

Study & Application on the Enzyme-Linked Immunosorbent Assay System of Grain in Southern China

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Abstract—With the acceleration of economic globalization, food security is not only related to the health of consumers, but also affects the international food and agricultural trade. It has become one of significant global focus. Aiming at the safety issues of chemical contamination in food processing, storage and transportation, the rapid detection technology of food security is studied in this paper, while four kinds of hapten with four kinds of antibodies were designed and screened. The effective detection reagents including PBDEs, imidacloprid, polybrominated diphenyl ether, bifenthrin, and fenvalerate were evaluated. The results show that the lowest detection limit (IC₁₀) for the detection of imidacloprid, polybrominated diphenyl ethers, fenvalerate and fenvalerate were 1.8ng/g, 0.1ng/g, 4.7ng/g, and 1.0ng/g. Through the correlation analysis, grain, rice and corn samples were highly correlated based on the average detection time, which was less than 10 minutes, faster than Gas Chromatography (GC) method.

Index Terms—detection reagents, IC₁₀, correlation analysis, GC method

I. INTRODUCTION

In 1967, Centno and Johnson first reported DDT and malathion antibody preparation. The report was marked as the beginning of the application of immunological techniques in pesticide analysis. In 1971, Engvail and Weerman established an enzyme-linked immunosorbent assay (ELISA): the antigen was coated on the stationary phase through direct or indirect competitive immunoreaction, and the marker reacted with antigens and antibodies. As an important means of food security,

the technology of pesticide detection received widespread attention from all over the world. In 1970s the Enzyme Linked Immunosorbent Assay (ELISA) was developed rapidly in terms of detection speed, sensitivity, accuracy, specificity, reducing detection cost along with the instrument analysis method of pesticide residues to highlight the advantages. Therefore, in recent years the ELISA method in food safety field has been widely recognized and used in pesticide residue analysis.

II. MATERIALS

Raw materials: iron powder, aluminum powder, iron oxide, oxygen scavenger, sodium chloride, sodium bicarbonate, calcium bicarbonate, sodium carbonate, sodium sulfite, anhydrous calcium chloride, wood carbon powder, diatomite, HRP labeled goat anti-mouse IgG, HRP labeled goat anti-mouse IgG, imidacloprid, thiamethoxam, HAT and HT selective medium, RPMI1640, fetal calf serum, horseradish peroxidase HRP (RZ \geq 3).

Test materials: grain, rice, wheat, Bal b/c mouse.

Sealing material: multi-functional film heat sealing machine, slot tube, hose, PA / PE five-layer co-extruded nylon film with permeability of 800ml/(m² 24h)

Test pest species: *sitophilus oryzae*, *castaneum*, rice moth

Equipment and facilities: OX-100a oxygen measuring instrument, CO₂ incubator, sealing machine, clean bench, liquid nitrogen cans, cell counting plate inverted microscope, 96 holes and 24 holes with flat cell culture plate, LD4-2 low speed centrifuge.

Manuscript received July 28, 2017; revised October 12, 2017.

III. METHODS

A. The Synthesis and Identification of Half Antigens

Using computer technology to simulate the molecular structure model to obtain the root mean square deviation of the compound, plane angle and molecular size and other information. According to the structural characteristics of all kinds of compounds, this study will synthesize the following major semi-antigens, semi-antigens and intermediate products are identified by mass spectrometry, infrared spectroscopy and nuclear magnetic resonance [1].

Half antigen: pyrethroid pesticide, 2,2,3,3- four methyl cyclopropane carboxylic acid; polybrominated diphenyl ether (PBDEs); imidacloprid (1- (6- Chloropyridine -3- pyridyl methyl) -N- Nitroimidazoline -2- based) amine (3-) and thiamethoxam (2- chloro -1,3- thiazole -5- methyl) -5- methyl -1,3,5- oxadiazine -4- based binary (nitro) amine).

B. Preparation and Purification of Antibodies

The hapten was conjugated to the carrier proteins BSA and OVA, using the mixed anhydride method and the active ester method. The immunogen was mixed with Freund's complete adjuvant (primary immunization) or incomplete Freund's adjuvant (booster immunization) to immunize New Zealand white rabbits and rabbits, and then polyclonal antiserum was prepared. Saturated ammonium sulfate and protein using an affinity chromatography column purification for antibodies.

C. The Establishment of ELISA Method

The optimal cultivation concentrations of the antigens and antibodies were screened by matrix method, and the inhibition test was carried out with different concentrations of target analyte. The standard inhibition curve was established, and the detection sensitivity and linearity range of ELISA were determined [2].

D. The Development Kit

Kit assembly: including a test of various conditions of the optimization of the configuration, the combination of the best process response, that is, to achieve the detection of standardization [3].

Quality Control: After the kit was assembled, all kinds of factors together, through the quality control of the whole kit, could help the quality evaluation of the various performance indicators, so as to improve the quality.

Precision: Determined by the comparison between the number of intra-and inter-variation coefficients.

Sensitivity: Analysis of the tests where the optical density value is two times the standard deviation (2SD) from the average of optical density value (X) [4].

Accuracy: Determined by quality test of low, medium and high concentrations of serum.

Specificity: The inhibition test was done by the chemical structure analogs, and the specificity of the kit was detected by the cross reaction.

Stability: The sensitivity of the kit was measured every other week, at least for 6 months, and during the effective period the kit was preserved at 4°C.

E. Sample Measurement

To detect rice paddy, a certain amount of the target analytes was added to the sample for the recovery test. First, the sample pretreatment method was conducted.

Extraction method: one or two kinds of organic solvents, including methanol, ethanol, dichloromethane, n-hexane, acetone and other organic solvents, were used with ultrasonic or manual oscillation where different time scales were set [5].

Effect of sample matrix on the detection of kit: the dilution method was used to eliminate the influence of matrix in the extraction product to optimize the detection sensitivity of the reagent kit.

Effect of organic solvents on the liquid-content analysis kit: different concentrations of organic solvents in the solution were analyzed.

Effect of ionic strength and pH value on the analysis kit: different NaCl concentration and pH values were studied to determine the influence of reagent kit and the allowable range of NaCl concentration and pH value.

IV. RESULTS

A. Immunoassay Analysis of Imidacloprid

Semi-antigenic structures:

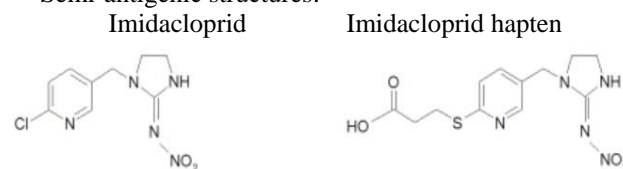


Figure 1. Imidacloprid and imidacloprid hapten structures

Preparation of coated antigens:

5.4mg Imidacloprid hapten (about 18μmol in Fig. 1) was dissolved in 200μl anhydrous DMF, and then 4.27μl (about 18μmol) tri-n-butylamine, and 2.34μl (about 18μmol) of isobutyl chloroformate were added to the sample in sequence. The solution was stirred at room temperature for 1h. Then 30mg OVA was dissolved in 2ml carbonate buffer solution dropping 100μl(???) for further reaction, stirring at room temperature at a slower rate for 3h. Then the solution went through dialysis, centrifugation and ultimately dry frozen at 4°C.

Screening of positive phage clones:

Linear random peptide library used by New England Biolabs America 7- company (Ph.D.-7)(???), with its huge storage capacity, containing about 1 x 10⁹ independent clones, was theoretically capable of screening to identify small peptide molecules. Specific phage peptide screening method was used to reduce the antigen and analyte concentrations to obtain competitive elution to improve affinity of phage peptide. Using the same technique, the results in Table I show that each round of screening phage titer increased with the improvement of the recovery rate, which indicates that positive phages were enriched, although the concentration of coated antigens was decreasing.

TABLE I. RESULTS OF PHAGE PEPTIDES AFTER EACH ROUND OF AFFINITY SCREENING

Panning (round)	Input phage (pfu)	Phage display (pfu)	Rate of recovery
1	5.4×10^{11}	3.2×10^4	5.9×10^{-8}
2	5.2×10^{11}	2.4×10^5	4.6×10^{-7}
3	4.6×10^{11}	3.5×10^6	7.6×10^{-6}
4	4.7×10^{11}	6.5×10^7	1.4×10^{-4}

After the fourth round of screening when the phage infected *E. coli* ER2738 after plate culture, 20 clones were randomly selected through a combination of competitive ELISA method for detection of phage and imidacloprid. 100ppb imidacloprid was added to the sample where 5 clones were coated with inhibited antigen BSA-IMI, and the inhibition ratio was 51.8 - 66.2% (Table II), showing that the positive ratio was 25%. With the highest inhibition efficiency of clones, 3 were amplified for the next step to establish phage ELISA method.

TABLE II. THE POSITIVE PHAGE AND IMIDACLOPRID CONCENTRATION PROFILE

Positive phage	OD=450nm			Inhibition rate (%)
	BSA-IMI (Oppb Imidacloprid)	BSA-IMI (100ppb Imidacloprid)	BSA	
1	0.921	0.561	0.226	51.8
2	1.014	0.599	0.318	59.6
3	1.127	0.605	0.339	66.2
4	0.966	0.547	0.252	58.7
5	0.969	0.601	0.328	57.4

Standard curve of ELISA method:

The main advantages of the immunoassay method are fast and convenient, though easy to be affected by various environmental factors, such as buffer pH value, ionic strength and organic solvent concentration. Under the optimal conditions, can increase the sensitivity of the analysis. Methanol is a commonly used organic solvent, and the solubility of Imidacloprid in methanol is high. Therefore, this study used methanol as the solvent of imidacloprid under optimal detection conditions.

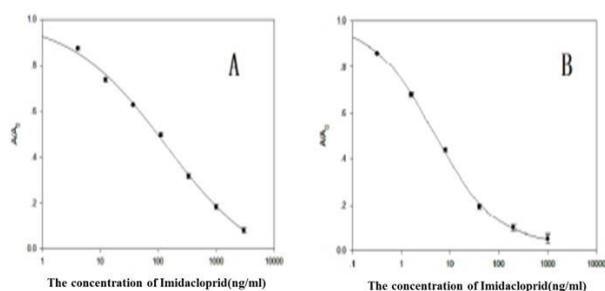







Figure 2. Phage ELISA method (A) and ELISA antibody (B) method standard curves (OD values of control hole without adding A)

In Fig. 2(A) (PBS buffer pH=7.4, NaCl concentration=8.0g/L, methanol content=5%), median inhibitory concentration (IC₅₀) was 96.6ng/mL, the linear detection range (IC₂₀ - IC₈₀) was 8 ~ 875ng/mL, and the minimum detection limit (IC₁₀) was 1.8ng/mL. Preserved by our laboratory monoclonal antibody based antibody ELISA method standard curve in Fig. 2 (B), ELISA IC₅₀ antibody was 5.1ng/mL, the detection range of was 0.6 ~ 43.4ng/mL, the lower detection limit was 0.2ng/mL. Compared with the monoclonal antibody ELISA method, due to limitation of low sensitivity of phage ELISA, screening results verified that the cyclic peptide library showed cyclic peptide affinity and specificity, and high detection sensitivity.

Cross reaction:

TABLE III. CROSS REACTION RATIOS OF BACTERIOPHAGE ELISA AND IMIDACLOPRID ANALOGUES

Test object	Chemical structure	IC ₅ (ng/mL)	Cross reaction ratio (%)
Imidacloprid		98	100
Thiamethoxam		14200	0.69
Worm amine		7480	1.31
Acetamiprid		5870	1.67
6-Chloronicotinic acid		875	11.16

To investigate the specificity of phage display peptide and Imidacloprid with the main metabolites of 6-chloronicotinic acid and nicotine pesticide imidacloprid were analyzed via cross reaction experiment. The results showed that phages display peptide analogues and imidacloprids participated in cross reactions [6]. The concentration ratio of imidacloprids reacted was very high as 100% (Table III). The results indicated that the possibility of cross reaction between the monoclonal antibodies with similar or the same structure was relatively small, and the minimum reaction ratio was as low as 0.69%. Antibodies and phage display peptides showed distinguishable cross reaction ratios, so ELISA could be used to detect different targets.

Additional recovery experiment:

Immune analysis method is a prevailing detection technology, it simplifies the sample pretreatment procedure to dilute the buffer solution, but the dilution method lowers the detection sensitivity. The sample pretreatment method requires a comprehensive reference on immunoassay and sensitivity of the concentration of analytes. In this study, the samples were recovered using phage ELISA method and antibody ELISA method. The sensitivity of the two methods was studied. The samples were diluted with PBS for 10 times each. The results showed that the recovery rate of phage ELISA was lower than that of antibody ELISA method (Table IV). A large amount of matrix such as esters and proteins promoted non-specific adsorption, and interfered with the binding

of antibodies and antigens. Although the sample was diluted 10 times, there was still a certain sample matrix interference. The results in Table IV showed that the recovery ratio of antibodies against substrate interference was significantly higher than that of phage display peptide.

TABLE IV. RECOVERY RATIOS OF THE TWO METHODS

Adding concentration (ng/g)	Phage -ELISA		Antibody -ELISA	
	Measured value (ng/g)	Recovery ratio (%)	Measured value (ng/g)	Recovery ratio (%)
1000	586	58.6	854	85.4
100	50.2	50.2	81.6	81.6

Although the screened positive phage display peptides and monoclonal antibody still had specific binding specificity, in complex environment, dilution was used to avoid interference of the matrix, especially the phage ELISA method. Thus it was necessary to adopt the method of sample purification to optimize the detection sensitivity. The recovery of bacteriophage ELISA method was 50.2-58.6%, and the recovery ratio of antibody ELISA was 81.6-85.4%, to meet the rapid screening demand of Imidacloprid in rice.

Dynamic changes of residual imidacloprid in rice:

Due to absolute absorption of imidacloprid, especially in seed processing and granule application, can early continuous pest control in cereal crops, currently have yet to develop rice paddy imidacloprid residue limits, the provisions of the maximum residue limit of 0.05mg/kg grains of imidacloprid. Analysis of dynamics of imidacloprid residue in paddy detection method used in this study established the basis for immunization, for our country the imidacloprid residue limits [7]. The concentration of imidacloprid in this experiment was 500ng/g, using ELISA method to analyze the change of antibody concentration in paddy residue of imidacloprid, the imidacloprid residue concentration decreased with time (Fig. 3).

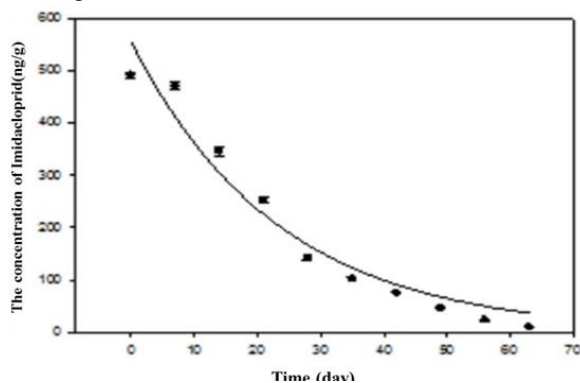


Figure 3. Dynamic changes of residual imidacloprid in paddy

Fig. 3 shows that the residual imidacloprid ratio in rice placed for 3 weeks was reduced to below 50% (245 ng/g); after 8 weeks' time, the ratio decreased to below 5% (23 ng/g), and after 10 weeks' time, the concentration became lower than the detection limit of 2 ng/g. Furthermore, it

could be speculated that the digestion of imidacloprid in rice might include photolysis, hydrolysis and biological degradation.

Monoclonal antibody technology is a major breakthrough in immunological detection technology. It has been widely used in clinical diagnosis, environmental monitoring and food safety testing fields for small substances, though emerging phage display technology with its unique advantages in other areas of biotechnology has made remarkable achievements.

B. Enzyme Linked Immunosorbent Assay for Polybrominated Diphenyl Ethers Residues

Design and synthesis of semi-antigen:

5 kinds of antigens B1, B2, B3, B4, and B5 were designed, and the structures are shown as follows:

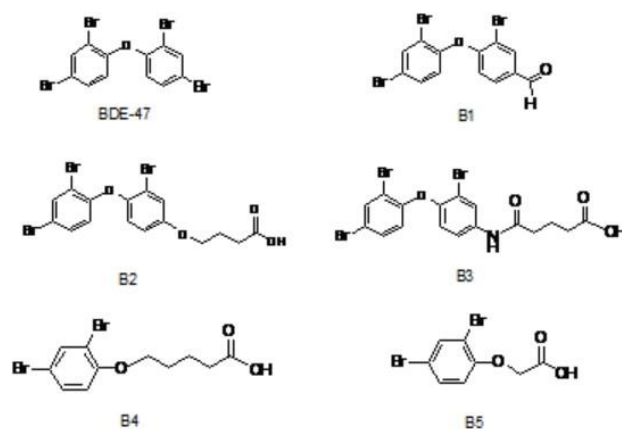


Figure 4. Five kinds of BDE47 semi-antigen

Establishment of direct competitive ELISA methods:

The combination of the supernatant of the positive cell line and the inhibition curve of the indirect competitive ELISA method was optimized by coupling the different semi antigens with the carrier protein OVA. The generated and purified antibodies were coated on the enzyme labeled plate, and the inhibition concentrations of the direct competitive ELISA method were determined by the combination of the different half antigens and the HRP conjugates (Table V).

TABLE V. COMPARISON OF IC50 VALUES FOR DIFFERENT ELISA METHODS

Indirect competitive ELISA method		Direct competitive ELISA method	
OVA-hapten	IC50 (ng*mL ⁻¹)	HRP-hapten	IC50 (ng*mL ⁻¹)
OVA-B1	22	HRP-B1	/ ^a
OVA-B2	34	HRP-B2	20
OVA-B3	36	HRP-B3	17
OVA-B4	73	HRP-B4	2.5
OVA-B5	88	HRP-B5	10
^a Not detected			

Standard direct competitive inhibition curve in Fig. 5, the detection conditions of 25% DMSO, the linear range

of detection (IC₂₀-IC₈₀) was 0.3-6.5 ng/ mL, and correspondingly, IC₅₀ = 1.4±0.05 ng /mL, while the minimum IC₁₀ detection limit of 0.1 ng/mL (n=6).

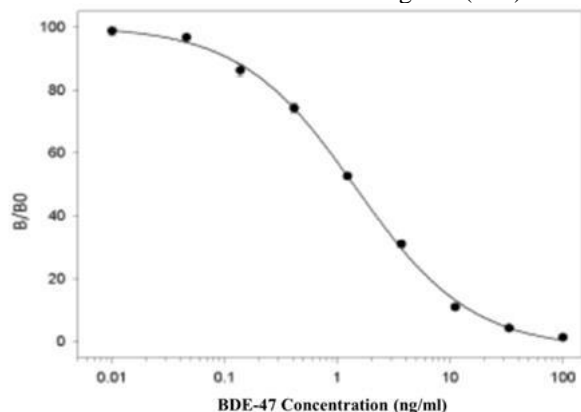


Figure 5. BDE47 monoclonal antibody standard inhibition curve

Antibody specificity:

Cross reactivity values of BDE47 monoclonal antibodies with other molecules were shown in Table VI.

The cross reactivity values of 5-MeO-BDE-47, BDE-99, BDE-100, 5-OH-BDE-47 and BDE-49 with BDE47 monoclonal antibodies were 5.8% and 2.4%, and for the other compounds were less than 0.1%.

C. Immunoassay Analysis of Ketelethrin

Synthesis of (±)-(cis)-3-[(Z)-3-chloro-4, 4, 4-trifluoro-1-ethyl-2, 2-YI] cyclopropylcarbonyloxy] phenoxy] benzene -propanoic acid:

Synthesis of 4-hydroxy phenyl methyl propionate (Cwb-1):

TABLE VI. CROSS REACTIVITY BETWEEN MONOCLONAL ANTIBODY 4F2 AND STRUCTURAL ANALOGUES

Compounds	Cross-reactivity (%)
BDE-15	<0.1
BDE-28	0.4
BDE-47	100
BDE-49	2.8
BDE-99	3.5
BDE-100	2.4
BDE-153	<0.1
BDE-154	<0.1
BDE-183	<0.1
BDE-209	<0.1
5-OH-BDE-47	3.3
5-MeO-BDE-47	5.8

Into a 250 mL three-mouth reactor mixed 14.4g p-hydroxyphenylethyl with 27mL propionic acid, 27mL benzyl alcohol, 15mL toluene, and 4 drops of 85% H₃PO₄ with reflux of water for 10h. After reaction, distillation was used to remove excessive toluene using vacuum distillation and therefore benzyl alcohol was derived in distillate product. The residue was then dissolved with saturated NaHCO₃ solution, and washed three times. Anhydrous MgSO₄ dry steam was introduced to bottom products. 234-236 4mm C (Hg-C) fractions were collected, with the colorless 16.1g 4-hydroxy benzyl propionate mucus at a yield of 72.5%.

Synthesis of 1- bromine -3- (two methyl) benzene (methyl) benzene (Cwb-2):

18.5g 3-bromobenzaldehyde, 12.2g trimethyl orthoformate, 15mL methanol and 2 drops of concentrated H₂SO₄ were fed into 250mL three-mouth reactor, stirring at room temperature for 3h. The mixture was dissolved in saturated Na₂CO₃ solution, and washed three times. Anhydrous MgSO₄ dry steam was introduced to bottom product. 102-104 6mm C (Hg-C) fraction, and 13.5g colorless 1- bromine -3- (two methyl) benzene liquid product was collected, at a yield of 57.9%.

Synthesis of 4- (3-) - formaldehyde () - benzene methyl propionate (Cwb-3):

Into a 100mL three-mouth reactor, 5.0g 4-hydroxy phenyl methyl and 30mL anhydrous DFM was placed in an ice bath to 5 °C, with addition of 1.0g NaH (50%), and after the bath, 2.7g 1-3-bromide (two methyl) benzene, 1.8g Cu₂Cl₂, 0.9g copper, and 4.8 mL pyridine were added. The reaction lasted for 8h with anti-flow reflux. The solution was cooled, diluted with 150mL ethyl acetate, and washed three times with 10% HCl. Then the solution was washed with 10mL 10% HCl three times. After drying, stripping and column chromatography, 1.27g 4-(3-formaldehyde phenoxy) phenyl methyl was collected, at a yield of 30.2%.

Preparation of polyclonal antibody:

The active ester method of hapten was coupled with bovine serum albumin conjugates, 2mg dissolved in 1mL normal saline, and 1mL mixture of complete Freund's adjuvant, injection of emulsified rabbit thigh, after every other week second immunization was done with incomplete Freund's adjuvant and immunogen mixture. The immune site was subcutaneous from the beginning of the third immunization after each week from serum titer of rabbit ear blood sample. A total of 5 times of immunization, a week after the last immunization from the rabbit carotid artery by blood, with 35% saturated ammonium sulfate salting out crude rabbit antiserum, and finally DE-52 was further purified by anion exchange chromatography, pure cyhalothrin polyclonal antibody was obtained.

Direct competitive ELISA method:

The samples were treated with methanol containing 50% PBS. The preparation of 0 ng/mL, 5 ng/mL, 10 ng/mL, 100 ng/mL, 1,000 ng/mL, 10,000 cyhalothrin standard solution of ng/mL, adding 50 L standard sample and filled each sample to each hole respectively, the standard sample with 2 to 4 repetitions, adding 50 μL antibody dilution, at 37°C for 30 minutes; then washed with diluted PBST 2 to 6 times, the ELISA plate was put upside down in the shoot dry absorbent paper; with 1:1,000 dilution ratio of enzyme, labeled Goat anti rabbit two - 100μL, incubated at 37°C for 30 minutes; the liquid pouring hole, washed with PBST in 2 ~ 6 times, pat dry; solution A and solution B were mixed together, with each hole plus 100μL, in the dark color of 10 ~ 15 minutes, in each hole reaction was stopped by adding 50μL to the mixture, at the wavelength of 450nm standard OD enzyme.

The OD value of standard solution containing 0 holes with the maximum OD value minus the concentration of standard solution hole is B0, while the OD value of the remaining holes is B; the B/B0 value for the vertical axis, the corresponding standard concentration log value as the abscissa and the drawing of Cyhalothrin standard inhibition curves (Fig. 6). Among them, the curve regression equation is $Y = -0.448X + 1.201$, $R^2 = 0.993$, when $IC_{50} = 37$ in the inhibition of $\mu\text{g/L}$, the minimum detection limit of $IC_{10} = 4.7 \mu\text{g/L}$.

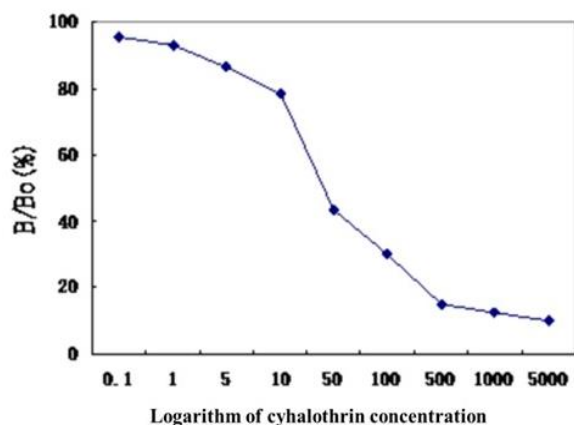


Figure 6. Standard curve of cyhalothrin

Kit specific experiments:

The commonly-used type II pyrethroid fenvalerate, fenpropathrin, deltamethrin, fluvalinate and cypermethrin (fluvalinate) (cypermethrin) were the objects to be measured, the concentration of inhibition of various substances in (IC_{50}) was measured.

Cross reaction (CR%) = IC_{50} (cyhalothrin) / IC_{50} (for test) * 100%.

Experimental results were shown in Table VII. Using the indirect ELISA method, the polyclonal antibody cross reactivity of type II pyrethroids was less than 3%, indicating that the specificity of the kit could guarantee the reliability of the results of Cypermethrin residues in samples.

TABLE VII. CROSS REACTIVITY VALUES OF ANALYTES

Target analytes	Structure	Cross reactivity (%)
fenvalerate		NI
fenpropathrin		< 0.05
deltamethrin		NI
fluvalinate		NI
cypermethrin		2.5
cyhalothrin		100
NI: when the measured substance concentration is 10mg/L, the inhibition ratio is less than 10%.		

D. Immunoassay Analysis of Fenvalerate

Synthesis of semi-antigens:

(1) Synthesis of 3-[(+)-(CIS) phenyl] methyl chloride-2-[3-3-D carbonyl-oxy] phenoxy] phenyl propionic acid and 1-bromo-3- (dimethoxymethyl) benzene:

18.5g (0.1 mol) of 3-bromobenzaldehyde was placed in a 100 mL three-necked flask, 5 mL of methanol was added, and the mixture was stirred at room temperature for about 2 minutes. Then, 12.2 g (0.118 mol) of trimethyl orthoformate and 10 mL methanol were added into the mixed solution, fully stirred after 2 drops of concentrated sulfuric acid as slightly exothermic. Stirring was carried out at room temperature, and TLC monitored the reaction progress. After the completion of the reaction, the reaction system was diluted with 40 mL of ether. The organic phase was washed with 10 mL of 5% aqueous Na_2CO_3 solution. After shaken, a large amount of white solid precipitated and the interface between the organic and aqueous phases was not clear. 30 mL of deionized water was used to wash the organic phase and then with 10 mL of deionized water. The organic phase was ultimately separated and dried over anhydrous MgSO_4 . The solution was distilled under reduced pressure, and fractions of 99 to 101 °C were collected at 0.5 mmHg, a colorless viscous liquid, about 16.16 g, with the yield of 70.3%.

Preparation of 4-hydroxy phenyl benzyl propionate:

16.6g (0.3 mol) of benzyl alcohol were placed in a 100mL single-necked flask, 50mL of anhydrous toluene was added thereto, and the mixture was stirred at room temperature. Then, 5 drops of 85% H_3PO_4 was added, and the mixture was heated. The reaction lasted for about 12 hours. At this time, the system was clarified as yellow and about 0.7mL of water was separated. The reaction stopped and was cooled down to room temperature. 50mL of ethyl acetate was added to the reaction system. The organic phase was washed twice with 2 portions of NaHCO_3 aqueous solution and the organic phase was washed three times with 10 mL of deionized water. The organic phase was separated and dried over anhydrous MgSO_4 . The distillate was collected by distillation at a pressure of 1 mmHg at a temperature of 216 to 217 °C, a light yellow viscous liquid 20.68g, with the yield of about 80.7%.

Preparation of 4- (3- two methyl) phenyl methyl benzene:

20.68g (0.08mol) of benzyl 4-hydroxybenzenepropanoate was placed in a 250 mL three-necked flask and dissolved in 120mL of dry DMF. The mixture was stirred with N_2 gas and 3.9g of 50% (Bubble), the system temperature increased by about 10 °C; plus NaH finished, stirring about 20min, then add the NaH into the dark, When the system color turned brown, 18.6g (0.08 mol) of I, and 50 mL of anhydrous pyridine were added to the flask, and then 0.87g of CuCl and 0.44g of Cu dust were added under stirring, at which time the system immediately turned dark brown; After the completion of heating to reflux, TLC monitoring reaction progress; to be complete reaction of raw

materials, stop heating, the system returned to room temperature; with 1500mL ethyl acetate dilution reaction system, then immediately a large number of brown gel insoluble precipitation, The filtrate was separated, and the insoluble matter was washed with ethyl acetate. The washings and the filtrate were combined to prepare an ethyl acetate solution of benzyl 4- (3-dimethoxymethyl) phenoxy-benzene-propanoate.

Preparation of polyclonal antibody:

Conjugated with bovine serum albumin by the active ester method. 2mg conjugate was dissolved in 1mL normal saline and mixed with 1 mL complete Freund's adjuvant. After fully emulsified, the rabbits were injected with thighs of New Zealand white rabbits. Two weeks to strengthen the immune time, for incomplete Freund's adjuvant and immunogen mixture, the immune site for the back of the neck subcutaneous, starting from the third immunization, each week after immunization from the rabbit ear vein blood serum titer. The whole blood was collected from rabbit carotid arteries one week after the last immunization. Rabbit antiserum was extracted by 35% saturated ammonium sulfate salting out, and then purified by DE-52 anion exchange chromatography to obtain pure fenvalerate polyclonal antibodies.

Direct competitive ELISA method:

After the samples were pretreated, the samples were stored in 50% methanol in PBS. Remove the vacuum bag and remove the plate, the balance at room temperature for 5 minutes standby. L, 0.5 μ g / L, 5 μ g / L, 50 μ g / L, 100 μ g / L and 200 μ g / L of fenvalerate standard solution were prepared by adding 50 μ L standard sample or treated sample to each well, The standard sample and the sample to do 2 to 4 repeat, add 50 μ L diluted antibody, 37 ° C incubation for 30 minutes; poured out the hole in the liquid, diluted with PBST wash 2 to 6 times, the ELISA plate inverted in water Add 1: 1000 dilution of a good enzyme-labeled goat anti-rabbit secondary antibody 100 μ L, 37 ° C incubation for 30 minutes; poured out the hole in the liquid, wash plate with PBST 2 to 6 times, pat dry; take A solution And B volume of the same volume, add 100 μ L per well, in the dark color 10 to 15 minutes, each well by adding 50 μ L stop solution to terminate the reaction, the microplate reader was measured at the wavelength of 450 nm OD value.

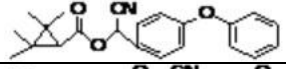
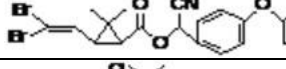
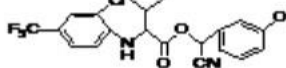
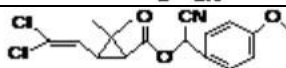
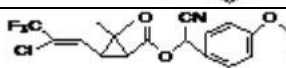
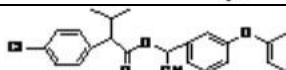
The OD value of the standard hole containing 0 minus the standard solution containing the maximum concentration of OD value as B0, the rest of the hole after the same method of correction of the OD value as B; B / B0 value of the ordinate, the corresponding standard The concentration of log value for the abscissa, draw the standard inhibition curve of fenvalerate. The regression equation was $y = -0.30x + 1.71$, $R^2 = 0.98$, the inhibition concentration was $IC_{50} = 10.7\mu\text{g} / \text{L}$, and the detection limit was 1.0 $\mu\text{g} / \text{L}$.

Kit specific experiment:

Fenpropathrin, deltamethrin, fluvalinate, cypermethrin and cyhalothrin were selected as the commonly used type II pyrethrin pesticides. IC_{50} of various substances were measured, and the cross-reactivity of the antibody to these substances was calculated by the following formula.

$$\text{Cross reaction (CR\%)} = \frac{IC_{50}(\text{beta})}{IC_{50}(\text{donor})} * 100\%.$$

TABLE VIII. CROSS REACTIVITY OF THE TARGET ANALYTES

Target analytes	Structure	Cross-reactivity (%)
fenpropathrin		< 0.1
deltamethrin		2.5
fluvalinate		NI
cypermethrin		2.5
cyhalothrin		NI
fenvalerate		100
NI: when the measured substance concentration is 10mg/L, the inhibition rate is less than 10%.		

The smaller the cross-reactivity, the stronger the specificity of the antibody to fenvalerate, and vice versa was poor. Experimental results shown in Table VIII, using the indirect ELISA method, the polyclonal antibody cross reactivity of type II pyrethroids was less than 3%, indicating the specificity of the kit could guarantee the reliability of the results of fenvalerate residues in samples.

The molecular structure model was simulated by computer technology to obtain the compound root mean square deviation, plane angle and molecular size information, design and screening of hapten. According to the structural characteristics of various compounds, the following major haptens, haptens and intermediates were identified by mass spectrometry, infrared spectroscopy and nuclear magnetic resonance. PBDEs; imidacloprid (1- (6-chloropyridine-3-pyridylmethyl) -N (3-pyridylmethoxy) benzoic acid; YI) amine) and thiamethoxam (3- (2-chloro-1, 3-thiazol-5-ylmethyl) -5-methyl-1,3,5-oxadiazol- (Nitro) amine), were the four kinds of hapten.

The hapten was conjugated to the carrier proteins BSA and OVA, using the mixed anhydride method and the active ester method. The immunogen was mixed with Freund's complete adjuvant (primary immunization) or incomplete Freund's adjuvant (booster immunization) to immunize New Zealand white rabbits and rabbits, and then polyclonal antiserum was prepared. Saturated ammonium sulfate and protein-A affinity chromatography column purification of antibodies. The polyclonal antibody, fenvalerate polyclonal antibody, imidacloprid monoclonal antibody and polybrominated diphenyl ether monoclonal antibody were prepared.

Factors affecting the results of the assay included pH of the buffer, ionic strength, and organic solvent. By optimizing these conditions, the sensitivity of the assay can be improved. The optimal conditions for the imidacloprid were as follows: PBS buffer pH = 7.4, NaCl concentration 8.0g/L, methanol content 5%. The optimal

detection conditions of PBDE were PBS buffer pH of 7.4, NaCl concentration 0.137mol / L and methanol 40%. The optimal conditions for the detection of the crude drug were PBS buffer pH of 7.4, NaCl concentration 5% and methanol content 50%. The optimal conditions for the determination of fenvalerate were: PBS buffer pH = 7.4, NaCl concentration 5%, methanol content 50%.

4 The average recoveries of imidacloprid in rice and rice samples were 74.6% -86.3% and 72.5%~ 93.6% respectively, compared with that of high performance liquid chromatography (HPLC). The results showed that the ELISA method was highly consistent with the high-performance GC method.

In comparison with gas chromatography-ion trap-mass spectrometry (GC-IT-MS), polybrominated diphenyl ethers (PBDEs) in rice were analyzed by competitive, noncompetitive methods and GC / ITMS, respectively, with the range of 2.3 to 58 ng g⁻¹, 2.1 to 64 ng g⁻¹, 2.9 to 52 ng g⁻¹, respectively. The polybrominated diphenyl ethers in corn were analyzed by competitive, noncompetitive and GC / ITMS were detected in the range of 1.8 ~ 28 ng g⁻¹, 2.2 ~ 32 ng g⁻¹ and 1.5 ~ 27 ng g⁻¹, respectively. Immunoassay and GC / ITMS correlation is good.

In comparison with GC method, in the immunoassay of rice, rice and maize, the contents of rice, rice and corn in the samples of rice, corn and corn were tested. The average recovery of ester was between 90% ~ 112%, 81% ~ 106% and 78% ~ 99.8%, respectively. The correlation analysis showed that the recovery of ELISA using the concentration of the actual concentration or the concentration of the instrument were highly correlated, indicating the use of ELISA residue analysis of permethrin was feasible.

V. CONCLUSIONS

Pesticide residue is the main pollutant of food pollution in Southern China area. The research and application of phage display technology was designed to obtain short peptide binding with imidacloprid, PBDEs, cyhalothrin, fenvalerate specific screening, so as to establish the corresponding phage immune analysis method. At the same time, the establishment of a conventional ELISA method of imidacloprid, PBDEs, cyhalothrin, fenvalerate monoclonal antibody based on the synthesis of hapten antibody preparation and purification, has shown relative sensitivity. ELISA kit detection sample analysis and monitoring analysis provide a scientific basis for safe grain storage in Southern China area. The main results are as follows:

(1) The design and screening analysis of 4 semi-antigens, hapten and carrier along with 4 kinds of antibody, were positively corresponding to the rapid detection of Cyhalothrin, fenvalerate, imidacloprid and PBDEs with detection reagent.

(2) Through the immune analysis of imidacloprid, PBDEs, cyhalothrin compared with gas chromatography (GC) of rice, grain and corn samples, we can see that the concentration was determined by the recovery of ELISA

concentration and the actual concentration of instruments were highly relevant using ELISA.

(3) Imidacloprid, PBDEs, cyhalothrin, fenvalerate range detection limit of detection antibody (IC₁₀) were 1.8ng/g, 0.1ng/g, 4.7ng/g, 1.0ng/g. Based on the results, the four kits were basically included in the range of 10-1000ppb. Outside this range, the optimum range of detection could be found by dilution of the sample.

(4) Each kit could detect 30-40 samples at the same time, while the detection time was about 1.5-2 hours, so the average detection time of each sample was less than 10 minutes; test strip detection sampling time was 10-15min. After application, the test cost of each sample could be 5-6 minutes.

(5) ELISA technology has been applied and developed in the detection of pesticide residues in food. Chemical immunoassay technology has strong specificity, high sensitivity, fast and convenient analysis of large capacity. Multiple samples could be detected at the same time, which resulted in lower cost and lower level of equipment requirements. In the complex samples via ultra-trace analysis and on-site rapid detection of pesticide residues, detection method has received steady improvement, especially high in affinity and antibody specificity, and has a broader application prospect and great potential.

ACKNOWLEDGEMENT

The authors wish to thank the collaborations of major projects (Item number: 2014Y2-00520, Guangzhou [2014] No. 351) and Guangdong Province public projects (Item number: 2014B070706001, Guangdong financial planning [2015] No.72) for fund support.

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