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Development of a Monoclonal Antibody-Based ELISA for the Detection of the Novel Insecticide Cyantraniliprole

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ABSTRACT

Cyantraniliprole is the newest anthranilic diamide insecticide acting on ryanodine receptors with higher efficacy, broader spectrum than chlorantraniliprole on pest control. To study effects of its residues on the environment and food samples, an enzyme-linked immunosorbent assay (ELISA) has been developed. The hapten was synthesized and conjugated with bovine serum albumin (BSA) and ovalbumin (OVA) as the immunogen and coating antigen, respectively. A sensitive and specific monoclonal antibody (mAb) was generated and was designated as mAb3B$_2$. mAb3B$_2$ was used to develop an indirect competitive ELISA (icELISA). The concentration of cyantraniliprole producing 50% inhibition (IC_{50}) was 1.57 μg L^{-1} and the effective range of icELISA was 0.43-6.15 μg L^{-1}. The icELISA showed low cross-reactivity with chlorantraniliprole, and very low or no cross-reactivity with the other cyantraniliprole analogues. Average recoveries from pakchoi (*Brassica rapa*) and tap water samples were 93.7-101.0% and 94.4%-100.6%, respectively. Meanwhile, to confirm the result of icELISA, high-performance liquid chromatography was applied for the determination of cyantraniliprole residues in pakchoi samples. The comparable results suggest that the ELISA is suitable for rapid detection of cyantraniliprole residues in environmental and agricultural samples.

KEYWORDS: monoclonal antibody, cyantraniliprole, ELISA, insecticide.

1. Introduction

The ongoing discovery and development of insecticides with novel mode of action is vital for ensuring the sustainability of crop protection strategies[1-2]. Compounds having diamide skeleton structure like insecticides chlorantraniliprole and flubendiamide could bind to the ryanodine receptors of insect pests and became a new class of insecticide. Chlorantaniliprole has been extensively employed to control *lepidoptera* pests on crops due to its excellent efficacy, low toxicity and unique action mode. However, the narrow control spectrum and no systemic character of chlorantaniliprole limit its application at some extent. To solve these two issues, DuPont discovered cyantraniliprole through replacing chlorine atom with cyano group on the benzene ring in chlorantraniliprole[3-5]. Cyantraniliprole[a], as the newest anthranilic diamide insecticide, also can activate the ryanodine receptors of insect pests, and cause calcium ions to pass through the muscle cells rapidly, resulting in feeding cessation, muscle paralysis, lethargy and regurgitation like chlorantraniliprole[6-7]. It could be against many important agricultural pests, such as Coleoptera, Diptera, Isoptera, and Hemiptera with low mammalian toxicity. Thus, cyantraniliprole can be applied on more crops to control pests in comparison with chlorantraniliprole and had the potential to reduce transmission of tomato spotted wilt virus[6].

Cyantraniliprole can be degraded easily and the final residual levels in pakchoi and soil were low[8]. Even though it is environmentally friendly, some studies reported that it still has some harmful effects, especially in agricultural system[9-10]. Furthermore, possible improper use during a harvest period could result in a level of residues being harmful to humans. Misuses could lead the rapid development of pest resistance. The levels of cyantraniliprole residues in food or the environment are, therefore, strictly regulated by regulatory authorities. The Pest Management Regulatory Agency (PMRA) prescribed 0.01 μg g^{-1} as the maximum

[a] *3-bromo-1-(3-chloro-2-pyridinyl)-N-[4-cyano-2-methyl-6-[(methylamino) carbonyl]phenyl]-1H-pyrazole-5-carboxamide*

concentration of cyantraniliprole residues in meat, egg and dairy products, and 30 μg g^{-1} on leafy brassica vegetables[11]. Such low maximum residue limits bring challenges to the inspection and monitoring. Therefore, it is indispensable to establish a quick determination method to monitor its residues and degradation rate in food and the environment. Analytical methods based on high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) methods have been established to measure the residue level[7-8], by which the limit of quantitation (LOQ) was 0.01 μg g^{-1} in four matrix groups of oily, watery, acidic, and dry crop[7]. The chromatographic and spectrophotometric methods show a good sensitivity and accuracy, while they are expensive, time-consuming, and usually need extensive sample preparation[12-13]. Therefore, it is of great significance to propose an efficient and cost-effective method to inspect cyantraniliprole residues in food and the environment. Immunoassays, as an alternative and complementary method for the analysis of agrochemicals[15-17], are based on the interaction of an analyte with an antibody that recognizes the analyte with high affinity and specificity[14]. Enzyme-linked immunosorbent assay (ELISA) is an immunoassay and has been extensively applied in the detection of environmental contaminants[12,18-20]. Compared to conventional instrumental methods, immunoassay is a rapid and sensitive method which needs small quantities of test materials and simple pretreatments. These features convert ELISA into very powerful tools for such pesticides that are difficult or costly to determine due to their physicochemical characteristics and for large monitoring programs[14].

To our knowledge, there is no report of monoclonal antibody (mAb)-based ELISA to monitor residual cyantraniliprole in the environment and food samples. In the present study, a

cyantraniliprole hapten was synthesized and a sensitive and selective indirect competitive ELISA was developed for monitoring cyantraniliprole degradation and residues.

2. MATERIALS AND METHODS

2.1. Materials

Cyantraniliprole standard (99.0% purity) and cyantraniliprole analogues were provided by the College of Science, China Agricultural University, Beijing, China. Formulation of cyantraniliprole (suspension concentrate SC, 2.5 %) was purchased from Shanghai DuPont (China). Reagents purchased from Sigma-Aldrich (St. Louis, MO) included 1-(3-dimethylaminepropyl)-3-ethylcarbodiimide (EDC), succinic anhydride, 4-dimethylamino-pyridine (DMAP), polyethylene glycol 2000, dimethyl sulfoxide (DMSO), hypoxanthine, aminopterin, and thymidine (HAT), penicillin, streptomycin, L-glutamine, complete and incomplete Freund's adjuvant, BSA, OVA, o-phenylenediamine (OPD), polyoxyethylene sorbitan monolaurate (Tween-20), N-hydroxysuccinimide (NHS), N,N-dimethylformamide (DMF), and N-iodosuccinimide (NIS). Goat anti-mouse IgG conjugated with horseradish peroxidase (IgG-HRP) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Cell culture medium (Dulbecco's modified Eagle's medium) and fetal bovine serum (FBS) were obtained from Gibco BRL (PaisLey, Scotland). Acetonitrile in chromatography grade was purchased from Fisher Scientific (New Jersey, USA). All other reagents were obtained from Beijing Chemical Reagents Co. (Beijing, China).

2.2. Apparatus

Cell culture plates and 96-well polystyrene microplates were purchased from Costar

(Corning, NY). The electric heating constant-temperature incubator was purchased from Shanghai Zhicheng Analytical Instrument Manufacturing Co. Ltd. (Shanghai, China). The automated plate washer (Wellwash 4 MK2), microplate reader (Multiskan MK3) and direct heat CO_2 incubator (Thermo, 311) were purchased from Thermo (Vantaa, Finland). The Agilent 1200 HPLC system and 5 μm × 4.6 mm × 150 mm Betasil C_{18} column (Agilent, USA) were used for pesticide analysis. The 0.22 μm syringe filters were purchased from Pall (Ann Arbor, MI).

2.3. Buffers and solutions

The buffers and solutions included coating buffer (0.05 M carbonate buffer, pH 9.6), phosphate-buffered saline (PBS) (0.1 M phosphate buffer containing 0.9% sodium chloride, pH 7.5), PBS with 0.1% (v/v) Tween-20 (PBST), PBST containing 0.5% (w/v) gelatin (PBSTG), citrate-phosphate buffer (0.01 M citric acid and 0.03 M monosodium phosphate, pH 5.5), substrate solution (4 μL of 30% H_2O_2 added to 10 mL of citrate-phosphate buffer containing 2 mg mL^{-1} OPD), a stop solution (2.0 M sulfuric acid) and extracting solution (0.1g butylated hydroxyltoluene added to the mixture of 100 mL distillated water and 400 mL absolute methanol). All reagents and solvents were analytical-grade. Deionized water used for making buffers and solutions was collected from a Millipore Water Purification System (Millipore Co., Billerica, MA).

2.4. Myeloma cell line and medium

The HAT-sensitive Balb/c mouse myeloma cell line SP2/0-Ag14 obtained from the China Institute of Veterinary Drug Control (Beijing, China) was used in fusion experiments. DMEM containing 10-20% (v/v) FBS was supplemented with 0.2 M glutamine, 50,000 U L^{-1}

penicillin, and 50 mg L^{-1} streptomycin, which was used for cultivating the myeloma and hybridoma cells.

2.5. Hapten synthesis

Hapten was synthesized[24-28] according to the synthetic route illustrated in Figure 1.

*Synthesis of 2-amino-5-iodine-3-methyl benzoic acid (**F_2**).*

2-Amino-3-methyl benzoic acid (**F_1**, 200 g, 1.32 mol) was dissolved in 700 mL of DMF in a 2 L flask equipped with a mechanical stirring, and then the solution was heated to 68 °C. *N*-Iodosuccinimide (NIS, 300 g, 1.33 mol) were slowly added into the flask, and then stirred at 75 °C for 3 h. The reaction mixture was slowly poured into 1 L of ice water and was stirred for 3 h. **F_2** as a gray solid (354 g, 72%) was obtained after filtration and drying.

δ_H (300 MHz, DMSO): 2.08 (3H, s, C<u>H</u>$_3$), 7.44 (1H, t, J = 1.1 Hz, Ar-<u>H</u>), 7.87 (1H, d, J = 2.2 Hz, Ar-<u>H</u>).

*Synthesis of 2-amino-5-cyano-3-methyl benzoic acid (**F_3**).*

F_2 (138.5 g, 0.5 mol) and cuprous cyanide (58.2 g, 0.65 mol) were added into 1 L of DMF in a 2 L flask, and the mixture was heated to at 145 °C and maintained for 6 h in an oil bath. After the reaction completed, most of the solvent was removed through reduced pressure distillation, and then the residue was treated with 1 L of water and 50 mL of ethylenediamine. The mixture was filtered, and the filtrate was acidified to pH 5 with 2 M HCl and stirred overnight. **F_3** as a gray solid (78.4 g, 89%) was obtained by filtration and drying.

δ_H (300 MHz, DMSO): 2.13 (3H, s, C<u>H</u>$_3$), 7.34 (2H, br, N<u>H</u>$_2$), 7.75 (1H, m, Ar-<u>H</u>), 7.97 (1H, t, J = 1.06 Hz, Ar-<u>H</u>), 13.10 (1H, br, COO<u>H</u>).

*Synthesis of 8-methyl-2,4-dioxo-2,4-dihydro-1H-benzo[d][1,3]oxazine-6-carbonitrile (**F_4**).*

F_3 (38 g, 0.22 mol) was dissolved in 400 mL of 1,4-dioxane in a 1 L flask, and heated to 102 °C with an oil bath. A solution of triphosgene (22.7 g, 0.075 mol) in 50 mL of dioxane was slowly added into the flask, and the mixture was kept at 102 °C for 4 h after addition. When reaction was completed, the mixture was cooled down to room temperature. A white solid **F_4** (37.8 g, 85%) was obtained after filtration and drying.

δ_H (300 MHz, DMSO): 2.35 (3H, s, C<u>H</u>$_3$), 8.00 (1H, t, J = 0.91 Hz, Ar-<u>H</u>), 8.21 (1H, d, J = 1.89 Hz, Ar-<u>H</u>), 11.46 (1H, s, N<u>H</u>).

Synthesis of 2-(3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl)-8-methyl-4-oxo-4H-benzo[d][1,3]oxazine-6-carbonitrile (F5).

A mixture of 3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazole-5-carbonyl chloride (0.64 g, 0.02 mol) and **F_4** (2.05 g, 0.1 mol) was dissolved in 20 mL of anhydrous pyridine in a 100 mL flask, then heated to 115 °C slowly with an oil bath and kept for 40 min. The reaction mixture was cooled down to room temperature, then was acidified to pH 5 with 2 M HCl, and collected precipitate through filtration. The obtained precipitate was recrystallized in mixed ethyl acetate and petroleum ether (1:3, v/v) to get a white solid as **F_5** (7.7 g, 87%).

δ_H (300 MHz, $CDCl_3$): 1.86 (3H, s, C<u>H</u>$_3$), 7.31 (1H, s, Ar-<u>H</u>), 7.75-7.97 (3H, m, Ar-<u>H</u>), 8.3-8.5 (2H, m, Ar-<u>H</u>).

Synthesis of 2-(3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazole-5-carboxamido)-5-cyano-3-methylbenzamido) butanoic acid (cyantraniliprole hapten).

F_5 (132 mg, 0.3 mmol), 4-aminobutyric acid (34 mg, 0.33 mmol)), and sodium

hydroxide (24.6 mg, 0.61 mmol) were added in 7 mL of DMF. The mixture was stirred at room temperature for 12 h. The reaction mixture was poured into 15 mL of water, acidified to pH 3 with 2 M HCl, and then extracted with ethyl acetate (5 mL × 3). The combined organic layers were washed with water (5 mL × 3), dried with anhydrous sodium sulfate, filtered and concentrated. The residue was recrystallized with ethyl acetate and petroleum ether to afford the cyantraniliprole hapten (**F_6**) as a white solid (129 mg, 79%).

δ_H (300 MHz, DMSO): 1.64-1.68 (2H, m, $CH_2CH_2CH_2$), 2.21-2.28 (5H, m, CH_2 and CH_3), 3.12-3.18 (2H, m, $NHCH_2$), 7.41 (1H, s, Py-H), 7.58-7.62 (1H, m, $CONHCH_2$), 7.78 (1H, d, J = 1.8 Hz, Ar-H), 7.88 (1H, d, J = 1.8 Hz, Ar-H), 8.14-8.17 (1H, m, Py-H), 8.42-8.46 (1H, m, Py-H), 8.48-8.50 (1H, m, Py-H), 10.52 (1H, br, s, NHCO), 12.03 (1H, br, s, COOH)

δ_C (75 MHz, DMSO): 17.82, 24.33, 31.27, 38.75, 109.39, 111.01, 118.20, 126.73, 126.96, 127.98, 129.80, 135.02, 135.30, 137.32, 137.88, 139.21, 139.36, 147.22, 148.45, 155.44, 165.44, 174.35.

HRMS (ESI): calc. $C_{22}H_{17}BrClN_6O_4$ $[M-H]^-$ for 543.0189, found 543.1047.

2.6. Preparation of cyantraniliprole-protein conjugates

The hapten was conjugated with BSA and OVA according to the carbodiimide method to produce immunogen (cyantraniliprole-BSA) and coating antigen (cyantraniliprole-OVA)[13]. The mixture was dialyzed against 3 L of PBS for 3 days with three changes per day, then lyophilized and stored at -20 °C. The structure of hapten conjugated to proteins was spectrophotometrically confirmed.

2.7. Immunization protocol, monoclonal antibody production, purification

Animal experiments were conducted under principles in good laboratory animal care, and approved by ethical committee for Laboratory Animals Care and Use of China Agricultural University. Six female Balb/c mice, 7 weeks old, were immunized with the immunogen cyantraniliprole-BSA. The protocols of immunization, fusion, antibody production, purification were as previously described[12].

mAbs were screened and identified as following:

Three days after the last injection of a mixture of immunogen and adjuvant, the mouse with the highest titer and best specificity was boosted intraperitoneally with 0.1 mg cyantraniliprole-BSA conjugate in 0.1 mL PBS and was used for the fusion. The spleen cells collected from the mouse were fused with the SP2/0 (obtained from China Institute of Veterinary Drug Control, Beijing, China) cell line using PEG-2000 at a ratio of 10:1 of spleen to myeloma cells. The plates were incubated at 37 °C in a CO_2 incubator (5% CO_2 in air). Selective growth of the hybrid cells in the DMEM supplemented with HAT. Seven days after fusion, the supernatant was tested by icELISA. Positive hybridomas were cloned by limiting dilution and clones were further selected by icELISA. The clone, designated as $3B_2$, having a high antibody titer and good sensitivity in the culture supernatant was expanded in mice for production of MAb in ascites. The $3B_2$ hybridoma cell line was archived in the Engineering Research Center of Plant Growth Regulator, China Agricultural University.

2.8. Indirect competitive ELISA

The microplate was coated with 100 μL of 1 mg mL^{-1} of cyantraniliprole-OVA in coating buffer at 37 °C for 3 h. The microplate was washed with PBST three times after coating, 50 μL of standard sample or analytes in PBSTG was added to each microplate well, followed by addition of 50 μL of mAb with a dilution ratio of 1:20000 in PBSTG. After incubated at 37

ºC for 0.5 h, the microplate was washed again with PBST three times, and then an aliquot of 100 μL per well of goat anti-mouse IgG-HRP with a ratio of 1:1000 diluted in PBSTG was added. After incubated at 37 ºC for 0.5 h, the plate was washed again with PBST three times, and then 100 μL per well of substrate solution was added. Finally, the reaction was terminated by adding 50 μL of the stop solution per well, and the absorbance was read with the microplate reader at 492 nm. Data were calculated with OriginPro 8.0 software.

2.9. Specificity of antibody

The specificity of the mAb was evaluated by cross-reactivity (CR) based on mass concentration with a set of structural analogs through icELISA. The relative CR was calculated by the formula: CR (%) = (IC_{50} of cyantraniliprole / IC_{50} of other compounds) × 100.

2.10. Preparation of pakchoi samples

Field trials were carried out in Shangzhuang experimental field of China Agricultural University in 2013. Each experiment field consisted of three replicate plots with an area of 3 m^2 each. Cyantraniliprole (SC, 2.5%) was dissolved in water and sprayed in the growing pakchoi using a knapsack sprayer at recommended dosage (60 g a.i. ha^{-1}) with interval of 7 days between each application for four times. The control was sprayed water without any insecticide. After the application, the pakchoi was sampled on day 7 after spraying, and each pakchoi sample was approximate 200 g. At the same time, 11 pakchoi samples were purchased from different local supermarkets. All collected samples were stored at -40 °C until analysis.

2.11. Preparation and extraction of samples

Chopped and homogenized pakchoi leaves (1 g) were accurately weighed into a 10 mL polytetrafluoroethylene centrifuge tube, followed by addition of 8 mL extracting solution. The mixture was sit at 4 °C overnight, and was centrifuged for 10 min at 8000 rpm. The supernatant was dried by a gentle stream of nitrogen gas to dryness. The residues were dissolved in PBSTG, and were diluted 200 or 500 folds for icELISA. Each analysis was performed in triplicate.

2.12. Sample fortification for recovery tests

One gram of pakchoi leaves was cut into pieces and then spiked with cyantraniliprole at concentrations of 0, 250, 500, 1000, 2000, and 4000 ng g^{-1}. Tap water was spiked at concentrations of 0, 1.25, 2.5, 5, 8, 10 μg L^{-1} cyantraniliprole in spiked samples were analyzed with the established icELISA.

2.13. HPLC analysis

Cyantraniliprole standards and pakchoi samples were analyzed with HPLC according to the procedure described by Schwarz[7] and Sun[8] with slight modification. An isocratic elution was used with 80% aqueous acetonitrile at a flow rate of 1 ml min^{-1}. The retention time of cyantraniliprole was about 5 min. The residue was dissolved in 2.0 mL of acetonitrile, and filtered with a 0.22 μm Millipore membrane. The filtered solution was used for HPLC analysis. The HPLC results were compared with those from icELISA to evaluate the reliability of the established immunoassay.

3. RESULTS AND DISCUSSION

3.1. Hapten design and synthesis, conjugation with proteins

It is known that hapten design plays a critical role on developing antibody for a small molecular weight compound. To obtain a sensitive and selective cyantraniliprole antibody, the aim of the hapten synthesis was to preserve the integrity of the parent structure and to introduce a linker and functional group so that the small molecule hapten could be conjugated with carrier proteins. In order to preserve the structural characteristics of cyantraniliprole at maximum extent, a carboxylic propyl was introduced into cyantraniliprole, and illuminated in synthetic route in Figure 1. The UV–vis spectra were used to monitor the conjugation of haptens and carrier proteins[12-13]. The obtained spectra showed that the haptens were successfully coupled with the carrier proteins. The molar ratios of cyantraniliprole hapten to proteins were estimated to be 15:1 and 8:1 for cyantraniliprole-BSA and cyantraniliprole-OVA, respectively.

3.2. Optimization of icELISA

In icELISA system, an IC_{50} value of the standard curve would be affected by the concentrations of coating antigen and antibody. The optimal concentrations of coating antigen and mAb were screened by checkerboard titration. The coating antigen (1 mg mL^{-1}) and purified $mAb3B_2$ (0.14 mg mL^{-1}) were at a dilution ratio of 1:100000 and 1:20000, respectively. The goat anti-mouse IgG-peroxidase conjugate (1 mg mL^{-1}) was at a dilution ratio of 1:1000. Figure 2 showed a cyantraniliprole standard curve in icELISA. A half maximal inhibition value (IC_{50}) of the assay was 1.57 μg L^{-1}, and a detection range (concentrations giving 80 and 20% B/B_0) was 0.43-6.15 μg L^{-1}.

3.3. Antibody specificity

To evaluate the specificity of mAb3B$_2$, chlorantraniliprole, flubendiamide and other cyantraniliprole analogues were used for testing the cross-reactivity. The results summarized in Table 1 showed their structures, IC_{50} and the cross-reactivity with cyantraniliprole. Flubendiamide, which was co-developed by Nihon Nohyaku and Bayer Crop Science, also acted on insect ryanodine receptors[29]. However, due to distinct differences between the chemical structures of flubendiamide and cyantraniliprole, the developed antibody as expected had no cross-reactivity with flubendiamide. Chlorantraniliprole showed low cross-reactivity with cyantraniliprole (CR, 9.0%), because its structure was very similar to cyantraniliprole. The other chemicals showed very low or no cross-reactivity with cyantraniliprole. The hapten we had designed was very reasonable, and the antibody specificity also ensured effective analysis of cyantraniliprole residues in food and environmental samples.

3.4. Recoveries of cyantraniliprole in pakchoi and tap water samples

The accuracy of an ELISA was commonly assessed by spiking matrix samples with the target analyte, and then evaluating the recovery[30]. The average recoveries of cyantraniliprole with the icELISA were 94-101% in both pakchoi and tap water (Table 2). The good recoveries showed that the established icELISA was a reliable and sensitive method to detect cyantraniliprole in pakchoi and tap water samples.

3.5. Comparison of icELISA and HPLC for analysis of cyantraniliprole in pakchoi samples

To be sure that icELISA was a reliable and sensitive method to detect cyantraniliprole in

pakchoi samples, HPLC had been employed for comparison (Table 3). The correlation coefficient (R^2) between icELISA and HPLC results was 0.997. We found that the residual cyantraniliprole in most of the samples purchased from the local supermarkets were not detectable by icELISA and HPLC. One reason may be that cyantraniliprole was degraded rapidly after application[8], and another reason may be that cyantraniliprole was lately registered in China and was indeed not applied on the samples. The maximum residue limit (MRL) of cyantraniliprole set by PMRA is 30 μg g^{-1} on leafy brassica vegetables and 0.01 μg g^{-1} in foods or fruits[11]. In the practical analyses in the present study, pakchoi samples were diluted by 250 or 500 fold. Therefore, if the residue of cyantraniliprole in leafy brassica vegetable samples reached 30 μg g^{-1} or higher, the samples should be diluted by more than 500 folds, which would have minimal background interference. It is noteworthy that 50 fold or less dilution would be sufficient in analyses of most food and fruit samples to minimize the background interference[31-32]. The limit of detection of the icELISA developed in the present study could reach 0.18 μg L^{-1}, which is 50 fold lower than the MRL of cyantraniliprole set by PMRA. So the sensitivity of the assay was sufficient for screening residual cyantraniliprole in real world samples. Overall, the results demonstrated that the developed icELISA could be used to quickly and reliably monitor cyantraniliprole residues in environmental and agricultural samples.

4. Conclusion

To our knowledge, this is the first paper on mAb-based icELISA for cyantraniliprole. The assay had low cross-reactivity with chlorantraniliprole, and very low or no

cross-reactivity with the other cyantraniliprole analogues. The icELISA using mAb3B$_2$ had an IC_{50} value of 1.57 μg L^{-1} of cyantraniliprole, and a working range of 0.43-6.15 μg L^{-1} based on 20-80% of inhibition. In addition, fortification tests of cyantraniliprole in pakchoi and tap water samples by icELISA had average recoveries of 94-101%. Moreover, analyses of pakchoi samples sprayed with cyantraniliprole and purchased from supermarkets suggested that the result by icELISA agreed well with that by HPLC. Overall, the results displayed that this new immunoassay could be used for the quick and convenient determination of residual cyantraniliprole in real world samples.

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Abbreviations

ELISA, enzyme-linked immunosorbent assay; icELISA, indirect competitive ELISA; HPLC, High performance liquid chromatography; FBS, fetal bovine serum; EDC, 1-(3-dimethylaminepropyl)-3-ethylcarbodiimide; DMAP, 4-dimethylamino-pyridine; DMSO, dimethyl sulfoxide; HAT, hypoxanthine, aminopterin, and thymidine; OPD, o-phenylenediamine; Tween-20, polyoxyethylenesorbitanmonolaurate; NHS, N-hydroxysuccinimide; N-iodosuccinimide (NIS); DMF, N,N-dimethylformamide; NIS, N-iodosuccinimide; IgG-HRP, Goat anti-mouse IgG; PBS, phosphate-buffered saline; PBST,

PBS with 0.1% (v/v) Tween-20; PBSTG, PBST containing 0.5% (w/v) gelatin and CR, cross-reactivity.

Appendix A. Supplementary data

δ_H, δ_C and HRMS spectrums of cyantraniliprole hapten (**F_6**). For review only.

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Figure captions

Figure 1. Route of cyantraniliprole hapten synthesis.

Figure 2. Standard inhibition curve of cyantraniliprole by icELISA. B_0 and B were absorbance in the absence and presence of competitors, respectively. The IC_{50} of icELISA was 1.57 μg L^{-1}. The working range of 20-80% inhibition was 0.43-6.15 μg L^{-1} of cyantraniliprole.

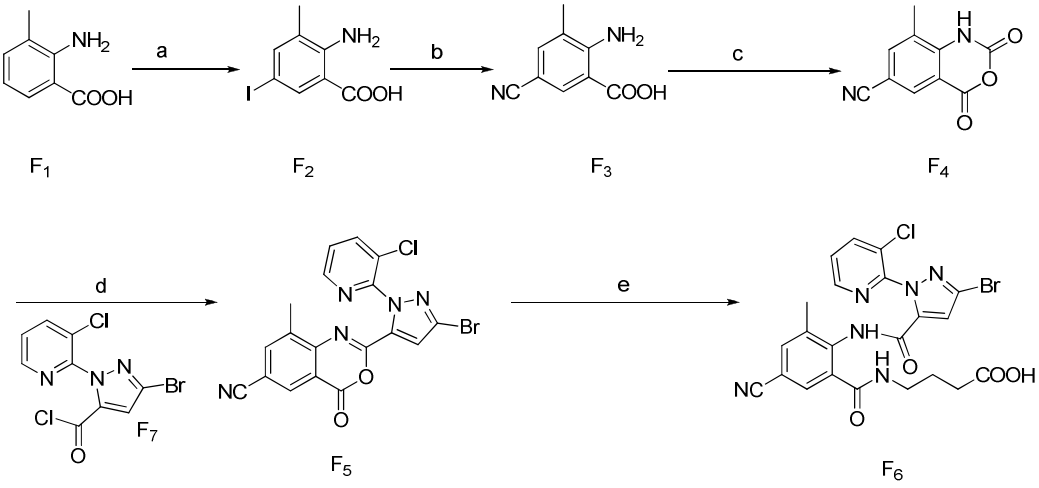


Figure 1. Route of cyantraniliprole hapten synthesis. Reagents and conditions (a) NIS, DMF, 68-75 ℃, 3 h; (b) CuCN, DMF, 145 ℃, 6 h; (c) triphosgene, 1,4-dioxane, 120 ℃, 4 h; (d) pyridine, 115 ℃, 40 min; (e) $NH_2(CH_2)_3COOH$, DMF, r.t, 12 h.

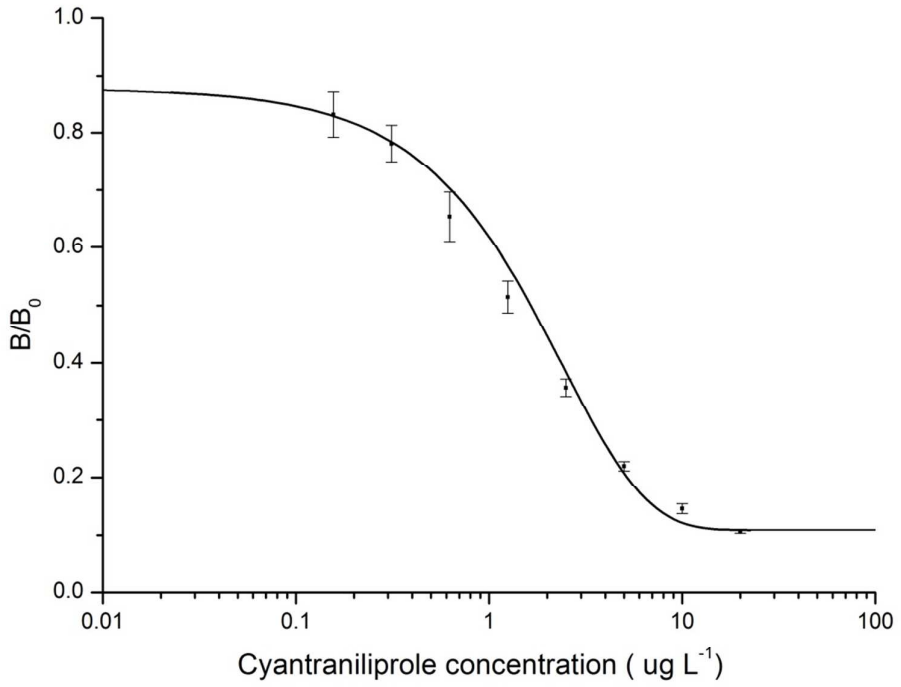
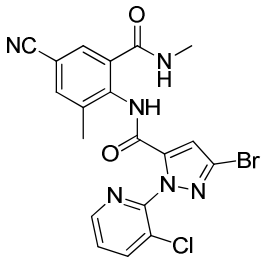
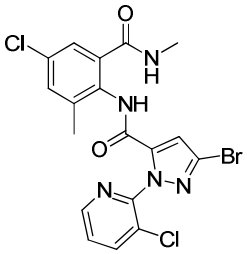
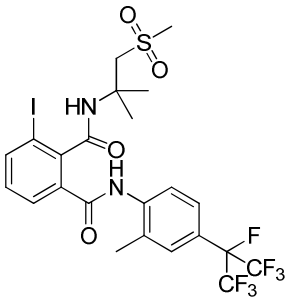
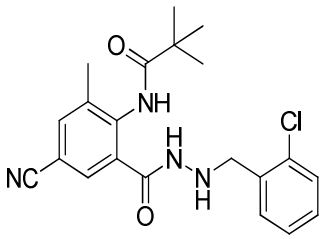
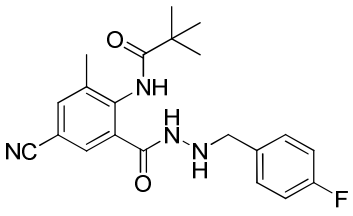
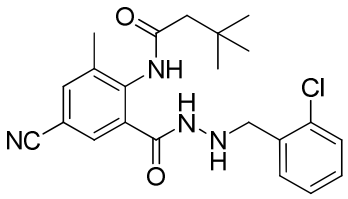
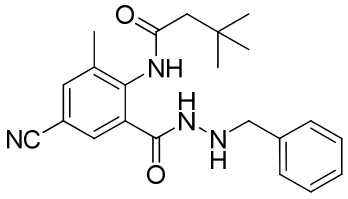
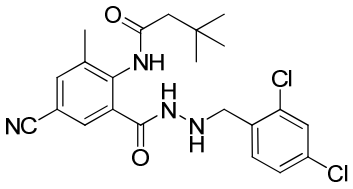
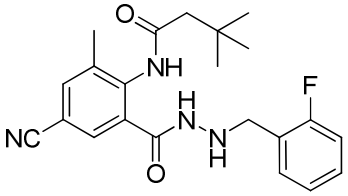
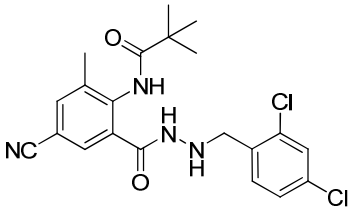


Figure 2. Standard inhibition curve of cyantraniliprole by icELISA. B_0 and B were absorbance in the absence and presence of competitors, respectively. Each point represents the mean of triplicate analyses. Vertical bars indicate ± standard deviations (SD) about the mean.

Table 1. Assay cross-reactivity of cyantraniliprole and its analogs

Chemical names	Structures	IC_{50} μg L^{-1}	CR (%)
3-bromo-1-(3-chloro-2-pyridinyl)-N-(4-cyano-2-methyl-6-((methylamino)carbonyl)phenyl)-1H-pyrazole-5-carboxamide(Cyantraniliprole)		*1.57*	*100*
3-bromo-N-(4-chloro-2-methyl-6-((meth-ylamino) carbonyl)phenyl)-1-(3-chloro-2-pyridinyl)-1H-pyrazole-5-carboxamide (chlorantraniliprole)		*17.3*	*9*
3-iodo-N'-(2-mesyl-1,1-dimethylethyl)-N-{4-(1,2,2,2-tetrafluoro-1-(trifluoromethyl]ethyl)-o-tolyl)} phthalamide **(**flubendiamide**)**		*NI*[a]	*<0.02*
N-(2-(2-(2-chlorobenzyl)hydrazinecarbonyl)-4-cyano-6-methylphenyl)pivalamide		*528*	*0.29*
N-(4-cyano-2-(2-(4-fluorobenzyl)hydrazinecarbonyl)-6-methylphenyl)pivalamide		*6250*	*0.03*

N-(2-(2-(2-chlorobenzyl)hydrazinecarbonyl)-4-cyano-6-methylphenyl)-3,3-dimethylbutanamide		*NI*[a]	*<0.02*
N-(2-(2-benzylhydrazinecarbonyl)-4-cyano-6-methylphenyl)-3,3-dimethylbutanamide		*1840*	*0.09*
N-(4-cyano-2-(2-(2,4-dichlorobenzyl)hydrazinecarbonyl)-6-methylphenyl)-3,3-dimethylbutanamide		*5080*	*0.04*
N-(4-cyano-2-(2-(2-fluorobenzyl)hydrazinecarbonyl)-6-methylphenyl)-3,3-dimethylbutanamide		*1620*	*0.10*
N-(4-cyano-2-(2-(2,4-dichlorobenzyl)hydrazinecarbonyl)-6-methylphenyl)pivalamide		*NI*[a]	*<0.02*

[a]No inhibition was observed up to 10,000 μg L^{-1} of the analytes

Table 2. Recovery of cyantraniliprole spiked to pakchoi samples and tap water

Samples	Concentrations (μg L^{-1})		Recovery (%)± SD[a]
	Spiked	Detected	
pakchoi	0	ND[b]	ND
	250	234.3	94 ± 1.1
	500	485.0	97 ± 1.1
	1000	944.0	94 ± 0.3
	2000	2020.0	101 ± 0.3
	4000	3840.0	96 ± 0.8
Tap water	0	ND	ND
	1.25	1.18	94 ± 1.0
	2.50	2.46	98 ± 2.8
	5.00	4.90	98 ± 3.4
	8.00	8.05	101 ± 0.9
	10.0	9.69	97 ± 1.2

a. Data were means ± SD of three determinations.

b. Not detected.

Table 3. Determination of pakchoi samples by icELISA and HPLC

Samples[a]	Concentrations of cyantraniliprole residues (ng g^{-1})	
	icELISA	HPLC
1	1474.1 ± 24.8[a]	1730.0 ± 31.7[b]
2	1437.0 ± 52.2	1587.0 ± 63.4
3	4431.4 ± 46.3	4787.0 ± 31.2
4	4670.0 ± 57.1	5039.0 ± 43.6
5	680.0 ± 18.2	1003.0 ± 25.3
6-15	ND[c]	ND

[a]Each sample was analyzed in triplicate.

[b]The data represented the mean ± SD.

[c]Not detected.

Sample 1-4: Pakchoi samples were sprayed with cyantraniliprole.

Sample 5-15: Pakchoi samples were bought from different local markets.

Conflict of Interest Statement

We declare that all authors have no financial and personal relationships with other people or organizations, which could have improperly effect on our work. There also is no professional or personal interest of service or companies that could influence the position presented in, or the review of, the manuscript entitled, “Development of a Monoclonal Antibody-Based ELISA for the Detection of the Novel Insecticide Cyantraniliprole”.