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Chemiluminescent Enzyme Immunoassay for Rapid Detection of Three α-Cyano Pyrethroid Residues in Agricultural Products

Niusha Taheri[1] · Meijing Lan[1] · Peng Wei[1] · Rui Liu[1] · Wenjun Gui[1] · Yirong Guo[1] · Guonian Zhu[1]

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Abstract An indirect competitive chemiluminescent enzyme immunoassay (CLEIA) using a new broad-specific monoclonal antibody was developed for the determination of α-cyano pyrethroids. Under optimum conditions, this assay exhibited high sensitivities toward fenpropathrin, deltamethrin, and λ-cyhalothrin, with a wide detection range of 0.16 to 100 ng/mL and half-maximal inhibition concentration (IC_{50}) of 1.9, 3.4, and 4.3 ng/mL, respectively. Cross-reactivity with other pyrethroids was not detected, except for cypermethrin with 5 %. The accuracy and repeatability of the established CLEIA were evaluated by intra-day and inter-day tests of spiked recovery. For agricultural samples such as orange, eggplant, and cowpea, the average recoveries ranged from 79.6 to 115.5 %, and the intra-day and inter-day coefficients of variation were from 4.7 to 12.2 % and 6.7 to 14.2 %, respectively. Furthermore, analysis of several real samples by the CLEIA was validated by gas chromatography equipped with an electron capture detector, showing good correlation between the two methods. Therefore, the CLEIA presented in this study is suitable as a rapid screening tool for the detection of three α-cyano pyrethroids in agricultural products.

Keywords α-Cyano pyrethroids · Chemiluminescent enzyme immunoassay · Monoclonal antibody · Agricultural products

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✉ Yirong Guo
yirongguo@zju.edu.cn

[1] Institute of Pesticide and Environmental Toxicology, Zhejiang University, Hangzhou 310058, People's Republic of China

Introduction

Pesticides are widely used in modern agriculture to control pests and diseases, as well as to improve crop yields. Pyrethroids constitute a class of synthetic insecticides similar to the natural pyrethrins found in the flowers of pyrethrums (*Chrysanthemum cineraria folium* and *Chrysanthemum coccineum*). To date, more than 70 pyrethroids worldwide have been registered, and 20 of these pyrethroids are widely used in agriculture, forestry, horticulture, public health, and households. The α-cyano pyrethroids such as deltamethrin, cypermethrin, λ-cyhalothrin, fenpropathrin, and fenvalerate are rapidly increasing in number (Hao et al. 2009). Although α-cyano pyrethroids are thought to be safe for humans, recent studies have reported that long-term exposure of humans to these pesticides causes reversible symptoms of poisoning and suppressive effects on the immune system; thus, the toxic effects of these pesticides on the ecological, environmental, and human health aspects have elicited increasing concerns (Kuivila et al. 2012; Palmquist et al. 2011). Therefore, the α-cyano pyrethroid residues in the environment and agricultural products should be determined for regulatory and monitoring purposes. Maximum residue limits (MRLs) for α-cyano pyrethroids have been established in standard regulations from different countries and areas. In China, the MRLs of α-cyano pyrethroids in fruits and vegetables mostly range from 0.05 to 1 mg/kg, according to the National Food Safety Standard MRLs for pesticides in food (China GB 2763–2014).

Gas chromatography equipped with electron capture detector (GC-ECD) or mass spectrometry is the major tool for pyrethroid residue detection (Lin et al. 2011; Zhu et al. 2014). This method is characterized by low limit of detection (LOD), high precision, and high sensitivity, but it involves shortcomings such as expensive instrumentation, complicated sample pretreatments, and time-consuming processes. Moreover, this

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method also produces a large amount of toxic organic solvent wastes, making it a less environment-friendly option. Compared with traditional instrumental methods, immunoassays offer advantages of desirable sensitivity and specificity, high-throughput sample analysis, and cost-effectiveness. Moreover, immunoassays are field-portable and do not require complicated instrumentation. The most popular immunoassay for pesticide residue analysis is enzyme-linked immunosorbent assay (ELISA). Several ELISAs for the residue detection of individual α-cyano pyrethroids in environmental matrices and agro-products have been reported, including deltamethrin in milk (Lee et al. 2003); cypermethrin in water (Lee et al. 2004); flucythrinate in crops (Nakata et al. 2001); cyhalothrin in water (Gao et al. 2006); fenpropathrin in water (Wengatz et al. 1998); esfenvalerate in water (Shan et al. 1999); and permethrin and cypermethrin in vegetables, fruits, and wine (Park et al. 2004).

For efficient surveillance purposes, an immunoassay that can detect multiple pesticides rather than a specific pesticide is preferred. Certain class-selective ELISAs have been developed for multi-residue analysis of α-cyano pyrethroids. However, most of these assays were based on polyclonal antibodies (Hao et al. 2009; Liang et al. 2013; Lu et al. 2010; Mak et al. 2005). Few works have used monoclonal antibodies (mAbs) for multi-residue detection of α-cyano pyrethroids (Chen et al. 2014; Wang et al. 2011). For instance, an extremely broad-selective and highly sensitive ELISA using a mAb derived from a generic hapten [3-(3-phenoxy-benzoyamino)-propionic acid] was developed for the detection of eight pyrethroids with the phenoxybenzene group (six α-cyano pyrethroids involved). This approach appears to be the most sensitive and class-selective immunoassay for pyrethroids, with half-maximal inhibition concentration (IC_{50}) values from 1.5 to 5.0 ng/mL (Wang et al. 2011). Based on a relatively specific hapten [(R,S)-cyano-3-phenoxybenzyl-2,2,3,3-tetramethyl cyclopropane carboxylic acid] for immunization, a broad-specific immunoassay was established for three α-cyano pyrethroids, with IC_{50} varying from 1.7 to 45.8 ng/mL (Chen et al. 2014). However, these methods were only applied to certain matrices such as water samples, and their linear ranges for determination were limited to two orders of magnitude. The relatively narrow dynamic range is attributed to the colorimetric measurement of ELISAs, which limits the applications of these methods (Schobel et al. 2000).

Chemiluminesent reagents can detect low concentrations of horseradish peroxidase, thereby reducing the optimal working concentrations of antibodies and antigens, resulting in high analytical sensitivities (Mickova et al. 2005). Recently, chemiluminescent enzyme immunoassay (CLEIA) has gained attention in different fields because of its ultra-high sensitivity, good specificity, wide range of applications, simple equipment, and broad linear range for detection (Gámiz-Gracia et al. 2005; Zhao et al. 2009). CLEIA based on a luminol/peroxide/enhancer system offers the possibility of improving the assay sensitivity and lowering the detection limit, in comparison with ELISA using the same antibody and antigen (Li et al. 2013; Li et al. 2015; Wang et al. 2015). To date, some publications have demonstrated the development of CLEIAs for some groups of pesticides, such as organophosphorus (Jin et al. 2012; Liu et al. 2013a; Soler et al. 2008; Xu et al. 2012), organochlorine (Botchkareva et al. 2003), neonicotinoid (Li et al. 2013; Liu et al. 2013b), and carbamate (Fang et al. 2015; Jin et al. 2013; Sun et al. 2010; Mickova et al. 2005). However, the use of CLEIA for the detection of pyrethroids has yet to be reported. The present study aims to develop a multi-target competitive CLEIA based on a new mAb for the broad-range detection of α-cyano pyrethroid residues in agricultural products such as orange, eggplant, and cowpea.

Material and Methods

Reagents and Chemicals

All pyrethroid pesticide standards used in this study (Fig. 1 lists their chemical structures) were obtained from the Agro-Environmental Protection Institute, Ministry of Agriculture (Tianjin, China). QuickAntibody adjuvant (Mouse 5 W) was supplied by Kang Biquan Biotechnology (Beijing, China). Ovalbumin (OVA), bovine serum albumin (BSA), rabbit anti-mouse immunoglobulin conjugated to horseradish-peroxidase (HRP), pristine, luminal, trometamol (Tris), N-

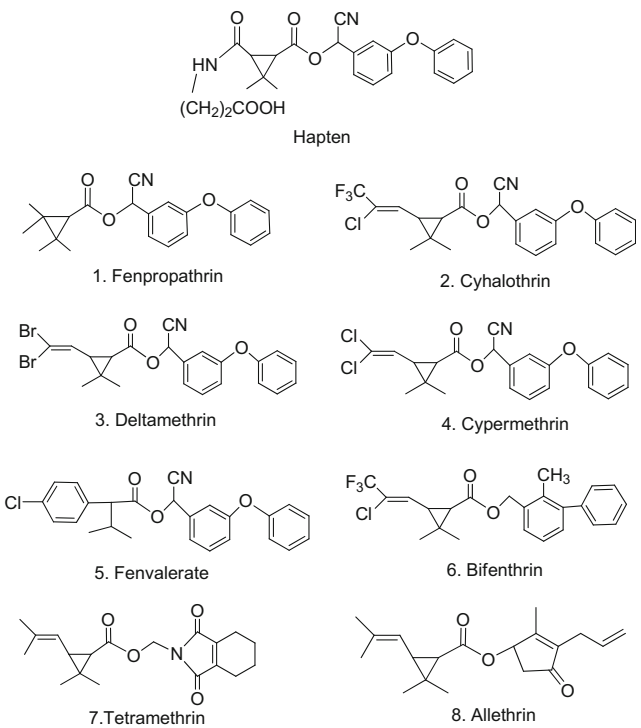

Fig. 1 Chemical structures of related compounds used in this study

hydroxysuccinimide (NHS), and N,N′-dicyclohexyl carbodiimide (DCC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *O*-phenylenediamine (OPD), *p*-iodophenol, polyoxyethylene sorbitan monolaurate (Tween-20), dimethyl sulfoxide (DMSO), N,N′-dimethylformamide (DMF), isobutyl choroformate, and other chemical reagents were purchased from Shanghai Chemical Reagents Co. All other chemicals and organic solvents were of analytical grade or better.

Buffers and Solutions

Phosphate-buffered saline (PBS; 0.01 M, pH 7.4, comprising 0.137 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 7 mM Na_2HPO_4) was self-prepared. Phosphate buffer (PB) and carbonate-buffered saline (CBS) were adjusted to various ionic strengths and pH values by changing the amounts of Na_2HPO_4 and KH_2PO_4 for PB and Na_2CO_3 and $NaHCO_3$ for CBS. The chemiluminescence-enhanced solution containing 0.5 mM luminol, 0.5 mM *p*-iodophenol, and 0.4 mM H_2O_2 in Tris–HCl buffer (0.1 M, pH 8.5) was freshly prepared, prior to use. Stock solution of each pyrethroid standard (1 mg/mL) was prepared in methanol and stored at −20 °C.

Preparation of Immunogen and Coating Antigen

The hapten for α-cyano pyrethroids, namely, 3-(2-carboxyethylcarbamoyl)-2,2-dimethyl-cyclopropanecarboxylic acid cyano-(3-phenoxy-phenyl)-methyl ester, was prepared as previously described (Liang et al. 2013). The immunogen (hapten-BSA conjugates) was prepared by the active ester method. Briefly, 100 μmol of the hapten was added to 1 mL of DMF containing 300 μmol of NHS and 150 μmol of DCC and then stirred at room temperature overnight. Precipitated urea was removed by centrifugation at 4000 rpm for 10 min, and 800 μL of supernatant was collected and added dropwise to 8 mL of CBS (0.01 M, pH 8.5) containing 160 mg of BSA; the mixture was stirred at room temperature for 6 h. The resultant conjugate was dialyzed against 5 % (*v*/*v*) methanol in distilled water (500 mL × 3), followed by PBS (0.01 M, pH 7.4) for 3 days, and the obtained immunogen was stored at −20 °C.

Coating antigen (hapten-OVA) was prepared by mixed anhydride method; that is, the hapten (100 μmol) was added to 1 mL of anhydrous DMF solution containing 24 μL of *n*-butyl amine and 12 μL of isobutyl chloroformate. The solution was stirred for 1 h at room temperature, and 400 μL of the mixed solution was then collected and added dropwise to 8 mL of CBS containing 160 mg of OVA. The reaction mixture was stirred for 2 h at room temperature. The succeeding steps were identical to those used for the preparation of immunogen.

Immunization

Three BALB/c female mice (6–8 weeks old, supplied by Laboratory Animal Research Center, Shanghai, China) were injected subcutaneously with a mixture (1:1, *v*/*v*) of immunogen (50 μg per mouse) in PBS and QuickAntibody adjuvant. After 3 weeks, the mice were booster-injected with the same immunogen dosage. Booster immunization was repeated at 2-week intervals, until the titer of mouse sera reached 1/10,000, which was checked by the non-competitive indirect ELISA with the coating antigen hapten-OVA (5 μg/mL). The sera were also tested for the recognition of α-cyano pyrethroids by competitive indirect ELISA. ELISA procedures were performed as previously described (Liang et al. 2013).

Production of Monoclonal Antibody

Among the antisera collected from the immunized mice, the one showing the best serum reactivity was selected for the spleen donator. Three days prior to cell fusion, the mice were booster-injected again with the same immunogen in PBS without adjuvant. Splenocytes were harvested and fused with SP2/0 myeloma cells to form hybridomas, according to the standard fusion protocol. After 12–14 days, culture supernatants were screened by indirect ELISAs for the presence of antibodies that could recognize the analytes. Selected hybridomas were subcloned by limiting dilution method. The most sensitive and stable hybridoma clone was injected into F1 hybrid mice for ascite production. The resultant mAb was purified by salting-out method with caprylic acid and ammonium sulfate. Isotype classification of the mAb was performed by using a commercially available kit from Thermo Scientific (Rockford, USA).

Immunoassay Procedure

CLEIA was performed as follows: high-binding black microtiter plates (Costar, USA) were coated with optimized concentrations of hapten-OVA in coating buffer by incubation for 2 h at 37 °C (all incubations were carried out at 37 °C). The plates were then washed thrice with washing buffer (PBST, 0.01 M, pH 7.4, PBS containing 0.05 % (*v*/*v*) Tween-20; 300 μL/well) by using a 96-channel washer (Highcreation, Shenzhen, China), and the free binding sites in the wells were blocked by incubation with 5 % (*w*/*v*) skim milk in PB (300 μL/well) for 1 h. After the plates were washed twice again, 50-μL/well aliquot of the analyte standard solution or sample extract and 50-μL/well aliquot of the diluted mAb were added to the wells and incubated for 30 min. After washing thrice, 120 μL/well of diluted rabbit anti-mouse IgG-HRP (1/20,000) with 2 % skim milk in PB was added to the plates. The plates were incubated for 50 min and subsequently washed five times.

Finally, 200 μL/well of the chemiluminescent-enhanced solution was added to the plates. Immediately after the addition of the substrate, chemiluminometric signals generated from the HRP-luminol-H_2O_2 system were measured by a SpectraMax L luminescence plate reader (Molecular Devices, Sunnyvale, CA, USA), and the results were expressed in relative light units (RLUs).

Assay Optimization

CLEIA was optimized using fenpropathrin as the analyte. The effects of organic solvents on the assay performance were tested by preparing the standard solution containing various percentages of methanol, ethanol, and DMF (10 to 50 %, *v*/*v*). To evaluate the effects of reaction buffers, both analyte and mAb were diluted in PB and Tris–HCl with varying pH values and salt concentrations. Similarly, the effects of coating buffers were investigated with CBS and PB at different pH levels and ionic strengths for antigen dilution. Afterward, the optimal reaction time for the competition step was determined by testing at different incubation times (30 to 120 min).

The experiments were carried out using the CLEIA protocol described above. IC_{50} was used as the primary criterion to evaluate the assay performance. The maximum of RLU (RLU_{max}), the ratio of RLU_{max} to IC_{50}, and the parameters of standard curves such as coefficient of determination (R^2) and the slope of linear equation were also examined.

Cross-Reactivity Measurement

To assess the specificity of the mAb, cross-reactivity (CR) data were obtained from standard inhibition curves of fenpropathrin and other structurally related compounds by using the optimized CLEIA system. CR values were calculated using the following formula:

$$CR = [(IC_{50}\text{of fenpropathrin})/(IC_{50}\text{of other compounds})] \times 100\ \%$$

Sample Pretreatment for CLEIA

Agricultural products such as orange, eggplant, and cowpea, for which fenpropathrin, λ-cyhalothrin, and deltamethrin are commonly used in China, were obtained from a local supermarket in Hangzhou, Zhejiang. Potential cross-reactant residues were verified by GC analysis to avoid contaminations.

Each homogenized sample (5 g) was separately fortified by adding aliquots of standard solutions of fenpropathrin, λ-cyhalothrin, or deltamethrin in methanol to form different spiked levels and left undisturbed at room temperature for 30 min prior to extraction. For CLEIA analysis, rapid extraction method was adopted from previous reports on ELISAs for the detection of pyrethroid residues in agricultural products (Nakata et al. 2001; Park et al. 2004). Samples were mixed with methanol (20 mL) in a 50-mL centrifuge tube and then vigorously shaken with a vortex mixer for 1 min. After centrifugation (5000 rpm, 4 °C) for 5 min and filtration through a 0.22-μm membrane filter, 4 mL of the supernatant was collected and mixed with 6 mL of PB. The prepared extract solutions were further diluted with methanol/PB (4:6, *v*/*v*) as needed, followed by CLEIA analysis.

Sample Pretreatment for GC Analysis

Samples were prepared with a modified QuEChERS treatment (Srivastava et al. 2011) as follows. A 5-g portion of the homogenized sample was individually spiked with aliquots of fenpropathrin, deltamethrin, or λ-cyhalothrin to form different fortification levels. After 30 min, 20 mL of acetonitrile was added and the mixture was homogenized at 10,000 rpm for 2 min by a high-speed homogenizer (IKA, T18BS25, Germany). After filtration, the filtrate was collected and transferred into a 50-mL centrifuge tube which was preloaded with 1 g of sodium chloride and 4 g of magnesium sulfate, and the mixture was manually shaken for 1 min. After centrifugation (5000 rpm, 4 °C, 5 min), 16 mL of upper layer was transferred into a flat bottom flask and concentrated to dryness in a rotary evaporator at 45 °C on a water bath. The residue was redissolved in 2 mL of ethyl acetate, filtered through a 0.22-μm membrane filter, and then transferred into an autosampler vial for GC analysis.

GC-ECD Conditions

GC-ECD analysis was conducted based on a previously reported method (Albadri et al. 2012) with some modifications. The apparatus was an Agilent 7890 GC equipped with a capillary column (30 m, 320 μm) containing a 0.25-μm-thick film of 5 % phenyl methyl siloxane (HP-5, Agilent). A HP model 5181 series auto-injector was used to inject 1 μL of sample. The injector temperature was set at 280 °C and the ECD temperature was 300 °C. The column temperature was programmed from 80 to 220 °C at the rate of 30 °C/min, held for 2 min, increased to 280 °C at the rate of 15 °C/min, and held for another 6 min. Nitrogen was used as carrier gas (12 psi).

Results and Discussion

MAb Production and Characterization

The development of class-selective immunoassay is mostly constrained by the availability of antibodies with appropriate

affinity and broad specificity. The selection of an appropriate hapten for immunization is the key step. According to our previous work (Liang et al. 2013), a broad-specific mAbs can be obtained by using a generic hapten that contains the common moiety of α-cyano pyrethroids without one side chain of the cyclopropane ring (see the structure in Fig. 1). After measuring the binding ability of antisera collected from the immunized mice, we found that the resulting antibody exhibited the best recognition toward fenpropathrin, with minor cross-reactivity to λ-cyhalothrin, deltamethrin, and cypermethrin. Thus, fenpropathrin was considered as the primary analyte for further tests. Finally, a stable cell line was found to secrete the target mAb with isotype of IgG1 heavy chain and *kappa* light chain. The purified mAb gave a titer of around 16,000 and showed the highest sensitivity toward fenpropathrin, with IC_{50} of around 20 ng/mL, as determined by competitive indirect ELISA based on 5 μg/mL of hapten-OVA. The cross-reactivities to three other α-cyano pyrethroids ranged from 10 to 50 %. These preliminary results indicated that the newly developed mAb seemed to be of broad specificity, which was ideal for establishing a class-selective CLEIA for multi-target detection.

Assay Optimization

Checkerboard titration was performed by competitive indirect CLEIA with a series of antibody and coating antigen dilutions. The combination of antibody and antigen dilutions, which provided the largest inhibition by the addition of free fenpropathrin, was confirmed for use in subsequent assays. For CLEIA optimization, the operation concentrations of the coating antigen and the mAb were 1.25 and 0.625 μg/mL, respectively.

Figure 2 presents the CLEIA tolerance to methanol, ethanol, and DMF. IC_{50} values tended to markedly decrease with the increasing content of organic solvents from 10 to 30 % and reached the lowest at 40 %. For RLU_{max}, methanol or ethanol did not significantly affect on the antibody-antigen binding, whereas the DMF amount exceeding 10 % showed negative interference. Considering IC_{50} value and RLU_{max} together, 40 % methanol was selected as the most suitable co-solvent. In addition, 0.05 M PB with pH 7.0 and 0.01 M CBS with pH 9.1 were correspondingly selected as the optimal reaction buffer and coating buffer conditions (Tables S1 and S2). Moreover, 30 min was selected as the optimal reaction time for the competitive step, due to the lowest IC_{50} value and the highest RLU_{max}/IC_{50} ratio, although RLU_{max} slightly decreased but was still acceptable (Fig. S1).

Characterization of the CLEIA

Under the established optimal condition, the consumption of coating antigen and antibody was tested again by checkerboard titration in a small concentration range. Finally, 0.3 μg/mL was selected as the optimal concentration for both coating antigen and antibody, yielding the best assay performance. Figure 3 shows the standard curves for fenpropathrin, deltamethrin, and λ-cyhalothrin, with the linear range of 0.16 to 100 ng/mL ($R^2 > 0.99$) and IC_{50} values of 1.9 to 4.3 ng/mL. The LODs (represented by IC_{20}, 20 % inhibitory concentration) for fenpropathrin, deltamethrin, and λ-cyhalothrin were calculated as 0.18, 0.33, and 0.39 ng/mL, respectively, according to the corresponding linear equations (the important parameters are listed in Table 1). Data were based on the mean values of nine replicates performed on different plates and on different days. Compared with the most feasible immunoassay that was previously reported for these pyrethroids (Wang et al. 2011), the CLEIA developed in the present study displayed similar sensitivities but considerably wider linear range for detection (three orders of magnitude). This finding was

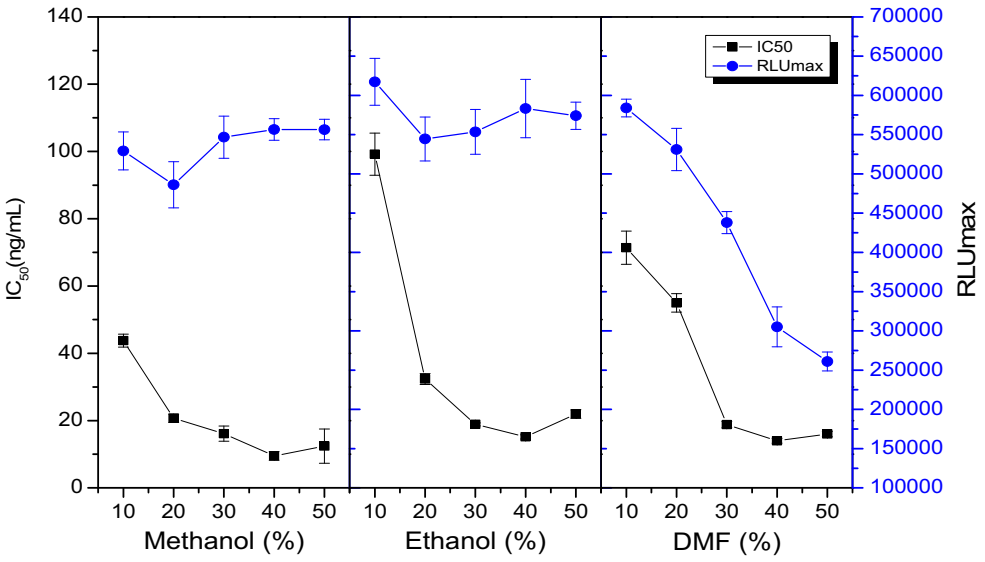

Fig. 2 Effects of different organic solvents on the assay performances ($n = 3$)

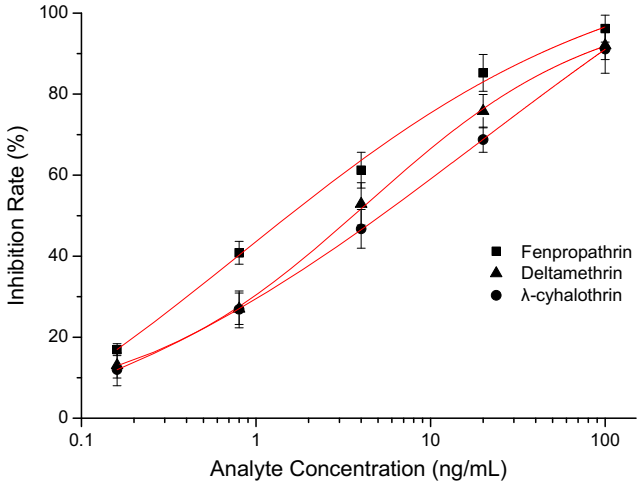

Fig. 3 Standard curves for fenpropathrin, deltamethrin, and λ-cyhalothrin by CLEIA ($n = 9$)

probably related to the high signal-to-noise ratio obtained from the chemiluminescent measurement, thereby offering a broad calibration range.

Cross-reactivities for eight common pyrethroid pesticides were measured to evaluate the assay selectivity. As shown in Table 1, the assay exhibited strong cross-reactivity with deltamethrin and λ-cyhalothrin but weak cross-reactivity with cypermethrin. Moreover, the developed CLEIA exhibited no evident cross-reactivity to the pyrethroids without α-cyano group, such as bifenthrin, allethrin, and tetramethrin. These results could be explained by the fact that the immunizing hapten owned the common moiety of α-cyano pyrethroids and its structure was the most similar to that of fenpropathrin. Additionally, changes in the side chain of the cyclopropane ring also had impacts on binding, which was similar to a previous report (Lu et al. 2010). Compared with the rabbit polyclonal antibody previously generated from the same immunizing hapten (Liang et al. 2013), the mAb in the current study exhibited lower group specificity. This difference can be explained by the facts that polyclonal antibodies can recognize multi-epitopes of an antigen, whereas a mAb can only recognizes a single epitope in a defined and specific manner. Moreover, the mAb-based CLEIA showed better sensitivities toward fenpropathrin, λ-cyhalothrin, and deltamethrin, which must be ascribed to the mAb's high affinity and the advantages of the chemiluminescent detection system.

Table 1 also included some related results from two other mAb-based class-selective immunoassays for pyrethroids. By comparison, this new CLEIA was not as broadly selective as the direct competitive ELISA developed by Wang et al. (2011), in which a generic hapten containing only the phenoxybenzene group of pyrethroids was employed. However, the CLEIA yielded superior performances to the indirect competitive ELISA reported by Chen et al. (2014). The immunizing haptens in both studies were similar, except that a longer spacer arm was attached to the dimethylcyclopropane terminal in the present study. These differences may contribute to the variety of mAb affinity to different α-cyano pyrethroids, as the antibody specificity and the final assay selectivity are mostly and inherently relevant to the immunizing hapten.

Furthermore, to estimate the assay reliability, different levels of spiked standards in the assay buffer were measured in four replicates on the same day, and the test was repeated for 3 days. The accuracy and precision of the method were represented by the recovery and the coefficient of variation (CV), respectively. For practical screening purposes, the ideal approach involves the use of a single standard curve for all target analytes in a broad-specific immunoassay, with relative CRs

Table 1 Assay cross-reactivities (CRs) to related pyrethroids and comparison with similar studies

Analyte	Standard equation parameters[a]			IC_{20} (ng/mL)	IC_{50} (ng/mL)	CR (%)	IC_{50} in Ref.[b] (ng/mL)	IC_{50} in Ref.[c] (ng/mL)	IC_{50} in Ref.[d] (ng/mL)
	Slope	Intercept	R^2						
Fenpropathrin	12.9	42.1	0.9909	0.18	1.9	100	3.0	14.0	14.5
Deltamethrin	12.8	34.4	0.9915	0.33	3.4	55.9	1.8	199.6	24.5
λ-cyhalothrin	12.4	31.9	0.9942	0.39	4.3	44.2	–	>1000	46.8
Cypermethrin	15.7	−6.5	0.9821	5.41	36.4	5.2	1.5	1.7	23.2
Fenvalerate	–	–	–	–	>2000	<0.1	2.0	298.5	–
Tetramethrin	–	–	–	–	>2000	<0.1	–	–	>1000
Alletrin	–	–	–	–	>2000	<0.1	–	–	>1000
Bifenthrin	–	–	–	–	>2000	<0.1	–	191.8	>1000

[a] The standard equation was defined as $y = a\ln(x) + b$ (obtained from Microsoft Office Excel 2010, using linear regression model), and data were based on the mean values of nine replicates performed on different plates and on different days

[b] Reference by Wang et al. (2011)

[c] Reference by Chen et al. (2014)

[d] Reference by Liang et al. (2013)

Table 2 Validation of the CLEIA by testing standards spiked in the assay buffer

Pesticide	Spiked (ng/mL)	Detected (ng/mL)[a]	Coefficients of variation (CV, %)		Mean relative recovery (%)	Mean actual recovery (%)[b]
			Intra-day ($n=4$)	Inter-day ($n=3$)		
Fenpropathrin	0.8	0.84 ± 0.10	8.6	11.9	105.0	105.0
	4	3.72 ± 0.23	4.7	6.2	93.0	93.0
	20	19.41 ± 1.29	5.8	6.6	97.1	97.1
Deltamethrin	0.8	0.48 ± 0.06	11.8	12.4	60.3	107.9
	4	2.13 ± 0.16	6.5	7.5	53.3	95.4
	20	11.03 ± 1.21	7.1	11.0	55.2	98.7
λ-cyhalothrin	0.8	0.37 ± 0.05	10.7	13.5	46.3	104.8
	4	1.71 ± 0.13	6.6	7.6	42.8	96.9
	20	8.37 ± 0.82	7.3	9.8	41.9	94.7

[a] Detected concentrations were all calculated from the standard curve of fenpropathrin, i.e., fenpropathrin equivalent, and data are mean ± standard deviation

[b] Mean actual recovery = mean relative recovery/CR

as reference values (Mak et al. 2005). In this study, the standard curve of fenpropathrin was used to measure λ-cyhalothrin and deltamethrin. The actual recovery was calculated by dividing the relative recovery by CR value. As presented in Table 2, the mean recoveries ranged from 93 to 108 %, and the intra-day and inter-day CVs were 4.7 to 11.8 % and 6.2 to 13.5 %, respectively. These results indicated that the broad-specific CLEIA was reliable, even based on a single standard curve for determination.

Matrix Interferences

Chemical compounds present in samples may affect the binding between the antibody and analyte, thereby reducing the sensitivity and reliability of immunoassays and possibly leading to false positive/negative results (Gabaldón et al. 1999). Usually, the matrix influence can be assessed by comparing a standard curve with a calibration curve obtained in the sample matrix.

In this study, matrices from different agricultural products (orange, cowpea, and eggplant) were rapidly extracted with methanol and adjusted with PB to obtain 40 % methanol. Subsequently, several dilutions (typically 1:5, 1:10, 1:20, and 1:40) of each sample extract were prepared in the competitive reaction buffer (40 % methanol in 0.05 M PB with pH 7.0). Fenpropathrin standard solutions were individually prepared in the above matrix buffers and in the normal matrix-free buffer as control. RLU_{max} and IC_{50} values of inhibition curves were considered as useful indicators to estimate the effects of different dilution ratios. As shown in Table 3, the matrix interferences could be eliminated by at least 1:10 dilution of the sample extracts in PB containing 40 % methanol (total dilution factor of 100 from the original sample). Thus, the limits of quantification (LOQs) for fenpropathrin, deltamethrin, and λ-cyhalothrin in these samples could reach 18, 33, and 39 ng/g, respectively, which were determined by multiplying the corresponding LOD with the total dilution factor for sample matrices (Vdovenko et al. 2014).

Table 3 Influence of matrix in orange, eggplant, and cowpea on the standard curves of fenpropathrin ($n=3$)

Sample	Indicator	Dilution fold				
		1:2 (*v*/*v*)	1:5 (*v*/*v*)	1:10 (*v*/*v*)	1:20 (*v*/*v*)	1:40 (*v*/*v*)
Orange	RLU_{max} ($\times 10^5$)	2.84 ± 0.29	3.47 ± 0.22	4.38 ± 0.18	4.42 ± 0.19	4.56 ± 0.21
	IC_{50} (ng/mL)	2.47 ± 0.35	3.11 ± 0.23	2.04 ± 0.19	1.98 ± 0.20	2.02 ± 0.17
Eggplant	RLU_{max} ($\times 10^5$)	2.57 ± 0.19	3.39 ± 0.27	4.31 ± 0.21	4.38 ± 0.23	4.47 ± 0.18
	IC_{50} (ng/mL)	2.36 ± 0.26	4.01 ± 0.36	2.19 ± 0.26	2.08 ± 0.14	2.11 ± 0.19
Cowpea	RLU_{max} ($\times 10^5$)	2.65 ± 0.24	3.28 ± 0.19	4.40 ± 0.13	4.47 ± 0.20	4.52 ± 0.28
	IC_{50} (ng/mL)	2.49 ± 0.23	3.72 ± 0.35	2.10 ± 0.16	2.09 ± 0.15	2.04 ± 0.13

For standard curves in matrix-free buffer, the RLU_{max} and IC_{50} values were $4.51 \pm 0.27 \times 10^5$ and 1.94 ± 0.18 ng/mL, respectively

Table 4 Recovery of fenpropathrin, deltamethrin, or λ-cyhalothrin spiked in orange, eggplant, and cowpea

Pesticide	Sample	Spiked (ng/g)	Detected (ng/g)[a]	CV (%)		Mean relative recovery (%)	Mean actual recovery (%)[b]
				Intra-day ($n=4$)	Inter-day ($n=3$)		
Fenpropathrin	Orange	50	53.8 ± 6.5	11.6	12.1	107.6	107.6
		200	190.4 ± 17.4	8.7	9.1	95.2	95.2
		1000	947.2 ± 61.8	5.8	6.5	94.7	94.7
	Eggplant	50	51.8 ± 6.9	11.8	13.3	103.6	103.6
		200	188.6 ± 17.0	7.5	9.0	94.3	94.3
		1000	923.7 ± 71.3	6.1	7.7	92.4	92.4
	Cowpea	50	54.3 ± 7.7	10.7	14.2	108.6	108.6
		200	191.5 ± 20.3	7.9	10.6	95.8	95.8
		1000	938.4 ± 66.4	5.2	7.1	93.8	93.8
Deltamethrin	Orange	50	30.6 ± 4.3	10.6	14.0	61.3	109.6
		200	105.2 ± 10.9	8.8	10.4	52.6	94.1
		1000	466.8 ± 37.8	5.7	8.1	46.7	83.5
	Eggplant	50	31.4 ± 4.0	10.7	12.7	62.8	112.4
		200	106.9 ± 9.7	6.2	9.1	53.4	95.6
		1000	463.4 ± 32.6	5.8	7.0	46.3	82.9
	Cowpea	50	29.0 ± 3.9	9.9	13.5	57.9	103.6
		200	104.9 ± 10.1	8.0	9.6	52.4	93.8
		1000	445.0 ± 29.9	4.7	6.7	44.5	79.6
λ-Cyhalothrin	Orange	50	25.5 ± 3.4	10.3	13.3	51.1	115.5
		200	91.5 ± 10.6	9.8	11.6	45.7	103.5
		1000	387.2 ± 32.9	6.8	8.5	38.7	87.6
	Eggplant	50	23.5 ± 3.3	12.2	14.0	47.0	106.4
		200	86.4 ± 9.2	9.1	10.7	43.2	97.7
		1000	371.3 ± 30.5	7.6	8.2	37.1	84.0
	Cowpea	50	24.0 ± 2.9	9.9	12.1	47.9	108.4
		200	84.7 ± 8.5	8.4	10.0	42.3	95.8
		1000	366.0 ± 31.7	7.1	8.7	36.6	82.8

[a] Detected concentrations were all calculated from the standard curve of fenpropathrin, i.e., fenpropathrin equivalent, and data are mean ± standard deviation ($n=12$)

[b] Mean actual recovery = mean relative recovery/CR

Analysis of Spiked Samples

For spiked recovery tests, fruit and vegetable samples were separately fortified with three different concentrations of fenpropthrin, deltamethrin, and λ-cyhalothrin. The spiked levels (0.05, 0.2, and 1 mg/kg) were selected between the assay LOQs for samples and the common MRLs of these pesticides in fruits and vegetables. The determination was performed in four replicates and continued for 3 days. As listed in Table 4, the mean recoveries for all samples were in the range of 79.6 to 115.5 %, with intra-day CVs of 4.7 to 12.2 % and inter-day CVs of 6.7 to 14.2 %. It was demonstrated that the newly developed CLEIA could be suitable for the rapid detection of trace levels of the three α-cyano pyrethroid pesticides in agricultural products, with a desirable sensitivity to reach the MRL requirement.

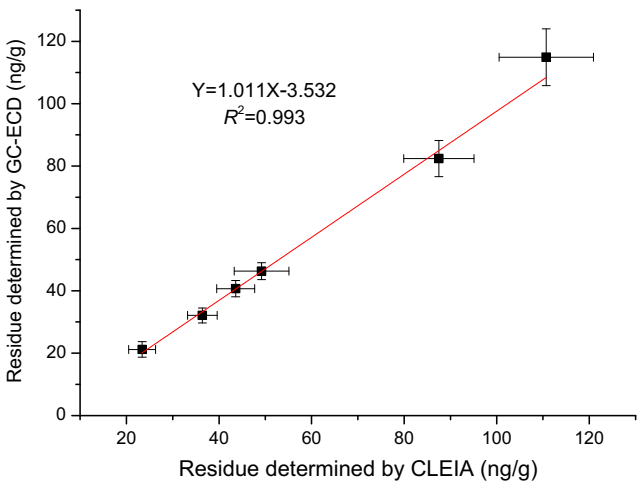

Fig. 4 Correlation between CLEIA and GC-ECD for real samples

Application Test for Real Samples

Given the high hydrophobicity of pyrethroids, they exist at extremely low residue levels in real water samples. Thus, it is more practical to detect these pesticide residues in agricultural products. Real samples (including orange, eggplant, and cowpea) were collected from five different markets in Hangzhou, Zhejiang. All 15 samples were tested using both GC-ECD and the developed CLEIA. As shown in Table S3, 6 out of the 15 samples were found to be positive, with concentrations of 21–115 ng/g (lower than related MRLs). However, the CLEIA was not able to distinguish fenpropthrin, deltamethrin, or λ-cyhalothrin, instead only indicating fenpropathrin equivalent levels. With the help of GC-ECD confirmation, the exact name of pyrethroid was identified. The linear regression analysis of actual residue values demonstrated good correlation between the two methods (Fig. 4). Therefore, the developed broad-specific CLEIA can be used as a screening tool to rapidly detect the three pyrethroid residues in large-scale real samples, thereby potentially reducing the sample amounts for further instrumental analysis.

Conclusions

A new mAb-based CLEIA for the rapid detection of α-cyano pyrethroids was developed, optimized, and characterized in this study. Under optimal conditions, the assay exhibited high sensitivities to fenpropathrin, deltamethrin, and λ-cyhalothrin with IC_{50} of 1.9, 3.4, and 4.3 ng/mL, respectively. For other tested analogs, only a minor cross-reactivity with cypermethrin was found at 5 %. The accuracy and repeatability of the assay were confirmed to be satisfactory by determining spiked standards in buffer solutions and samples such as orange, eggplant, and cowpea, using intra-day and inter-day tests. Moreover, the assay showed good correlation with GC-ECD method by analysis of real samples. Overall, the results suggested that the proposed CLEIA was feasible for screening the three α-cyano pyrethroid residues in agricultural samples, owing to high sensitivity, wide calibration range, short analysis time, and high sample throughput. To the best of our knowledge, this study is the first to report on CLEIA method for the detection of α-cyano pyrethroids, which will contribute to the control of pesticide contamination in agricultural products.

Compliance with Ethical Standards

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Conflict of Interest Niusha Taheri declares that she has no conflict of interest. Meijing Lan declares that she has no conflict of interest. Peng Wei declares that he has no has no conflict of interest. Rui Liu declares that she has no conflict of interest. Wenjun Gui declares that he has no conflict of interest. Yirong Guo declares that she has no conflict of interest. Guonian Zhu declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human subjects. Studies on animals were performed under the guidance of the animal welfare committee of Zhejiang University in China.

Informed Consent Not applicable.

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