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Analytical Methods

Monitoring of organophosphorus pesticides in vegetables using monoclonal antibody-based direct competitive ELISA followed by HPLC–MS/MS

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ABSTRACT

A multi-analytes method for monitoring of organophosphorus pesticides (OPs) using a combination of broad-specificity direct competitive enzyme-linked immunosorbent assay (dcELISA) and high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) was developed. The reaction formats of dcELISA as well as the matrix effects of vegetable samples by different treatments were studied. The dcELSIA based on horseradish peroxidase-labelled monoclonal antibody and solid-phase extraction can analyse 42 samples in duplicate simultaneously for 12 OPs with a limit of detection at 20 μg L^{-1} within 40 min, with good accuracy and reproducibility. For screening purpose, the dcELISA can distinguish positive samples from hundreds of negative samples at a rapid, high-throughput and low cost manner. The positive samples can be following confirmed by HPLC–MS/MS for the kinds and the relative amounts of OPs. The method is suitable for monitoring of OP contamination in vegetables samples with high-efficiency and low cost.

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1. Introduction

Pesticide residues present in food have potential detrimental effects on human health. Many governmental and industrial programs have been implemented for the regulatory analysis of pesticide residues in food (Adou, Bontoyan, & Sweeney, 2001). Multi-analytes methods, such as GC–MS or LC–MS, are usually applied in pesticide monitoring because several compounds can be targeted simultaneously, which reduces labour and material costs (Adou et al., 2001; Obana, Kikuchi, Okihashi, & Hori, 1997; Sharmaa, Nagpala, Pakadeb, & Katnoria, 2010). Although instrumental methods offer high sensitivity and specificity, the associated high-costs and time-consuming labour requirements have inhibited the application of these methods. Therefore, the need for rapid, simple and high-throughput analysis of pesticides in a low cost manner is rapidly growing.

Immunoassays, which are simple, robust, and low-cost techniques, are widely used for simple and high-throughput analysis in a broad range of applications (González-Techera et al., 2007). They have been developed as an alternative to the labourious and expensive instrumental methods used to quantify pesticides (Morozova, Levashova, & Eremin, 2005; Suri et al., 2009). Immunoassays that can detect a group of closely related analytes are favored because they can identify more than one target, and detect positive samples from hundreds of negative samples in one simple test (Spinks, 2000). Several works on the development of broad-specificity enzyme-linked immunosorbent assay (ELISA) for triazine herbicides (Bruun et al., 2001; Winklmair, Weller, Mangler, Schlosshauer, & Niessner, 1997), organophosphorus pesticides (Alcocer et al., 2000; Johnson, Van Emon, Pullman, & Keeper, 1998; Piao et al., 2009; Xu et al., 2010), and pyrethroid insecticides (Mak et al., 2005; Watanabe, Shan, Stoutamire, Gee, & Hammock, 2001) have been reported. However, there are several problems associated with the application of broad-specificity ELISA for pesticides in food samples. Firstly, the commonly used indirect competitive ELISA (icELISA) always involves two incubation and washing steps as well as the use of a labelled secondary antibody. Secondly, the matrix effects of food samples were always conquered by employing a large dilution of samples (Mak et al., 2005; Piao et al., 2009), which resulted in significant reduction of assay sensitivity. Thirdly, the quantification of single pesticide within the same sample is not possible with a broad-specificity ELISA because the affinity of the antibodies varies between different compounds and the relative amounts of the compounds are unknown in real samples (Degelmann, Wenger, Niessner, & Knopp, 2004).

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In the present study, one step direct competitive ELISA (dcELISA) for multi-analytes determination of organophosphorus pesticides (OPs) was developed based on a monoclonal antibody (MAb) with broad-specificity for OPs. The MAb, coating hapten and coating antigen were labelled with horseradish peroxidase (HRP) as tracers to develop different format of dcELISA. The matrix effects of vegetable samples on ELISA performance were evaluated using different treatments, including liquid–liquid extraction (LLE) and solid-phase extraction (SPE). To make the broad-specificity dcELISA available in the determination of real-world samples, the corresponding high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) for multi-analytes analysis of selective OPs was developed and used to further confirm the positive samples which were screened out by the dcELISA.

2. Materials and methods

2.1. Materials and instruments

The analytical OP standards were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The MAb with broad-specificity for OPs, the coating hapten (4-((diethoxyphosphorothioyl)amino)butanoic acid), and the coating antigen (hapten–ovalbumin (OVA)) were self-prepared, as described previously (Xu et al., 2010). Horseradish peroxidase (HRP), *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), and 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from Sigma (St. Louis, USA). Sodium metaperiodate ($NaIO_4$) and sodium borohydride ($NaBH_4$) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shenyang, China). A dual-layer (primary secondary amine (PSA) in combination with graphitized carbon black (GCB)) SPE column (500 mg/500 mg/6 mL) was purchased from Shanghai ANPEL Scientific Instrument Co., Ltd. All other chemicals and organic solvents were of analytical grade obtained from a local chemical supplier (Yunhui Trade Co., Ltd., Guangzhou, China).

The ELISA was carried out in 96-well polystyrene microplates (Xiamen Yunpeng Technology Development Co., Ltd., Xiamen, China). ciELISA plates were washed using a Multiskan MK2 microplate washer (Thermo Labsystems, USA). Absorbance was measured at a wavelength of 450 nm using a Multiskan MK3 microplate reader (Thermo Labsystems, USA).

2.2. Preparation and characterization of HRP-labelled tracers

The MAb–HRP tracer and antigen–HRP tracer were prepared using modified $NaIO_4$ method described by Tsang, Greene, and Pilcher (1995). Briefly, 5 mg of HRP (M_w 40,000) or 5 mg of antigen (M_w 45,000) was dissolved in 500 μL of CH_3COONa–CH_3COOH buffer (0.2 mol L^{-1}, pH 5.6) and 100 μL of $NaIO_4$ (0.1 mol L^{-1}) was then added. The mixture solution was stirred for 30 min at 4 °C and 500 μL of glycol (2.5%, w/v) was added dropwise. After reaction for 30 min at room temperature, 5 mg of MAb (M_w 162,000) dissolved in phosphate-buffered saline (PBS, 0.01 mol L^{-1}, pH 7.4) was added dropwise and stirred for 30 min at 4 °C. The mixture solution was dialysed against $NaHCO_3$–Na_2CO_3 buffer (0.05 mol L^{-1}, pH 9.6) overnight before 100 μL of $NaBH_4$ (5 g L^{-1}) was added and stirred for 2 h at 4 °C. The mixture was dialysed against PBS (0.01 mol L^{-1}, pH 7.4) overnight. Ammonium sulphate was added to reach a saturation concentration of 45% and kept stirring for 30 min at 4 °C. The mixture was centrifuged at 5000*g* for 10 min at 4 °C and then the precipitate was collected and dissolved in PBS to obtain MAb–HRP or antigen–HRP.

The hapten–HRP was synthesized by the activated ester method (Kolosova, Shim, Yang, Eremin, & Chung, 2006). Briefly, 11.5 mg (100 μmol) of NHS, 20.5 mg (100 μmol) of DCC and 6.38 mg (25 μmol) of hapten were dissolved in 1 mL of absolute DMF, then the reaction mixture was incubated (keep stirring) overnight at room temperature and finally centrifuged at 3075*g* for 5 min. Two milligrams (0.05 μmol) of HRP was dissolved in 1 mL of carbonate buffer (0.05 mol L^{-1}, pH 9.6) and cooled at 4 °C for 1 h. The supernatant of the active ester (20 μL) was slowly added dropwise to the HRP solution. The mixture was kept for 2 h at room temperature and overnight at 4 °C. Subsequently, the mixture was dialysed against PBS (0.01 mol L^{-1}, pH 7.4) for 3 days to obtain the hapten–HRP.

The obtained tracers were characterized using a chequerboard titration. MAb was coated for characterization of hapten–HRP and antigen–HRP, while coating antigen (hapten–OVA) was coated for characterization of MAb–HRP.

2.3. Development of dcELISA

Three dcELISA formats (A, B and C) were developed and compared for their sensitivity for OPs in this study (Fig. 1).

2.3.1. Format A (MAb–HRP)

The 96-well microtiter plate was coated with the coating antigen (50 μg L^{-1}, 100 μL $well^{-1}$) in carbonate buffer (0.05 mol L^{-1}, pH 9.6) overnight at 4 °C. The wells were washed five times with PBST (0.01 mol L^{-1} PBS containing 0.05% Tween® 20, pH 7.4), and blocked with 5% skim milk in PBS (200 μL $well^{-1}$) for 2 h. After washing two times with PBST, the plates were dried at 37 °C overnight. OP standards or samples in PBS (0.02 mol L^{-1}, pH 6.2, 50 μL $well^{-1}$) were added to the wells followed by addition of the PBS (0.02 mol L^{-1}, pH 6.2) diluted (1/24,000) MAb–HRP (50 μL $well^{-1}$). After incubation for 40 min at 37 °C, the plates were washed five times with PBST, and the TMB solution (100 μL $well^{-1}$) was added to the wells and incubated for 10 min at 37 °C. The reaction was stopped by addition of 2 mol L^{-1} H_2SO_4 (50 μL $well^{-1}$), and the absorbance was recorded at 450 nm. Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentration. The sigmoid curves were generated by using Originpro 7.5 software (OriginLab Corp., Northampton, MA, USA).

2.3.2. Format B (hapten–HRP)

The plate was coated with MAb (1/1000, 100 μL $well^{-1}$) in carbonate buffer (0.05 mol L^{-1}, pH 9.6) overnight at 4 °C. The wells were washed five times with PBST and blocked with 5% skim milk for 2 h. After washing two times with PBST, the plates were dried at 37 °C overnight. OP standards or samples (50 μL $well^{-1}$) were added to the wells followed by addition of the diluted (1/2400) hapten–HRP (50 μL $well^{-1}$). Other steps were the same as those of format A.

2.3.3. Format C (antigen–HRP)

Performance of format C was similar to that of format B, except the coating dilution of MAb of 1/2000 and the dilution of antigen–HRP of 1/4800.

2.4. Cross-reactivity study

The specificity of the dcELISA assays was determined using 18 *O,O*-diethyl OPs and 13 *O,O*-dimethyl OPs under the optimised dcELISA conditions. The cross-reactivity (CR) values were calculated according to the following equation: [IC_{50} (hapten, μmol L^{-1})/IC_{50} (cross-reactant, μmol L^{-1})] × 100%.

2.5. Matrix effects

Three vegetable samples including leek, cowpea and Chinese cabbage were purchased from a local market. The samples were confirmed to be OP-free by high-performance liquid chromatog-

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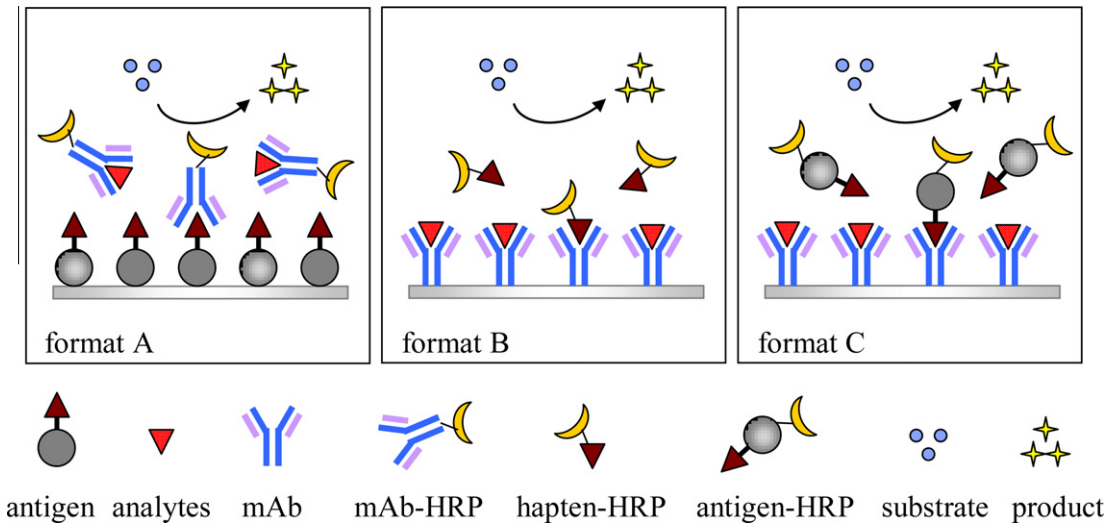


Fig. 1. A schematic representation of three formats of dcELISA in this study.

raphy–tandem mass spectrometry (HPLC–MS/MS) analysis. For matrix effect investigation, three sample treatments (A, B and C) were performed.

2.5.1. Treatment A (methanol extraction)

To 5.0 g of a finely chopped vegetable sample was added 5 mL of methanol, and the mixture was oscillated for 10 min with a vortex mixer. Then, 0.5 g of sodium chloride and 2.0 g of anhydrous magnesium sulphate were added, followed by 5 min of oscillation. After centrifugation (3075*g*, 5 min), the supernatant was diluted with PBS (0.02 mol L^{-1}, pH 6.4) for 10, 20, and 50 times, with a final methanol concentration of 10%. The parathion standard was prepared with diluted samples extraction solutions to obtain a dose dependent standard curve, which was then compared to a standard curve of the parathion standard prepared in PBS (0.02 mol L^{-1}, pH 6.2) containing 10% of methanol.

2.5.2. Treatment B (acetone–dichloromethane extraction)

To 5.0 g of a finely chopped vegetable sample was added 5 mL of water and 10 mL of acetone, and then the mixture was oscillated for 10 min with a vortex mixer. After centrifugation (3075*g*, 5 min), 10 mL of the supernatant was transferred and then evaporated to about 2 mL at 40 °C under vacuum. Then 5 mL of sodium chloride (20%, w/v) was added and mixed. Finally, the mixture was extracted with 10 mL of dichloromethane for two times. The dichloromethane layer was collected and dehydrated by anhydrous magnesium sulphate, and then evaporated at 40 °C under vacuum. The residue was dissolved into 3.3 mL of methanol and then diluted with PBS (0.02 mol L^{-1}, pH 6.4) for 10, 20, and 50 times, with a final methanol concentration of 10%. Other steps were the same as those of treatment A.

2.5.3. Treatment C (SPE extraction)

To 5.0 g of a finely chopped vegetable sample was added 10 mL of acetonitrile, and the mixture was oscillated for 10 min with a vortex mixer. Then, 1.5 g of sodium chloride was added, followed by 2 min of oscillation. The mixture was centrifuged at 3075*g* for 5 min. The acetonitrile layer was collected and then dehydrated by anhydrous magnesium sulphate. The acetonitrile (5 mL) was evaporated to about 1 mL under nitrogen stream at 40 °C. The residue was passed through the GCB/PSA SPE column (preconditioned with 5 mL of acetonitrile:methylbenzene (3:1, v/v)). The column was washed with 15 mL of acetonitrile:methylbenzene (3:1, v/v) and the eluant was evaporated under vacuum at 40 °C. The residue was dissolved with 0.5 mL of methanol and diluted with 4.5 mL of PBS (0.02 mol/L, pH 6.4). Other steps were the same as those of treatment A.

2.6. Analysis of spiked samples by dcELISA

Five grams of finely chopped vegetable sample was spiked with three concentrations of six selected OPs (parathion, phoxim, triazophos, phosalone, parathion-methyl and phorate), covering the assay linear range, respectively. After being set aside for 2 h, the samples were treated by SPE extraction mentioned above. Sample extraction (50 μL) was analysed by dcELISA. Each analysis was done in triplicate.

2.7. HPLC–MS/MS multi-analytes analysis

HPLC–MS/MS for multi-analytes analysis of 11 selective OPs (coumaphos, parathion, phoxim, quinalphos triazophos, dichlofenthion, azinphos-ethyl, phosalone, isochlorthion, parathion-methyl and phorate) was developed. The 1200 series HPLC system (Agilent Technologies, USA) was used for the separation of these pesticides on a Hypersil BDS C8 column (100 mm × 2.1 mm i.d. and 2.4 μm particle size; Thermo Scientific, USA). Mobile phase A consisted of 0.2% acetic acid and 10 mmol L^{-1} ammonium acetate in water, mobile phase B consisted of 0.2% acetic acid in acetonitrile, and they were used in the following gradient profile: 0 min, 55% A and 45% B; 8 min, 10% A and 90% B; then 8.1–14 min 55% A and 45% B. For analysis of individual parathion, the mobile phase was containing 65% A and 35% B. The flow rate of the mobile phase was 0.2 mL min^{-1} and an aliquot of 10 μL of each sample was injected into the HPLC system. The mass spectra were obtained by an Agilent 6410 Triple Quad mass spectrometer (Agilent Technologies, USA). The electrospray ionization (ESI) technique was used. All pesticides were analysed in the positive ionization (PI) mode.

For HPLC–MS/MS analysis, samples were treated by the normalized procedures of China National Standards (GB/T20769–2008). Briefly, 20.0 g of each spiked sample was homogenized with 40 mL of acetonitrile for 1 min, and then 5.0 g of sodium chloride was added and homogenized for another 1 min. The mixture was centrifuged at 3075*g* for 5 min and 20 mL of the acetonitrile phase was transferred and evaporated to about 1 mL at 40 °C under vacuum. The residue was then transferred onto a Sep-Pak Vac column

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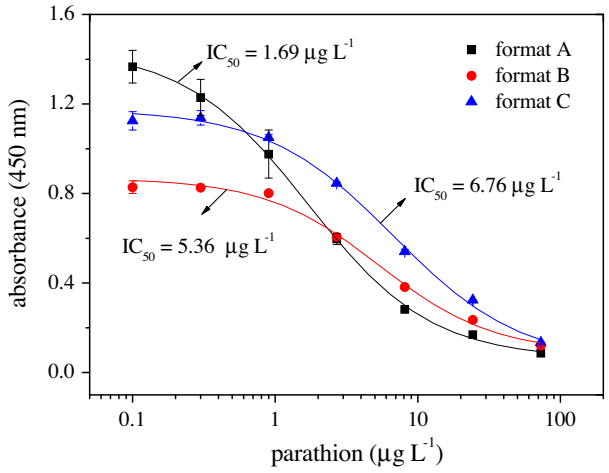


Fig. 2. The comparative standard curves of three dcELISA formats for parathion.

(added with 2 cm of anhydrous sodium sulphate and preconditioned with 5 mL of acetonitrile:methylbenzene (3:1, v/v), Waters, Germany). The column was washed with 25 mL of acetonitrile:methylbenzene (3:1, v/v) and the eluant was evaporated under vacuum. 1 mL mixture of acetonitrile:water (3:2, v/v) was used to dissolve the residue and the samples were used for HPLC–MS/MS analysis.

3. Results and discussion

3.1. Comparison of dcELISA formats

Usually there are three formats of dcELISA, including antigen-coated format, antibody-coated format and the second antibody-coated format (Zhu et al., 2008), can be developed for small molecules. The second antibody-coated format involves the used of second antibody and the addition of three reagents (sample, antibody and hapten–HRP tracer) (Zhu et al., 2008), therefore, it is not considered in this study. The antigen-coated format (format A) was first considered, where the analytes competed for limited MAb–HRP with solid phase antigen (Fig. 1). For antibody-coated format, both hapten and antigen were labelled with HRP as tracers, where the analytes competed for limited hapten–HRP (format B) or antigen–HRP (format C) with solid phase antibody (Fig. 1).

Prior to the development of the standard curves for OPs, the dcELISA conditions such as coating concentration, tracer dilution, incubation time, ionic strength, pH, Tween® 20 and organic solvents were carefully optimised, as described in the development of icELISA (Xu et al., in press). The results indicated that an incubation of 40 min was enough for obtaining equilibrium of antibody–antigen interaction, because both maximum absorbance (A_{450nm}) and sensitivity (IC_{50}) were not improved with the increasing of incubation time. The highest sensitivity was obtained when the ionic strength and pH of reaction buffer was 20 mmol L^{-1} and 6.2,

Table 1
OPs that can be detected at a concentration below 20 μg L^{-1}.

Analytes	IC_{50}	%CR[a]	LOD[b]	Linear range[c]
1. Hapten	45.28	100.0	4.05	13.71–335.65
2. Coumaphos	0.26	21764.6	0.03	0.07–1.17
3. Parathion	1.69	2688.4	0.19	0.44–8.48
4. Phoxim	10.87	428.1	0.86	2.33–44.56
5. Quinalphos	20.09	231.6	1.95	4.64–103.10
6. Triazophos	20.52	238.2	2.13	5.13–140.52
7. Azinphos-ethyl	33.32	161.7	3.80	9.39–170.47
8. Dichlofenthion	47.06	104.5	4.22	9.80–103.40
9. Phosalone	76.47	75.0	4.91	13.15–459.37
10. Isochlorthion	94.34	49.2	7.80	20.95–1276.68
11. Parathion-methyl	169.25	24.2	18.88	43.78–1379.68
12. Phorate	226.97	17.9	19.14	28.97–1112.01

[a] CR is the cross-reactivity.
[b] LOD is the limit of detection (IC_{10}).
[c] Linear range is the lower (IC_{20}) and upper (IC_{80}) limit of quantification.

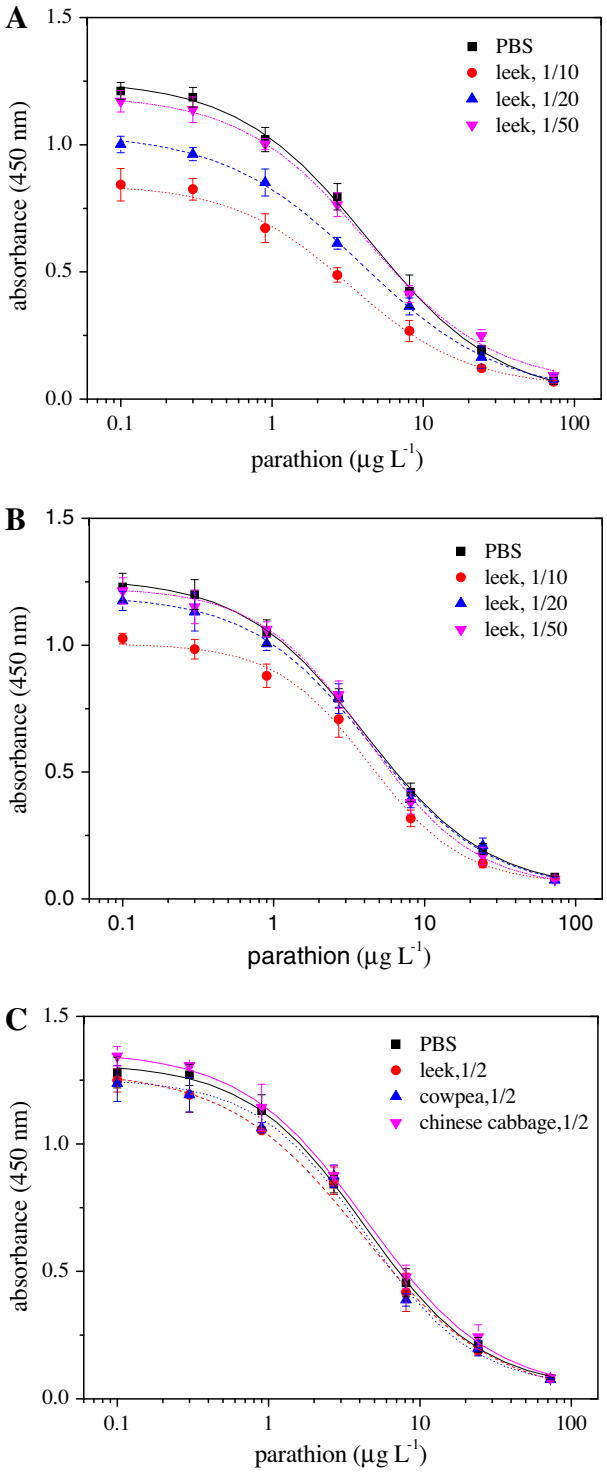


Fig. 3. Matrix effects on dcELISA caused by vegetable samples after different treatments.(A) methanol extraction; (B) acetone–dichloromethane extraction; (C) SPE extraction. Note: leek, 1/10 in (A), (B) and (C) means that the leek sample was diluted 10 times with PBS.

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respectively. The present of Tween® 20 in reaction buffer was proved to be not good for improving the assay sensitivity. Therefore, 20 mmol L^{-1} of PBS (pH 6.2) containing no Tween® 20 was the optimum buffer for both standard and antibody diluent. Methanol caused the least negative effects on ELISA performance and the tolerance of methanol concentration was 10%. The optimum coating concentration and tracer dilution for different dcELISA formats are presented in Section 2.

The typical standard curves of the three dcELISA formats for parathion are given in Fig. 2. It is clear that format A had the best performance in sensitivity for parathion (IC_{50} of 1.69 μg L^{-1}) than format B (IC_{50} of 5.36 μg L^{-1}) and format C (IC_{50} of 6.76 μg L^{-1}). Theoretically, format B might be the optimum dcELISA format for obtaining higher sensitivity because the unconjugated hapten can be separated from the tracer (hapten–HRP) by dialysis. For format A and C, the unconjugated antibody or antigen cannot be separated from the tracers (MAb–HRP or antigen–HRP), which might influence the sensitivity of developed dcELISA. However, in this study, the titer of hapten–HPR was considerably lower than that of MAb–HRP, even the ratio of hapten and HRP was increased to 50:1 or 100:1 during the preparation of hapten–HRP. Due to the lower titer of hapten–HRP, the corresponding higher concentration of coating antibody was need, which should be the main reason for lower sensitivity of format B. Since format A exhibited the highest sensitivity, it was chosen to develop the dcELISA for OPs in this study.

3.2. Assay sensitivity and specificity

Based on the optimised format A, the typical standard curves for 18 *O,O*-diethyl OPs and 13 *O,O*-dimethyl OPs were developed. The dcELISA showed cross-reactivities (CRs) higher than 100% for seven OPs (coumaphos, parathion, phoxim, quinalphos, triazophos, azinphos-ethyl and dichlofenthion) and higher than 20% for three OPs (phosalone, isochlorthion and parathion-methyl), as shown in Table 1. The CRs for the other OPs were lower than 20% (data not shown). Eleven OPs can be determined at concentrations ranging from 0.03 to 20 μg L^{-1}. The broad-specificity and sensitivity of the described dcELISA for OPs were better than those previous reports (Alcocer et al., 2000; Johnson et al., 1998; Piao et al., 2009; Xu et al., 2010).

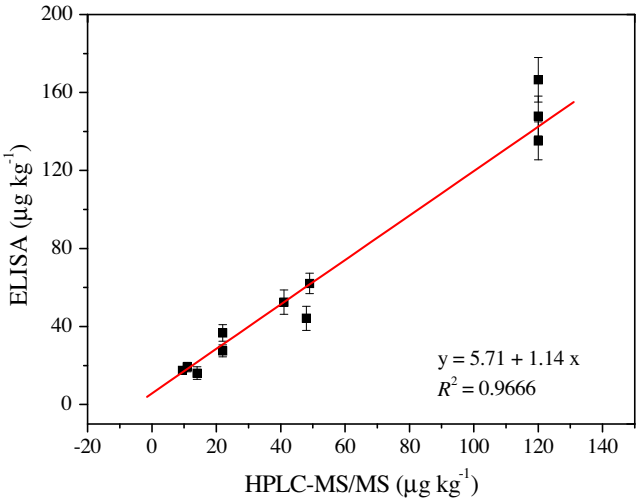


Fig. 4. Comparison of dcELISA and HPLC–MS/MS for parathion determination in leek samples. Note: ELISA was performed using SPE treatment.

In China, the maximum residue levels (MRLs) for the selective 11 OPs in foodstuff (such as rice, vegetable and fruits) are set as follows according to the Chinese National Standard (GB 2763-2005): 10–100 μg kg^{-1} for parathion and parathion-methyl, 100–1000 μg kg^{-1} for phosalone, 200–500 μg kg^{-1} for quinalphos, 50–100 μg kg^{-1} for triazophos, 50 μg kg^{-1} for phoxim, 20–100 μg kg^{-1} for phorate. No values are available for other OPs. The limit of detections (LODs) of current used screening method (cholinesterase-based spectrophotometric methods, GB/T 5009.199-2003) for OPs in China are in the level of mg kg^{-1} (e.g. 1.0 mg kg^{-1} for parathion and 0.3 mg kg^{-1} for phoxim). Since the LODs of the developed dcELISA for all the 11 OPs are below 20 μg kg^{-1}, the proposed method is suitable for the screening of positive samples from a large number of negative vegetable samples.

3.3. Study of matrix effect

The matrix effect is one of the main factors that restrict the application of immunoassays in pesticides analysis (Nunes,

Table 2
Recovery of three selected OPs from three spiked vegetable samples ($n = 3$).[a]

Analytes	Added	Leek			Cowpea			Chinese cabbage		
		Found	%*R*	%CV	Found	%*R*	%CV	Found	%*R*	%CV
Parathion	2	2.8	141.5	19.1	1.9	94.5	21.1	1.5	77.0	22.4
	4	4.6	114.5	8.9	5.1	128.0	11.1	4.2	104.5	9.7
	10	11.5	115.1	12.3	8.1	81.4	14.2	8.8	87.9	16.3
Phoxim	10	10.6	105.9	10.6	9.4	94.0	9.1	11.1	111.4	10.2
	20	23.1	115.7	15.4	22.1	110.7	10.9	18.0	89.9	9.9
	50	61.7	123.4	18.5	51.5	102.9	13.8	45.3	90.5	15.8
Triazophos	20	26.7	133.3	14.7	21.3	106.5	11.2	14.0	70.0	15.9
	40	40.6	101.5	11.5	39.3	98.3	18.5	44.7	111.8	10.2
	100	89.3	89.3	10.1	101.3	101.3	9.7	82.7	82.7	14.3
Phosalone	100	104.5	104.5	16.5	95.6	95.6	12.1	101.7	101.7	6.7
	200	249.5	124.8	11.2	217.5	108.8	15.3	162.3	81.2	12.7
	500	612.4	122.5	14.6	493.1	98.6	9.7	490.5	98.1	17.4
Parathion-methyl	100	95.6	95.6	12.1	112.0	112.0	18.9	75.9	75.9	15.9
	200	245.7	122.8	9.5	257.4	128.7	22.1	221.3	110.7	20.2
	500	435.9	87.2	18.9	468.3	93.7	14.3	571.0	114.2	13.6
Phorate	100	124.3	124.3	14.3	85.6	85.6	10.9	109.5	109.5	9.7
	200	209.5	104.8	17.1	204.7	102.4	6.7	191.6	95.8	10.9
	500	586.7	117.3	10.5	587.1	117.4	14.5	478.6	95.7	18.6

R is the recovery (%). CV (coefficient of variation), and the data was obtained from three determinations performed on the same ELISA plate.
[a] Analyte concentrations are in units of μg kg^{-1}.

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Toscano, & Barceló, 1998). In the development of immunoassays for pesticides in food samples, direct extraction of pesticides from food samples with organic solvents such as methanol are commonly used (Esteve-Turrillas, Abad-Fuentes, & Mercader, 2011; Lee, Ahn, Park, Kang, & Hammock, 2001; Piao et al., 2009; Zhang et al., 2008). However, they caused a strong matrix effect on immunoassays performance. In practice, a dilution of 20–100 times of methanol extraction should be done to reduce matrix effect prior to immunoassays. But the dilution can cause a reduction of assay sensitivity, and overlarge dilution would cause inapplicable of the immunoassays.

In the present study, three sample treatments were used to compare the matrix effects on dcELISA performance. The direct extraction with methanol (treatment A) gave a similar result (Fig. 3A) as the previous reports (Esteve-Turrillas et al., 2011; Lee et al., 2001; Piao et al., 2009; Zhang et al., 2008). A dilution of 50 times was needed to eliminate the matrix effect (Fig. 3A). Due to the large dilution, this treatment was not considered in this study. Liquid–liquid extraction (LLE) was further used for sample treatment using acetone and dichloromethane as extracted solvents (treatment B). A dilution of 20 times was needed to eliminate matrix effect (Fig. 3B). The reduce effect of matrix by treatment B (compared with treatment A) might be due to the removal of unwanted matrices in different solvents (water, acetone and dichloromethane). However, this treatment was only applicable only when the detected concentration range remains on the inside of the dynamic range of the calibration curve. To further reduce the matrix effect, solid-phase extraction (SPE, treatment C) was then applied. A dual-layer SPE, a primary secondary amine (PSA) in combination with graphitized carbon black (GCB), was used. The matrix effect of samples treated by SPE is presented in Fig. 3C. When all the three samples were diluted to 1/2 (v/v) with PBS, the interference of matrix to the standard curve became insignificant. This suggested the SPE treatment was useful for removing the interference of matrix of vegetable samples. It is reported that GCB is very useful for removal of colouring substances (i.e. pigments) and sterols, while PSA has been found as the most effective sorbent for removal of various matrices and significantly reducing matrix-enhancement effect (Shimelis, Yang, Stenerson, Kaneko, & Ye, 2007). Therefore, SPE treatment was applied for the following studies.

3.4. Analysis of spiked vegetable samples

To study the reproducibility of the developed dcELISA, the recoveries of six representative OPs (parathion, phoxim, triazophos, phosalone, parathion-methyl and phorate) from three vegetable samples (leek, cowpea and Chinese cabbage) were studied. The results are presented in Table 2. The recovery ranged from 70.0% to 133.3%, and the coefficients of variation (CV) ranged from 6.7% to 22.4%. The mean recovery was 113.6%, 103.4% and 94.9% for leek, cowpea and Chinese cabbage, respectively. The vegetable matrix caused an inhibition on antibody–antigen reaction (Fig. 3). The relative higher recovery for leek sample should

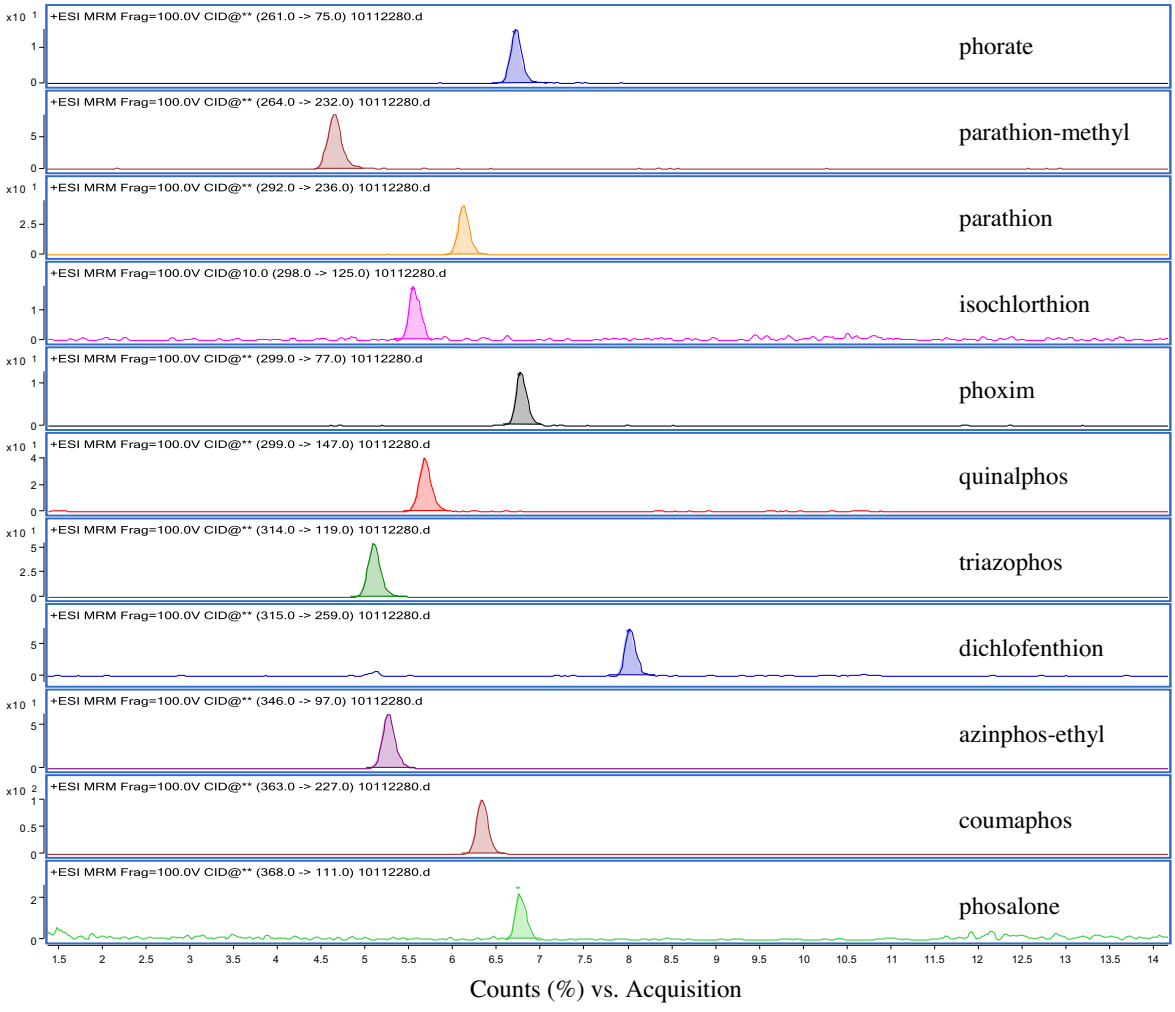


Fig. 5. Multiple reaction monitoring chromatogram of a vegetable sample spiked with pesticides at the 10 μg kg^{-1} level.

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be due to its stronger matrix than cowpea and Chinese cabbage. The samples free from OPs (blank samples) were also determined and no OPs were detected in all the cases. Since the mean recoveries ranging between 90% and 120%, and the mean CV (13.7%) was below 20%, it indicated that the dcELISA developed can be used for detecting of OPs with good reproducibility (Zhu et al., 2008).

3.5. Comparison of dcELISA and HPLC–MS/MS analysis

To study the accuracy of the developed dcELISA, 24 leek samples were separated into four groups and then spiked with parathion at 12.5, 25, 50 and 150 μg L^{-1}, respectively. Half of the samples were determined by dcELISA (n = 5), while the remainder half were determined by HPLC–MS/MS (a lost of samples spiked at 25 μg L^{-1} by accident). The relationship between the data from dcELISA and HPLC–MS/MS is displayed in Fig. 4. Good correlation (R^2 = 0.9666, n = 11) between the results of dcELISA and HPLC–MS/MS was obtained. Overall, the dcELISA gave higher recoveries (mean recovery of 117.8%) for parathion from leek samples than that of the HPLC–MS/MS (mean recovery of 88.0%). The higher mean recovery of ELISA should be due to its susceptibility to the matrix effect, while the lower mean recovery of HPLC–MS/MS should be due to the lost of analyte during the sample pretreatment.

3.6. Screening of real-world samples

Immunoassays with broad-specificity are unable to quantify single pesticide within the same sample because the affinity of the antibodies varies between different compounds and the relative amounts of the compounds are unknown in real samples (Degelmann et al., 2004). To solve the problem, we developed the broad-specificity dcELISA for OPs as a semi-quantitative screening method. We used percent inhibition to determine whether a sample contained OPs. The percent inhibition was calculated using the following equation: $I(\%) = [(A_0 - A_x)/A_0] \times 100$, where A_0 is the absorbance of the control and A_x is the absorbance of a sample at 450 nm. When samples showed a percent inhibition lower than 10%, they were regarded as negative, and they were considered positive when the percent inhibition was higher than 10%. The sample was more positive the higher the value of percent inhibition. To further confirm the positive samples, HPLC–MS/MS multi-analytes analysis for the 11 OPs was developed. The multiple reaction monitoring (MRN) chromatogram of leek sample spiked with 11 OPs at the 10 μg L^{-1} level is presented in Fig. 5. The limit of quantification (LOQ) of HPLC–MS/MS ranged from 0.1 to 1.0 μg L^{-1} for the tested pesticides (coumaphos, parathion and parathion-methyl, 0.25 μg L^{-1}; phoxim, quinalphos, triazophos, phosalone and azinphos-ethyl, 0.1 μg L^{-1}; dichlofenthion and phorate 0.5 μg L^{-1}; isochlorthion 1.0 μg L^{-1}).

Thirty vegetable samples (including leek, lettuce, cowpea, Chinese cabbage and cucumber) were randomly collected from three markets of Guangzhou and then analysed by the developed dcELISA. All the vegetable samples were free from the selective OPs by dcELISA (average percent inhibition ranging from −2.7% to 8.6%). The following HPLC–MS/MS analysis further confirmed the results. Thus, the developed dcELISA exhibited good accuracy and reproducibility, and is ideally suited as a fast, high-throughput and low-cost screening test for OP residue prior to chromatographic analysis.

4. Conclusions

The development of a monoclonal antibody (MAb)-based direct competitive enzyme-linked immunosorbent assay (dcELISA) for organophosphorus pesticides in a broad-specific manner was described. Three horseradish peroxidase-labelled tracers were synthesised and used to evaluate their effect on the sensitivity of dcELISA. The MAb–HRP based dcELISA with solid-phase extraction was developed and applied to detect OPs in vegetable samples. The recovery ranged from 70.0% to 133.3% with the coefficients of 8.9–22.4%. A good correlation (R^2 = 0.9666) between the results of dcELISA and HPLC–MS/MS was obtained, which indicated the good accuracy and reproducibility of the developed dcELISA. The HPLC–MS/MS multi-analytes analysis for selective OPs was developed and used to further confirm the positive samples screened out by the described dcELISA. The combination of broad-specificity dcELISA with HPLC–MS/MS multi-analytes analysis ensures the effective monitoring of the OP contaminants in vegetables in a rapid, high-throughput and low cost manner.

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References

Adou, K., Bontoyan, W. R., & Sweeney, P. J. (2001). Multiresidue method for the analysis of pesticide residues in fruits and vegetables by accelerated solvent extraction and capillary gas chromatography. *Journal of Agricultural and Food Chemistry, 49*, 4153–14160.

Alcocer, M. J. C., Dillon, P. P., Manning, B. M., Doyen, C., Lee, H. A., Daly, S. J., et al. (2000). Use of phosphonic acid as a generic hapten in the production of broad specificity anti-organophosphate pesticide antibody. *Journal of Agricultural and Food Chemistry, 48*, 2228–22233.

Bruun, L., Koch, C., Jakobsen, M. H., Pedersen, B., Chirstiansen, M., & Aamand, J. (2001). Characterization of monoclonal antibodies raised against different structures belonging to the *s*-triazine group of herbicides. *Analytica Chimica Acta, 436*, 87–101.

Degelmann, P., Wenger, J., Niessner, R., & Knopp, D. (2004). Development of a class-specific ELISA for sulfonylurea herbicides (sulfuron screen). *Environmental Science and Technology, 38*, 6795–6802.

Esteve-Turrillas, F. A., Abad-Fuentes, A., & Mercader, J. V. (2011). Determination of fenhexamid residues in grape must, kiwifruit, and strawberry samples by enzyme-linked immunosorbent assay. *Food Chemistry, 124*, 1727–1733.

González-Techera, A., Kim, H. J., Gee, S. J., Last, J. A., Hammock, B. D., & González-Sapienza, G. (2007). Polyclonal antibody-based noncompetitive immunoassay for small analytes developed with short peptide loops isolated from phage libraries. *Analytical Chemistry, 79*, 9191–9196.

Johnson, J. C., Van Emon, J. M., Pullman, D. R., & Keeper, K. R. (1998). Development and evaluation of antisera for detection of the *O,O*-diethyl phosphorothionate and phosphorothionothiolate organophosphorus pesticides by immunoassay. *Journal of Agricultural and Food Chemistry, 46*, 3116–31123.

Kolosova, A. Y., Shim, W.-B., Yang, Z.-Y., Eremin, S. A., & Chung, D.-H. (2006). Direct competitive ELISA based on a monoclonal antibody for detection of aflatoxin B1. Stabilization of ELISA kit components and application to grain samples. *Analytical and Bioanalytical Chemistry, 384*, 286–294.

Lee, J. K., Ahn, K. C., Park, O. S., Kang, S. Y., & Hammock, B. D. (2001). Development of an ELISA for the detection of the residues of the insecticide imidacloprid in agricultural and environmental samples. *Journal of Agricultural and Food Chemistry, 49*, 2159–2167.

Mak, S. K., Shan, G., Lee, H.-J., Watanabe, T., Stoutamire, D. W., Gee, S. J., et al. (2005). Development of a class selective immunoassay for the type II pyrethroid insecticides. *Analytica Chimica Acta, 534*, 109–120.

Morozova, V. S., Levashova, A. I., & Eremin, S. A. (2005). Determination of pesticides by enzyme immunoassay. *Journal of Analytical Chemistry, 60*, 202–217.

Nunes, G. S., Toscano, I. A., & Barceló, D. (1998). Analysis of pesticides in food and environmental samples by enzyme-linked immunosorbent assays. *Trends in Analytical Chemistry, 17*, 79–87.

Obana, H., Kikuchi, K., Okihashi, M., & Hori, S. (1997). Determination of organophosphorus pesticides in foods using an accelerated solvent extraction system. *Analyst, 122*, 217–220.

Piao, Y. Z., Kim, Y. J., Kim, Y. A., Lee, H.-S., Hammock, B. D., & Lee, Y. T. (2009). Development of ELISAs for the class-specific determination of organophosphorus pesticides. *Journal of Agricultural and Food Chemistry, 57*, 10004–10013.

Sharmaa, D., Nagpala, A., Pakadeb, Y. B., & Katnoria, J. K. (2010). Analytical methods for estimation of organophosphorus pesticide residues in fruits and vegetables: A review. *Talanta, 82*, 1077–1089.

Shimelis, O., Yang, Y., Stenerson, K., Kaneko, T., & Ye, M. (2007). Evaluation of a solid-phase extraction dual-layer carbon/primary secondary amine for clean-up of fatty acid matrix components from food extracts in multiresidue pesticide analysis. *Journal of Chromatography A, 1165*, 18–25.

中国科技论文在线 http://www.paper.edu.cn

Spinks, C. A. (2000). Broad-specificity immunoassay of low molecular weight food contaminants: New paths to Utopia! *Trends in Food Science and Technology, 11*, 210–217.

Suri, C. R., Boro, R., Nangia, Y., Gandhi, S., Sharma, P., Wangoo, N., et al. (2009). Immunoanalytical techniques for analyzing pesticides in the environment. *Trends in Analytical Chemistry, 28*, 29–39.

Tsang, V. C. M., Greene, R. M., & Pilcher, J. B. (1995). Optimization of the covalent conjugating procedure ($NaIO_4$) of horseradish peroxidase to antibodies for use in enzyme-linked immunosorbent assay. *Journal of Immunoassay, 16*, 395–418.

Watanabe, T., Shan, G., Stoutamire, D. W., Gee, S. J., & Hammock, B. D. (2001). Development of a class-specific immunoassay for the type I pyrethroid insecticides. *Analytica Chimica Acta, 444*, 119–129.

Winklmair, M., Weller, M. G., Mangler, J., Schlosshauer, B., & Niessner, R. (1997). Development of a highly sensitive enzyme-immunoassay for the determination of triazine herbicides. *Fresenius' Journal of Analytical Chemistry, 358*, 614–622.

Xu, Z.-L., Zeng, D.-P., Yang, J.-Y., Shen, Y.-D., Beier, R. C., Lei, H.-T., et al. (in press). Monoclonal antibody-based broad-specificity immunoassay for monitoring of organophosphorus pesticides in environmental water samples. *Journal of Environmental Monitoring*. doi:10.1039/C1EM10331H.

Xu, Z.-L., Shen, Y.-D., Zheng, W.-X., Beier, R. C., Xie, G.-M., Dong, J.-X., et al. (2010). Broad-specificity immunoassay for *O*,*O*-diethyl organophosphorus pesticides: Application of molecular modeling to improve assay sensitivity and study antibody recognition. *Analytical Chemistry, 82*, 9314–9321.

Zhang, Q., Sun, Q., Hu, B., Shen, Q., Yang, G., Liang, X., et al. (2008). Development of a sensitive ELISA for the analysis of the organophosphorous insecticide fenthion in fruit samples. *Food Chemistry, 106*, 1278–1284.

Zhu, G., Jin, M., Gui, W., Guo, Y., Jin, R., Wang, C., et al. (2008). Development of a direct competitive enzyme-linked immunoassay for carbofuran in vegetables. *Food Chemistry, 107*, 1737–1742.