microtiter plates were coated with the optimised concentrations of antigens in CBS (100 μl/well) by incubation for 2 h. The plates were washed and then blocked by incubation with 2% of degreasant milk in PBS (300 μl/well) for 30 min. Serial dilutions (50 μl/well) of the analyte in methanol–PBS were added, followed by adding 50 μl/well of a previously determined McAb concentration. After incubation for 1 h, 100 μl per well of diluted (1/10000) peroxidase-labelled goat

anti-mouse IgG was added. After incubation for another 1 h, the plates were washed and 100 μl/well of a OPD solution (10 mg of OPD and 30 μl of 30% H_2O_2 diluted with 25 ml of phosphate–citrate buffer, pH 5.4) was added. After incubation for 15 min, the reaction was stopped by adding 50 μl of 2 M H_2SO_4, and absorbance at 490 nm was read and recorded.

2.4. Molecular modelling

Molecular modelling was performed using the Hyper-ChemPro 6.0 software package (Hypercube, Gainesville, FL, US). Conformations of low energies for each compound were evaluated using the molecular mechanic (MM+) method and then refined using the semi-empirical mechanic (PM3) method. Finally, the conformation of the lowest energy was refined, and the electronic distribution was obtained.

2.5. Cross-reactivity determinations

To evaluate the specificity of the McAb, parathion and several related compounds were tested for cross-reactivity (CR). CR was calculated by the following equation: [IC_{50} (parathion)/IC_{50} (compound)] × 100%. Here, IC_{50} value (analyte concentration that reduces 50% maximum absorbance of the competitive ELISA) was determined by performing competitive immunoassay. Competitive curve was obtained by plotting absorbance against the logarithm of analyte concentration. Sigmoid curve was simulated by means of Microsoft Excel 2000.

2.6. Sample analysis

Standards and samples were run in triplicate wells. Determination of spiked samples was performed by interpolating their mean absorbance values in the standard curve run in the same plate.

For water sample, the selected ELISA was used for parathion determination in two water samples, pond water (Huajiachi Pond, Hangzhou, China), river water (Tiesha River, Hangzhou, China). Prior to the ELISA determination, 4.5 ml of water sample (filtered with filter paper) was spiked with 0.5 ml of parathion standard in methanol, and diluted with 10% methanol–PBS.

For soil sample, purple clayed paddy soil (Hangzhou, China), silt-loamy paddy soil (Hangzhou, China), were collected, dried and passed through a 60 mesh sieve. Five grams of soil sample, free of parathion residues tested by gas–liquid chromatography, was spiked with 0.5 ml of parathion standard in acetonitrile. The soil was mixed thoroughly with a stainless steel spatula for 1 min. Acetonitrile (9.5 ml) was added and vortexed for 1 min, and then the fortified sample was centrifuged for 5 min (4000 rpm). One millilitre of the superstratum was transferred and evaporated by gently blowing nitrogen to move off the solvent. The residue was dissolved with 10% methanol–PBS. After vigorously shaken for 5 min by a rotation shaker, it was determined by the developed ELISA.

For cucumber sample, 10.0 g of a finely chopped sample of cucumber was added 1 ml of an acetonitrile fortification solution. After being set aside for 30 min, 9 ml of acetonitrile was added and vigorously shaken for 1 min by hand. 1 g of NaCl and 4 g of $MgSO_4$ were added and vigorously vortexed for 1 min. Then, it was centrifuged for 5 min (4000 rpm) to separate the acetonitrile from water. The following steps were just same as for soil sample.

For rice sample, 5.0 g of comminuted sample, 1.5 ml of water was added and then intermixed. 0.5 ml of parathion standard in methanol was spiked. After being set aside for 30 min, 9.5 ml of acetonitrile was added and vigorously shaken for 1 min by hand. The following steps were just same as for cucumber sample.

3. Results and discussion

3.1. McAb production and characterisation

Three cell fusion experiments were performed. Growing hybridomas, which were observed in 90% of the wells, were screened for parathion antibodies. The culture supernatants were tested by noncompetitive indirect ELISA against hapten **1**-OVA, OVA, and BSA. Amongst the 1744 wells with growing hybridomas, 25 wells with hybridomas produced antibodies that recognised only the conjugate but not BSA or OVA. Hybridomas from these 25 wells were cloned by limiting dilution. Competitive ELISA was performed to evaluate the ability of these 25 antibodies to detect parathion. Twenty monoclonal antibodies presented no competition with parathion in the conditions of the assay; the five others, named 5A11, 3F10, 5H2, 5H7 and 7H7, showed a significant competition in the presence of parathion. Table 2 summarised the characterisation of these five antibodies in terms of class, subclass, IC_{20} and IC_{50} value (analyte concentration that reduces 20% and 50% maximum absorbance of the competitive ELISA), demonstrating that the antibodies are of G_1 and G_{2a} subclass with κ light chain. With the antibody (5H7), we established the calibration curve with lower IC_{20} and IC_{50} value. The hybridomas (5H7) were injected intraperitoneally into mineral oil-primed mice to produce ascites, which was purified and selected for further study.

3.2. Competitive indirect ELISA

Competitive ELISA formats can be divided into homologous and heterologous formats (Schneider & Hammock, 1992). In homologous format the same hapten is used for immunization and assay purposes whereas in heterologous format the immunizing hapten and the competitor hapten (ELISA coating hapten) differ in their molecular structures. Hapten heterology is commonly used to eliminate problems associated with the high affinity of the antibodies to the spacer arm that leads to no or poor inhibition by the target compound (JÜlicher, Mussenbrock, Renneberg, & Cammann, 1995). Heterologous assay, especially heterology in structure of hapten would largely improve the sensitivity of ELISA assay (Kim, Cho, Lee, & Lee, 2003; Lee et al., 2002). Thus, on an initial step we explored the influence of the degree of hapten heterology on ELISA.

Table 1 showed the structures of parathion and the haptens used in this study. When hapten **1** was used as immunizing hapten, other designed haptens were set as competitors. Because the phosphorothioate OP pesticides such as parathion have a thiophosphate group in common and only differ in the structure of their aromatic rings, it is desirable to synthesise haptens that have a bridge at the thiophosphate group in order to preserve the aromatic ring unique to parathion. Haptens **1**–**4** preserved the special nitro group of parathion and the position of nitro group was changed to form haptens **5**–**8**. Additionally, the types of substituent of the special nitro group were changed to form haptens **9**–**13**. Hapten **14**, which was selected as the optimal competitor based on the polyclonal antibody (Liu et al., 2007), was also used as the competitor in this work.

Table 2
Identification and characterisation of anti-parathion McAbs.

Hybridoma	Class and subclass	Type	IC_{20} (ng/ml)	IC_{50} (ng/ml)
5A11	IgG_1	κ	24.16	99.34
3F10	IgG_{2a}	κ	16.87	90.25
5H2	IgG_1	κ	19.52	157.04
5H7	IgG_1	κ	7.04	47.79
7H7	IgG_1	κ	26.85	178.36

Coating antigens (hapten-OVA conjugates) were tested by non-competitive indirect ELISA format. Haptens **1**–**4** preserved the special nitro group of parathion could be recognised by the McAb, but haptens with different position or substituent of the nitro group could not be recognised except hapten **10**. We had observed previously (Liu et al., 2007) conjugates prepared with these 14 haptens could be all recognised by the polyclonal antibody, especially with haptens **9**–**11**, **13** and **14** where the sensitivity (IC_{50}) could be improved from 81.70 ng/ml (homologous) to less than 10 ng/ml (heterologous). Some authors had also discussed the competitor heterology effect of the immunoassay-based on polyclonal antibody (Galve et al., 2002; Kim, Cho, Lee, Lee, Gee, et al., 2003; Liu et al., 2007). In those studies, competitors could be more or less recognised. In this work, the lack of recognition could attribute to a more definite specificity of the monoclonal antibody than the polyclonal antibody.

Homologous and heterologous competitive ELISAs in indirect format were used to characterise reactivities of the McAb to these five hapten conjugates (Table 3). The data of IC_{50} and IC_{20} values were analysed by ANOVA (analysis of variance), followed by Tukey's multiple comparison test ($P < 0.05$) using the SPSS 13.0 software package (SPSS, Chicago, US). Statistically significant differences were noted amongst homologous and heterologous immunoassays. Compared with the homologous result of hapten **1**, the heterologous system showed lower IC_{50} and IC_{20} values for hapten **3**, followed hapten **2**, and then hapten **4**, and hapten **10**. Concerning the reproducibility, average intra-assay CVs of the IC_{50} and IC_{20} values were 7.42% and 12.71%, respectively.

3.3. Molecular modelling

Since antigen–antibody recognition is based on steric criteria and interactions resulting from the electronic properties of the molecules, molecular modelling may be helpful to determine the volumes and charges of the compounds (Bertoncini, Pichon, & Hennion, 2003). Some authors had demonstrated that the immunoassay specificity and detectability could be modulated by the chemical structure of the competitor hapten (Nichkova, Galve, & Marco, 2002), and the best performance occurred when the immunizing hapten and the competitor hapten had the highest heterology (Galve et al., 2002). To obtain the optimal competitor structures, a hypothesis was recommended: the more heterologous of the competitor to the analyte, the better detectability of the immunoassay established by the corresponding competitor and the already available antibody. The similarity or dissimilarity could be identified by the electronic parameters which came from the aromatic ring, the substituent and the common part of the competitors and the analyte.

The resulting electronic parameters were shown in Fig. 1. Regarding the charge distribution of aromatic ring, we could define the following heterology order: hapten **10** > hapten **4** > hapten **1** > hapten **2** > hapten **3**. Since haptens **1**–**3** preserved the nitro group of parathion and the substituent of the aromatic ring was unchanged, the charge distribution of substituent was parallel to parathion, whilst the difference between hapten **4** and **10** was significant (hapten **10** > hapten **4**). Moreover, compared with the charge distribution of common part of parathion, hapten **3** showed the most similarity, whilst other competitors showed significant difference. These results suggested that hapten **10** was the greatest heterology with parathion, and also was the optimal competitor theoretically according to the hypothesis. This theoretical result was consistent with the experimental result.

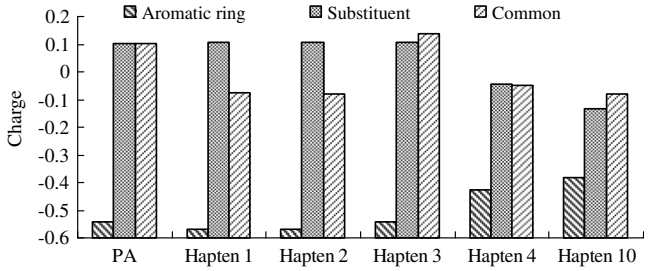


Fig. 1. Charge of aromatic ring, substituent and common part of parathion (PA) and competitors.

3.4. Cross-reactivity studies

Assay specificity was evaluated by using a set of organophosphorus insecticides and metabolites with similar structures to parathion. Several chemically related pesticides were also used due to their widespread agricultural and domestic uses. The detailed cross-reactivity (CR) data for each compound were given in Table 4. No cross-reactivity was observed except for parathion-methyl and fenitrothion, and each IC_{50} value was greater than the highest concentration of compounds used (10,000 ng/ml). So it can be concluded that these immunoassays development for parathion in this study were highly specific.

Moreover, these results gave information about the epitope recognised by the McAb. Since reduced parathion and fenthion showed no cross-reactivity, the presence of the nitro group of the molecule was important in antibody recognition. If the specificity of the McAb was based only on the nitro group of the molecule,

Table 3
The results of homologous and heterologous indirect competitive ELISA.

Competitor	IC_{50} value (ng/ml)[a]	CV[b] (%)	IC_{20} value (ng/ml)[a]	CV[b] (%)
Hapten **1**[c]	(a) 33.36 ± 3.19	7.69	(a) 4.94 ± 0.70	11.41
Hapten **2**	(b) 13.10 ± 0.96	5.88	(b) 3.02 ± 0.38	10.12
Hapten **3**	(b) 15.46 ± 1.94	10.12	(b) 3.30 ± 0.70	17.15
Hapten **4**	(c) 8.00 ± 0.82	8.23	(c) 2.24 ± 0.41	14.90
Hapten **10**	(d) 2.94 ± 0.19	5.21	(d) 0.70 ± 0.09	9.94

[a] Within a column, means denoted by similar letters (a–d) were tukey test results ($p < 0.05$).
[b] CV (coefficient of variation): data obtained from five determinations performed in different ELISA plates.
[c] The result of competitor hapten **1** came from the homologous indirect competitive ELISA.

Table 4
Cross reactivity of parathion-related compounds, their metabolites and other extensively used pesticides.

Compound	CR (%)				
	$Hapten_1$-OVA	$Hapten_2$-OVA	$Hapten_3$-OVA	$Hapten_4$-OVA	$Hapten_{10}$-OVA
Parathion	100	100	100	100	100
Parathion-methyl	3.26	2.83	16.82	1.17	8.69
Paraoxon	<0.33[a]	<0.13[a]	<0.15[a]	<0.08[a]	<0.03[a]
Diazinon	<0.33[a]	<0.13[a]	<0.15[a]	<0.08[a]	<0.03[a]
Reduced parathion	<0.33[a]	<0.13[a]	<0.15[a]	<0.08[a]	<0.03[a]
4-Nitrophenol	<0.33[a]	<0.13[a]	<0.15[a]	<0.08[a]	<0.03[a]
Diethylchlroro-thiophosphate	<0.33[a]	<0.13[a]	<0.15[a]	<0.08[a]	<0.03[a]
Triazophos	<0.33[a]	<0.13[a]	<0.15[a]	<0.08[a]	<0.03[a]
Fenthion	<0.33[a]	<0.13[a]	<0.15[a]	<0.08[a]	<0.03[a]
Fenitrothion	<0.33[a]	<0.13[a]	0.88	<0.08[a]	0.72
Chlorpyrifos	<0.33[a]	<0.13[a]	<0.15[a]	<0.08[a]	<0.03[a]
Chlorpyrifos-methyl	<0.33[a]	<0.13[a]	<0.15[a]	<0.08[a]	<0.03[a]
Atrazine	<0.33[a]	<0.13[a]	<0.15[a]	<0.08[a]	<0.03[a]

[a] 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 10,000 ng/ml.

Table 5
Recoveries of parathion from spiked samples.

Sample	Spiked (ng/ml)	Measured (ng/ml)	Recovery (%)	CV (%)
Pond water	5.00	3.77 ± 0.34	75.37	7.27
	10.00	8.76 ± 1.78	87.61	16.35
	20.00	18.47 ± 2.26	92.37	9.86
River water	5.00	5.15 ± 0.65	102.95	10.22
	10.00	8.26 ± 0.92	82.59	8.98
	20.00	17.52 ± 1.06	87.62	4.87
Purple clayey soil	10.00	7.82 ± 0.79	78.19	8.14
	20.00	17.16 ± 2.01	85.81	9.41
	50.00	47.55 ± 8.15	95.09	13.81
Silt-loamy paddy soil	10.00	10.78 ± 1.32	107.76	9.86
	20.00	17.66 ± 3.40	88.30	15.50
	50.00	51.87 ± 3.53	103.73	5.49
Cucumber	10.00	7.42 ± 1.37	74.23	14.87
	20.00	20.38 ± 3.27	101.91	12.90
	50.00	48.98 ± 6.86	97.96	11.27
Rice	20.00	15.53 ± 2.31	77.64	17.01
	50.00	44.62 ± 4.02	89.21	10.28

4-nitrophenol and paraoxon would present cross-reactivity. Whilst in this study they showed no cross-reactivity, which indicated that the presence of the phosphorothionate part of the molecule was also important in antibody recognition.

3.5. Sample analysis

According to the results obtained (see above), the optimal competitor was hapten **10**, which gave the lowest IC_{50} values for parathion and showed negligible CR of parathion-related compounds, when the monoclonal antibody was produced by hapten **1**. Therefore, the combination of the McAb with coating antigen hapten **10**-OVA was selected for sample analysis.

The spiked samples were applied to indirect competitive ELISA without any sample clean-up procedure, as described in Section 2.6. Control samples without parathion were also systematically included in the analysis, the degree of sample matrix interference followed this order: rice > cucumber > soil > water. Some authors have suggested that dilution of sample was an effective means to circumvent matrix interference, especially at the lower concentrations that are to the left of the IC_{50} value (Kim et al., 2007; Zhu et al., 2008). However, dilution of samples causes reduction of assay sensitivity due to the shifting of the dynamic range. Therefore, concerning the sensitivity and the dynamic range of the immunoassay, water, soil, cucumber and rice samples were diluted to 1:3 (v/v), 1:10 (v/v), 1:10 (v/v) and 1:20 (v/v), respectively.

Results of the spiked recovery experiments were showed in Table 5. The mean recoveries over three spiked levels were 85.12%, 91.05%, 86.36%, 99.93% and 91.37% for pond water, river water, purple clayey soil, silt-loamy paddy soil and extract samples of cucumber. These data proved that acceptable recoveries were obtained for this immunoassay. Additionally, rice samples were also analysed by the ELISA after being fortified with parathion at 20.00 and 50.00 ng/ml. Since the maximum residue limits (MRLs) of parathion in rice and vegetables set by Ministry of agriculture of People's Republic of China is 0.01 mg/kg. Improvement of the immunoassay was necessity for determination of parathion in rice samples.

4. Conclusions

A specific monoclonal antibody (5H7) for parathion was produced. The influence of the competitor structure on this McAb-based immunoassay was investigated. According to the results obtained, when the specific monoclonal antibody was used in the ELISA experiment, competitors should have a certain degree of homology with the immunizing hapten for immunoassays. On the other hand, the best performance occurred when the competitor hapten had the highest heterology with the analyte. With the most suitable competitor and the McAb, an indirect competitive ELISA was developed. Results of the spiked recovery experiments showed that this assay was suitable for the detection of parathion in environmental and agricultural samples.

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