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Biomimetic Enzyme-linked Immunosorbent Assay Using a Hydrophilic Molecularly Imprinted Membrane for Recognition and Fast Determination of Trichlorfon and Acephate Residues in Vegetables

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Abstract This study describes the development of a new direct competitive enzyme-linked immunosorbent assay method with a hydrophilic imprinted film as biomimetic antibody for determining multi-pesticide residues. The imprinted film, which can selectively recognize trichlorfon and acephate, was synthesized directly on the surface of a 96-well plate using 4-(dimethoxyphosphorothioylamino) butanoic acid as the template molecule. This film exhibited antibody-like recognition ability, fast adsorption–desorption dynamics and good stability. Under optimal conditions, the sensitivity (IC_{50}) of the biomimetic enzyme-linked immunosorbent assay (BELISA) method was 12.0 mg/L for trichlorfon and 30.0 mg/L for acephate. The limit of detection (LOD, IC_{15}) was 8.0 μg/L for trichlorfon and 12.0 μg/L for acephate. The developed method was applied to the determination of the trichlorfon and acephate in the spiked asparagus and cucumber samples with recoveries ranging from 72.1 to 92.0 % for trichlorfon and 70.0 to 85.0 % for acephate.

Keywords Molecular imprinting · Hydrophilic imprinted membrane · Biomimetic antibody · Enzyme-linked immunosorbent assay · Multi-pesticide residues · Asparagus · Cucumber

Introduction

Pesticide application to grain crops increases production and maximizes resource utilization rates (Bojacá et al. 2013). Although these benefits of pesticide use could reduce global food shortages, the soil and water pollution and the human health problems caused by pesticides misuse should not be underestimated (He and Ming 2012; Susan et al. 2009).

Pollution from residual pesticides, especially organophosphorus pesticides, has attracted attention in recent years. Consequently, many techniques have been used for determination of pesticide residues. Current organophosphorus pesticide determination methods include gas chromatography (Dugo et al. 2005), high performance liquid chromatography (Seebunrueng et al. 2014), gas chromatography coupled to mass spectrometry (Sun et al. 2011), and liquid chromatography coupled to mass spectrometry (Li et al. 2013). Although these detection methods have high sensitivity and accuracy, they suffer from complex sample pretreatment processes, portability limitations because of instrument size, lengthy run times and high costs, and are not suitable for rapid, on-site testing (Suri et al. 2009). Therefore, it is necessary to establish a rapid and simple technique for pesticide residue detection.

Immunoassays provide pesticide residue detection with high sensitivity and efficiency (Wang et al. 2011). In 1971, enzyme-linked immunosorbent assay (ELISA) was first applied in the determination of pesticide residues by Ercegovich et al. (1972). In recent years, this technology has been widely used for agricultural products, fruit juice, and wine (Argarate et al. 2010). However, in traditional ELISA technology, the monoclonal and polyclonal antibodies have low stability and short preservation times (Liang et al. 2013). Furthermore, this technique is time-consuming, expensive, and difficulties

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associated with antibody production (Wang et al. 2009). These problems have restricted the application of ELISA in the determination of pesticide residues.

To overcome these issues, many studies have investigated to design and synthesis of antibody-like receptors that can imitate the molecular recognition ability of biological antibodies. Among the approaches used, molecular imprinting technology has been recognized as one of the most attractive for synthesizing biomimetic mimics (Guan et al. 2012). The resulting molecularly imprinted polymers (MIPs) have good rigidity, stability, and high specific recognition ability, and can be prepared simply and inexpensively. MIPs have been widely applied in separation technology (Sun et al. 2012), sensor preparation (Zhang et al. 2012), catalysis, and enzymes mimics (Haupt 2013). However, using MIPs as artificial antibodies in immunoassay analysis is one of the most exciting applications of MIPs (Vlatakis et al. 1993; Sellergren 1997; Surugiu et al. 2001).

In recent years, many biomimetic enzyme-linked immunosorbent assay (BELISA) methods have been reported (Surugiu et al. 2000; Wang et al. 2009; Meng et al. 2011; Sun et al. 2014). However, the MIPs prepared by traditional method can only selectively recognize the template molecule, and the adsorption capacities toward other analytes are low (Alexander et al. 2006; Whitcombe et al. 2014). This has restricted the development and application of BELISA in pesticide multi-residues determination. Our previous report showed that 4-(dimethoxyphosphorothioylamino) butanoic acid shares a common structure and functional groups with organophosphorus pesticides, and it can be used as a template to prepare MIP that can selectively recognize multi-pesticides (Wang et al. 2013). Trichlorfon and acephate are two kind of broad-spectrum insecticides, and have been widely used in agricultural production. In this paper, the objective was to synthesize a novel hydrophilic MIP film that could selectively recognize trichlorfon and acephate in aqueous environment with 4-(dimethoxyphosphorothioylamino) butanoic acid as the template molecule. Using the imprinted film as an artificial antibody, a sensitive direct competitive biomimetic BELISA method was developed. The parameters affecting the performance of the BELISA method are optimized in detail. The applicability and accuracy of this presented method are also evaluated.

Materials and Methods

Chemicals and Materials

Trichlorfon, acephate, omethoate, monocrotophos, atrazine and cyfluthrin were obtained from the Institute for the Control of Agrochemicals of the Ministry of Agriculture (Beijing, China) and were >99 % pure. *O,O*-Dimethyl phosphorochloridothioate and ethylene glycol dimethacrylate (EGDMA) were purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). 4-Aminobutyric acid was purchased from TCI Development Corp. (Shanghai, China). Methacrylic acid and 2,2-azobis(isobutyronitrile) were purchased from Tianjin Chemical Reagent Factory (Tianjin, China), and purified before use. 3,3′,5,5′-Tetramethylbenzidine, horseradish peroxidase, N-hydroxysuccinimide, *N*,*N*′-dicyclohexylcarbodiimide, dimethyl sulfoxide, and other organic chemicals used for hapten synthesis were supplied by Sigma-Aldrich. Double deionized water (DDW, 18.2 $M\Omega cm^{-1}$) was obtained from a Water Pro water system (Labconco Corp., Kansas City, MO, USA).

Microlon ELISA 96-well plates (Costar®, Corning) were purchased from Beijing Biolead Biology Sci & Tech Co., Ltd. (Beijing, China).

Solutions

Solutions of phosphate-buffered saline (PBS, 50 mmol/L sodium phosphate, 154 mmol/L NaCl, pH 7.0), PBS with 0.05 % Tween-20 (PBS/T), substrate solution (1.25 mmol/L 3, 3′, 5, 5′-tetramethylbenzidine-1.6 mmol/L hydrogen peroxide in acetate buffer, pH 5.0), and stopping solution (1.25 mmol/L H_2SO_4) were used in this study.

Instrumentations

A SS-35 scanning electron microscope (SEM, Shimadzu, Kyoto, Japan) set at 20.0 kV and a DTG-60AH thermogravimetric analyzer (TGA, Shimadzu) were used. Immunoassay absorbance was read in dual-wavelength mode (450–650 nm) with a Multimode Plate Reader (Molecular Devices, Sunnyvale, CA).

Methods

Synthesis of 4-(Dimethoxyphosphorothioylamino) Butanoic Acid

In this study, 4-(dimethoxyphosphorothioylamino) butanoic acid was synthesized following the method of Wang et al. (2013). Firstly, 1.032 g of 4-aminobutyric acid (10 mmol) was added to a stirred solution of 10 mL 2.5 mol/L NaOH in an ice-bath. The mixture was continuously stirred for 30 min, and then 1.215 mL of *O,O*-dimethyl phosphorochloridothioate was added to the mixture. After another 6.0 h of stirring at room temperature, the mixture was washed with diethyl ester to remove impurities, and the pH of the reaction solution was adjusted to 2.0 by addition of 1.0 mmol/L HCl. Finally, the

mixture was extracted with diethyl ester (3×25 mL), and the organic layers were combined and dried over Na_2SO_4. The product was obtained by reducing the organic extract to dryness under rotary evaporation.

Preparation of Hydrophilic Molecularly Imprinted Film on the Surface of a 96-Well Plate

The hydrophilic molecularly imprinted film was prepared on the 96-well plates directly as follows. 4-(Dimethoxyphosphorothioylamino) butanoic acid (0.908 g, 4 mmol) and 1.033 g of methacrylic acid (12 mmol) were dissolved into a mixed solution of 4.0 mL of DDW, 4.0 mL of dimethyl sulfoxide, and 12 mL of acetonitrile. After stirring at room temperature for 15 min, 3.236 g of EGDMA (16 mmol) and 100 mg of 2,2-azobis(isobutyronitrile) were added. The mixture was continuously stirred for 60 min and sonicated for 10 min. Then, 200 μL of the mixture was placed in each well of a 96-well plate, and the plate was sealed in nitrogen gas at 38 °C for 18 h in the dark. When the polymerization ended, the plate was firstly extracted with 300 mL of methanol/acetic acid (3:1, *v*/*v*) for 12 h and then 300 mL of methanol for 6 h. Finally, the 96-well plate was dried at 37 °C for 4 h.

For comparison, a non-imprinted film was polymerized following the above procedure without the addition of 4-(dimethoxyphosphorothioylamino) butanoic acid.

Characterization of Imprinted Film

To measure the adsorption capacity, the novel imprinted or non-imprinted film was equilibrated with 200 μL of a 15 % methanol PBS solution containing trichlorfon or acephate at various concentrations (30–230 mg/L). After the plate was shaken for 60 min at room temperature, the supernatants were analyzed with a UV spectrometer for unextracted trichlorfon at 195 nm and acephate at 220 nm, and the adsorption capacities (μg/well) were calculated.

The adsorption kinetics of the imprinted film toward trichlorfon or acephate at 200 mg/L was also evaluated. After shaking for 30, 60, 90,120, 150, 180, 210, and 240 min at room temperature, the supernatant was analyzed, respectively.

Preparation of Enzyme Conjugate

Solution A was prepared by dissolving 6.81 mg of 4-(dimethoxyphosphorothioylamino) butanoic acid (0.03 mmol) in 618 μL of DMF solution, and preserved at the temperature of −20 °C. Solution B was prepared by dissolving 3.4 mg of *N*-hydroxysuccinimide (0.03 mmol) and 12.4 mg of *N*,*N*′-dicyclohexylcarbodiimide (0.06 mmol) into solution A, and stirring at room temperature for 4 h. After centrifugation, the supernatant was preserved as solution B. Solution C was prepared by adding 10 mg of horseradish peroxidase to 2 mL of 50 mmol/L K_2HPO_4 solution. In an ice-bath, solution B was added dropwise to solution C, and the mixture was shaken gently by hand. Then, the mixture was placed in a 4 °C environment over-night. The enzyme conjugated solution was then dialyzed against PBS (pH 7.0) for 3 days and then stored at 4 °C before use.

Direct Competitive BELISA Procedure

The direct competitive BELISA was performed as follows. First, the plate was washed with PBS/T solution three times. Then, 5 % PBS was added to the blank (200 μL) and control (100 μL) wells. Gradient standard solutions or sample extracts were applied to the allocated wells (100 μL/well). Then, 100 μL of enzyme conjugate was added immediately to all wells except for the blank wells. After that, the 96-well plate was shaken (200 times/min) for 1 h at room temperature. After washing with PBS/T five times, 150 μL of substrate solution was added to each well. After incubation at room temperature for 30 min, the reaction was terminated by adding 50 μL of stopping solution. The UV absorbance was recorded using a Labsystems 96-well plate reader in dual-wavelength mode (450–650 nm), and the inhibitions were calculated. Finally, the plate was extracted with 300 mL of methanol/acetic acid (3:1, *v*/*v*) for 4 h, followed with 300 mL of methanol for 2 h, for the next BELISA procedure.

Sample Preparations

Asparagus and cucumber samples were purchased from the Taishan Yaxiya Food Co., Ltd. (Taian, China) in April 2014. To evaluate the accuracy of the developed method, spiked asparagus and cucumber samples were prepared. Before spiking, these samples were determined by gas chromatography to be free of trichlorfon and acephate. Briefly, 2.0 g of the asparagus and cucumber sample were weighed into a 25-mL beaker, and then spiked with 1.0 mL of trichlorfon standard solution (10, 15, and 20 mg/L) or acephate standard solution (10, 30, and 50 mg/L), respectively. After incubation for 4 h, the spiked samples were extracted by ultrasonication with 3×10 mL of PBS for 30 min. The resulting extracts were collected and made up to a constant volume of 50 mL with PBS. After filtration through a 0.45-μm membrane, the filtrates were analyzed by the BELISA method.

Leek sample obtained randomly from a market was prepared according to the above process without the addition of richlorfon or acephate standard. The richlorfon and acephate levels were calculated.

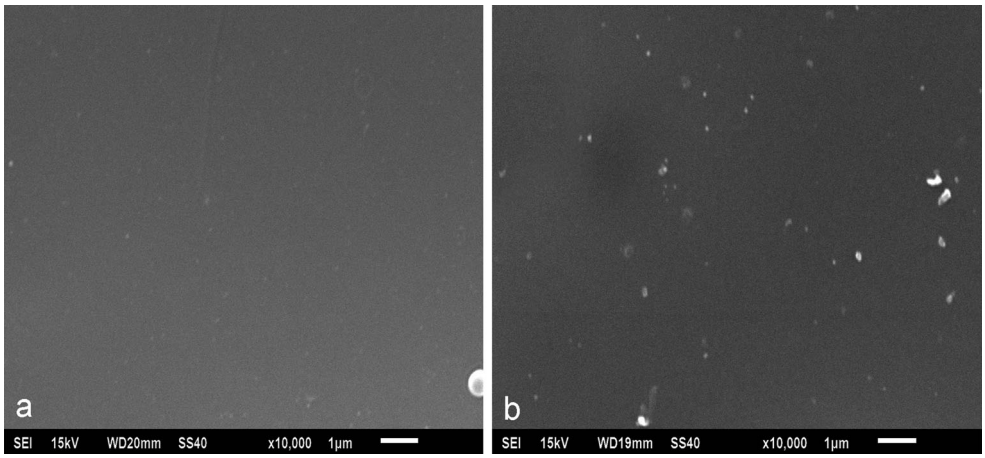


Fig. 1 SEM images of the imprinted film (**a**) and non-imprinted film (**b**) (×10,000)

Results and Discussion

Characterization of the Hydrophilic Imprinted Film

The structure of the imprinted polymer film on the well surface of a 96-well plate was visualized using SEM (Fig. 1). The SEM images showed that both the imprinted film (a) and non-imprinted film (b) had flat and smooth surfaces. Therefore, the imprinted film was successfully synthesized.

The TGA was applied to investigate the thermal stability of the imprinted film. A typical TGA curve is depicted in Fig. 2. There was little mass loss between 0 and 320 °C, and an obvious mass loss occurred when the temperature was over 320 °C. These results indicated that the MIP film had high thermostability.

The isothermal adsorptions of the imprinted and non-imprinted films toward trichlorfon (a) and acephate (b) at 50–250 mg/L concentrations in a 5 % methanol PBS solution were investigated (Fig. 3). Results showed that the amounts of trichlorfon and acephate extracted by the imprinted and non-imprinted films increased as the initial concentrations of the pesticides increased. However, the imprinted film exhibited a higher adsorption ability for trichlorfon and acephate than the non-imprinted film, and the adsorption capacities of the imprinted film toward trichlorfon and acephate (1.25 and 1.09 μg/well) were more than 1.7 times those of the non-imprinted film (0.71 and 0.62 μg/well) at a 250 mg/L concentration.

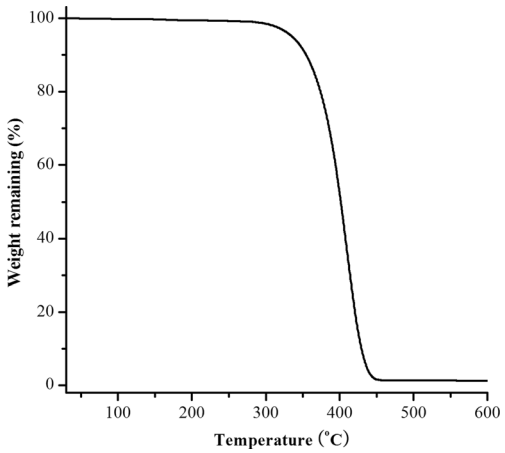


Fig. 2 TGA curves of the MIP film on heating the samples at 10 °C/min from 25 to 600 °C in nitrogen atmosphere

The adsorption kinetics of the imprinted film toward trichlorfon and acephate at 200 mg/L were also tested. Adsorption capacities of 0.78 and 0.67 μg/well for trichlorfon and acephate, respectively, were obtained after shaking for 30 min, which were 63.41 % (trichlorfon) and 65.68 % (acephate) of the maximum adsorption capacity of the imprinted film. The adsorption was almost approached equilibrium within 180 min. The fast adsorption kinetics of the hydrophilic imprinted film is an advantage for its application as a recognition element in BELISA analysis.

Optimization of the BELISA Conditions

To improve the precision and sensitivity for the BELISA method, the enzyme conjugate concentration, preparation solution, and pH were optimized.

The enzyme conjugate concentration affects the sensitivity of the ELISA method. As the quantity of the enzyme conjugate increased, the color intensity increased and the sensitivity of ELISA may decrease. The concentration of enzyme conjugates was optimized by titration of enzyme tracers to yield absorbance values ranging from 0.7 to 1.2. In this study, the enzyme conjugate was diluted to 1:5000 before use.

The solvent used for the preparation of the standard solution and samples can affect the recognition ability and selectivity of the imprinted film toward the target molecules, and this directly affects the sensitivity of BELISA method. To investigate the influence of the preparation solution on the assay performance of BELISA, different working solutions including PBS, PBS/T, 5 % methanol PBS (*v*/*v*), and DDW were investigated (Fig. 4). When 5 % methanol PBS was selected as the preparation solution, the BELISA method

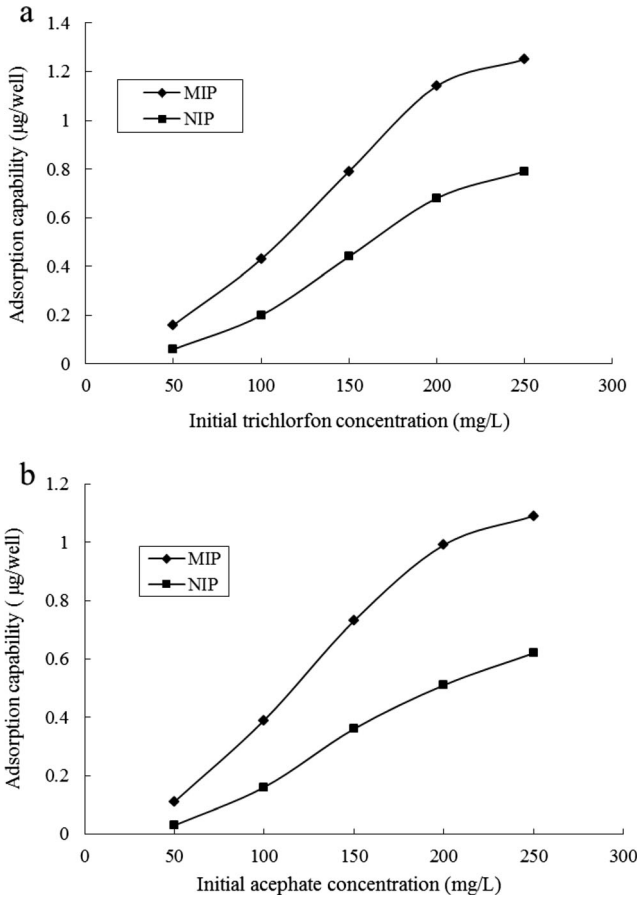


Fig. 3 Adsorption isotherms of the imprinted and non-imprinted films toward trichlorfon (**a**) and acephate (**b**)

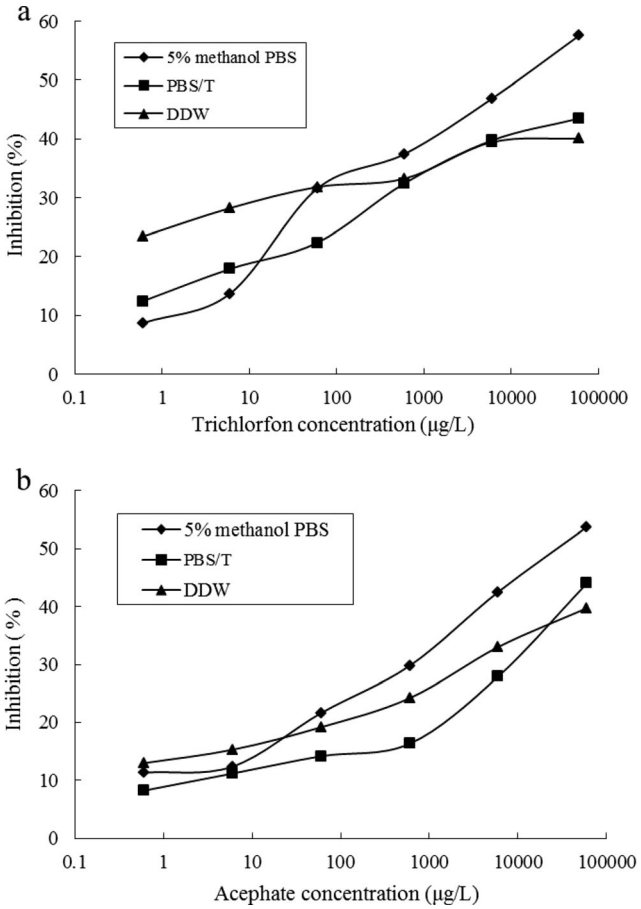


Fig. 4 BELISA standard curves of trichlorfon (**a**) and acephate (**b**) using imprinted film as the artificial antibody in 5 % methanol PBS, PBS/T, and DDW solutions, respectively

had higher sensitivity for both trichlorfon (a) and acephate (b) than when the other solutions were used. Thus, 5 % methanol PBS was chosen as the preparation solution in subsequent experiments.

To improve the sensitivity of the BELISA method, the pH of the 5 % methanol PBS solution was optimized in the range 3.74–7.37 (Fig. 5). The better results were obtained when the pH was 7.37 for trichlorfon (Fig. 5a) and 6.30 for acephate (Fig. 5b). Therefore, 5 % methanol PBS at pH 7.37 was chosen as the working solution for trichlorfon and 5 % methanol PBS at pH 6.30 was chosen as the working solution for acephate.

Parameters of the BELISA Method

Under the optimal conditions, standard curves (Fig. 6) were obtained using the imprinted film as the artificial antibody with trichlorfon and acephate standard solution concentrations between 60,000 μg/L and 0.6 μg/L in 5 % methanol PBS solution. From the standard curves, the sensitivity (IC_{50}, concentration giving 50 % inhibition of color development) of the developed BELISA was 12.0 mg/L for the trichlorfon and 30.0 mg/L for the acephate, and the limit of detection (LOD, IC_{15}) was 8.0 μg/L for trichlorfon and 12.0 μg/L for acephate. The limit of quantitation (LOQ) for trichlorfon and acephate was 26.6 and 40.0 μg/L, respectively. The linearity of the BELISA method was 0.1 to 100,000 μg/L. According to the "Japanese Positive List System," the maximum residue level of trichlorfon and acephate in the primary products is 10 and 20 μg/L, respectively. This method meets the need of sensitivity of trace trichlorfon and acephate determination. These results demonstrate that the developed BELISA method can effectively detect multi-pesticides at a wide range of concentrations in samples.

In this study, the MIP film that prepared with 4-(dimethoxyphosphorothioylamino) butanoic acid as the template molecule had broad selectivity toward organophosphorus pesticides. Therefore, when applied as the artificial antibody, the sensitivity (IC_{50}) of the BELISA method for trichlorfon and acephate was lower than reported results (Wang et al. 2009). These results indicate that the selectivity of the MIP had an important effect on the sensitivity of the BELISA method.

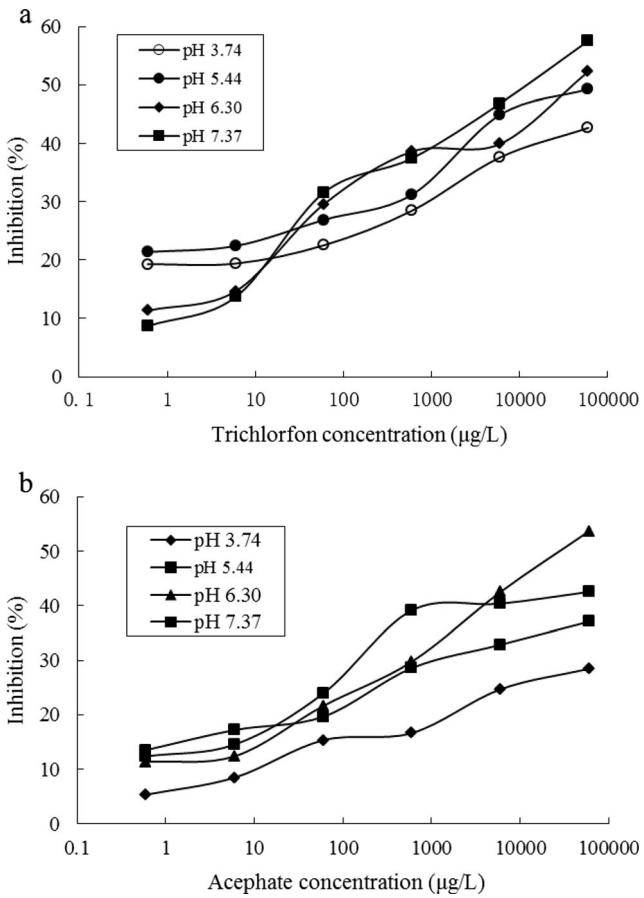


Fig. 5 BELISA standard curves of trichlorfon (**a**) and acephate (**b**) using 5 % methanol PBS as the preparation solution at pH of 3.74–7.37

Selectivity of BELISA Method

The specificity of the developed BELISA was also evaluated using competitive assays with related compounds. For evaluation of interfering compounds, omethoate and monocrotophos were selected as organophosphorus pesticides with similar molecular structures to the template; atrazine and cyfluthrin were selected as non-organophosphorus pesticides with different structures to the template. Calibration curves were established and the cross-reactivity (CR) was calculated as the percentage between the IC_{50} value for trichlorfon and that for acephate or other interfering compound with the following the equation:

$$\%CR = \left\{IC_{50}(\text{trichlorfon}) \Big/ IC_{50}(\text{structural analogue})\right\} \times 100\%. \quad (1)$$

The results are shown in Table 1 and Fig. 6. The imprinted film had broad selectively toward organophosphorus pesticides because of the imprinting effect, and high CR values (>20 %) were obtained for both omethoate and monocrotophos. However, the CR values for atrazine and cyfluthrin were less than 12 %, which indicated that the imprinted film had low sensitivity toward non-organophosphorus pesticides because of their different structures. These results confirmed that the structure of the target molecule plays an important role in the recognition and competitive immunoassay.

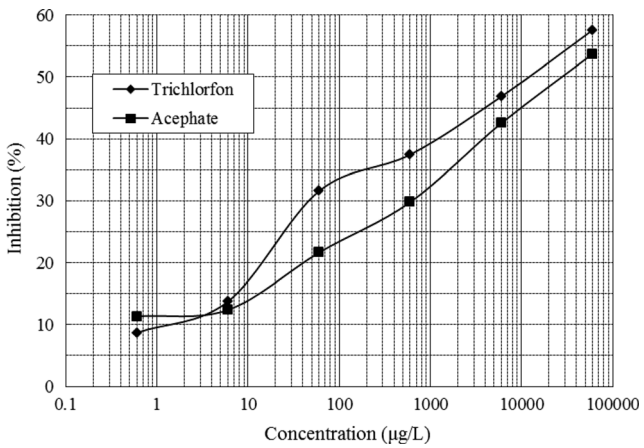


Fig. 6 Standard BELISA competition curves for trichlorfon and acephate with concentrations from 60,000 to 0.6 μg/L in a 5 % methanol PBS solution

Accuracy Evaluation of the BELISA Detection

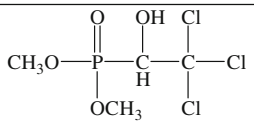
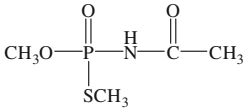
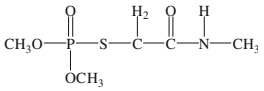
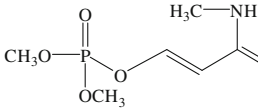
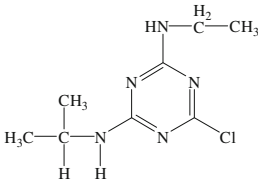
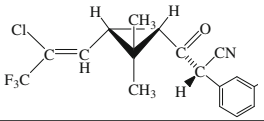
To evaluate the accuracy and applicability of the BELISA method, the intra-, inter-day precisions, and recovery tests were performed by spiking asparagus and cucumber samples with trichlorfon or acephate at three levels. The data are shown in Table 2. For each concentration, measurements were repeated for five times, and good recoveries were achieved for trichlorfon (72.1–92.0 %) and acephate (70.0–85.0 %) of asparagus and cucumber samples. Intra-day (five times), inter-day (1, 3 or 5 day), and inter-laboratory precisions ranged from 5.5 to 7.9 %, 7.7 to 9.4 %, and 5.1 to 7.5 % were obtained, respectively. These results indicated that the developed BELISA method had good accuracy and precision for determination of trace organophosphorus multi-pesticides in agricultural products.

To evaluate the applicability of the BELISA method, a leek sample was analyzed. The acephate has not been found. The acephate level in the leek sample was 2.15±0.08 mg/kg, which was higher than the maximum residue limits (MRLs) of the Japanese Positive List System (0.1 ppm). Therefore, more effort should be done to control the pesticide residues in agricultural products.

Merits and Advantages

Compared with the traditional ELISA and chromatography methods, the operation procedure of the developed

Table 1 The cross-reactivity (CR) ratio of four kinds of organophosphorus pesticides

Pesticides	Pesticide structure	IC_{50} (mg/L)	CR (%)
Trichlorfon		12	100
Acephate		30	40
Omethoate		41	29
Monocrotophos		45	27
Atrazine		>100	<12
Cyhalothrin		>100	<12

method is simpler, and no huge instruments are required. The matrix effect on the BELISA method was minimal because of the high selectivity of the MIP and the pretreatment procedures such as SPE are not needed. Furthermore, the BELISA method can selectively recognize and detect trichlorfon and acephate. More importantly, the developed method can be reused for more than 10 times without sensitivity lost. Therefore, the cost per analysis of this new method is low.

Conclusion

In this study, a rapid BELISA method that can determine trichlorfon and acephate residues was developed. This assay is simple, inexpensive, and provided good preliminary results. Therefore, it is an attractive tool for rapid detection of multi-pesticides. This study established a methodology for the preparation of a MIP that can selectively recognize many structural analogs. This could lead to further research into the

Table 2 Analysis results and the recoveries of BELISA method for the determination of spiked trichlorfon and acephate in asparagus and cucumber samples (n=5)

Samples	Spiking levels of trichlorfon (μg/g)	Analysis results (μg/g, ±SD)	Recovery (%, ±RSD)	Spiking levels of acephate (μg/g)	Analysis results (μg/g, ±SD)	Recovery (%, ±RSD)
Asparagus	5.0	3.75±0.0162	75.0±2.7	5.0	3.50±0.0242	70.0±4.6
	7.5	6.50±0.0284	86.7±5.3	15.0	10.95±0.0346	73.0±5.6
	10.0	9.20±0.0176	92.0±4.4	25.0	21.25±0.0226	85.0±6.3
Cucumber	5.0	3.61±0.0326	72.1±5.9	5.0	3.70±0.0221	74.0±4.0
	7.5	6.23±0.0165	83.0±3.4	15.0	11.4±0.0121	76.0±2.5
	10.0	8.90±0.00349	89.0±8.4	25.0	21.05±0.0219	84.2±4.9

applicability of MIP-based biomimetic immunoassays for multi-pesticide residues analysis.

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Conflict of Interest Chen Shi, Xueyan Liu, Lingyu Song, Xuguang Qiao, and Zhixiang Xu declare that they have no conflict of interest.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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