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Sensitive detection of thiacloprid in environmental and food samples by enhanced chemiluminescent enzyme immunoassay

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A fast and sensitive enhanced chemiluminescent enzyme immunoassay (ECL-EIA) was developed based on horseradish peroxidase detected with a luminol-based substrate for neonicotinoid insecticide thiacloprid in environmental and food samples. Toward this goal, variety conditions of chemiluminescent substrate solution including the reacting buffer, the concentrations of p-iodophenol, luminol and H_2O_2 were optimized. Under the optimal conditions, the sensitivity (the 50% inhibitory concentration value) was 1.80 ng/mL. The ECL-EIA was 5.5 times more sensitive compared to the colorimetric-EIA. The average recoveries of thiacloprid from spiked ten samples were estimated to range from 79.7 to 119 %, with relative standard deviations of 4.2 to 11.2 %. The dissipation of thiacloprid applied to real tomato samples was monitored with the ECL-EIA and HPLC methods. The ECL-EIA results agreed well with the HPLC results (R^2=0.993). These results suggested that the thiacloprid in the samples could be simply, rapidly and accurately detected by

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ECL-EIA.

1. Introduction

Thiacloprid [(Z)-3-(6-chloro-3-pyridylmethyl)-1, 3-thiazolidin-2-ylidenecyanamide] belongs to neonicotinoid insecticides and is widely used in agriculture as insecticides owing to their broad spectrum of activity and their low bioaccumulation potential.[1,2] However, its aspects of leaching into surface water and ground water, contaminating agricultural products, and toxicity to beneficial organisms, remain to be elucidated.[3] Because of its extensive application, thiacloprid residue problems have been arisen in both agricultural products and the environment. To protect consumers from risks related to thiacloprid residue, the maximum residue limits (MRLs) of thiacloprid have been established by European Union (0.2 mg/kg), China (1 mg/kg) and Japan (1 mg/kg). [4] So, a suitable method for analyzing thiacloprid residues is of great significance.

Conventional methods for the detection of thiacloprid residues are high-performance liquid chromatography (HPLC) [5,6,7] and high-performance liquid chromatography-mass spectrometry (HPLC-MS). [4] However, these methods are costly and time-consuming. Immunoassays as rapid, low cost and high throughput tests are becoming a reliable analytical tool for pesticide residues. The sensitivity of an immunoassay strongly depends on the affinity of specific antibodies and on the sensitivity of the detection method.[8] Enzyme labels detected by chemiluminescent (CL) substrates (dioxetane-based substrates for alkaline phosphatase or the luminal-peroxide-enhancer system for horseradish peroxidase), represent the most

sensitive detection system in immunoassay development. [8,9,10] In addition, the light intensity of CL reaches the maximum 1-2 min after substrate addition, thus shortening the overall analytical procedure when compared with conventional colorimetric assays.[8,9] These advantages of CL techniques make it useful detection system for residue analysis. To date, several chemiluminescent methods have been successfully established for the analysis of pesticides,[11,12] veterinary drugs[13,14] and environmental contaminates.[15,16]

Recently, we reported on the development of enzyme-linked immunosorbent assay (EIA) for thiacloprid based on polyclonal antibody. [1] But, the sensitivity of published EIA was still not high enough for some applications. In the present work, for the first time, we adapted the enhanced chemiluminescent (ECL) detection to the development and optimization of a sensitive EIA (ECL-EIA) for the detection of thiacloprid in environmental and food samples. In addition, the ECL-EIA performance was evaluated by HPLC using real tomato samples.

2. Materials and methods

2.1 Reagents

Pesticide-grade thiacloprid with a purity of 98.0% was obtained from Nanjing Jiangsu Flag Chemical Industry Co., Ltd. (Jiangsu, China). Bovine serum albumin (BSA), ovalbumin (OVA), goat anti-rabbit immunoglobulin horseradish peroxidase conjugate (GAR−HRP), luminol, and p-iodophenol (PIP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All reagents and solvents were analytical grade. All animals used in this study and animal experiments were approved by Department of

Science and Technology of Jiangsu Province. The license number was SYXK (SU) 2010-0005.

2.2 Buffers and solutions

The following buffers were used: (A) coating buffer, 0.05 mol/L carbonate-buffered saline (CBS), pH 9.6; (B) blocking buffer, 0.01 mol/L phosphate-buffered saline (PBS), pH 7.4; (C) washing buffer, PBS containing 0.05% Tween-20 (PBST); (D) substrate buffer, 0.1 mol/L Tris-HCl buffer, pH 8.6.

2.3 Instruments and equipments

Microtiter plates used were 96-well white microplates (Corning, MA, USA) with high binding capacity (Maxisorp and Costar). The chemiluminescence emission was measured with a SoftMax Pro 5.4.1 Microplate Luminometer (Molecular Devices, USA). Thiacloprid was detected using an Agilent 1200 HPLC chromatograph (Agilent, USA).

2.4 Preparation of antigens and polyclonal antibody

The structures for thiacloprid and thiacloprid hapten are shown in Fig. 1. Thiacloprid hapten was synthesized according to the previous report,[1] and coupled to BSA and OVA to produce immunogen and coating antigen by activated ester method.[17]The conjugates were dialyzed in PBS at 4 °C for 72 h and stored at -20 °C.

The polyclonal antibodies were obtained from immunized New Zealand white rabbits. The immunization schedule was conducted according to Liu et al. [17] The rabbits had free access to drinking water and commercial standard laboratory diet (CZZ, Nanjing,

China). It was housed according to the EEC 609/86 Directives regulating the welfare of experimental animals. The rabbits were bled after 8 days of the last injection. The antiserum was centrifuged and then purified via the caprylic acid–ammonium sulfate precipitation method and freeze-dried. [18,19]

2.5 Coating of the microplate wells

White microplates were coated with coating antigen (100 μL per well, in CBS) overnight at 4 °C. After washing three times with PBST, the binding sites not occupied by coating antigen were then blocked with 200 μL of 5 % skim milk per well for 1 h at 37 °C. The plates were stored at 4 ºC in sealed packages.

2.6 Sample preparation

A total of ten environmental and food samples including three water samples (pond water, rice field water and canal water), three soil samples (rice field soil, vegetable field soil and park soil) and four food samples (pear, tomato, cabbage and apple) were collected from the suburb and local supermarket of Zhenjiang (China). Each analysis was performed in triplicate.

For water samples, simple filtration with filter paper was needed. The filtered water samples were spiked with thiacloprid standard solution at 0.5, 1 and 10 ng/mL. The spiked water samples were directly analyzed by ECL-EIA.

Before spiking, soil samples were dried, homogenized and sieved, while vegetable and fruit samples were finely chopped and triturated. Soil, pear, tomato, cabbage and apple samples (10 g) were spiked with thiacloprid at 2.5-50 ng/g. It was mixed with

10 mL PBST containing 50% methanol. After ultrasonic extraction for 10 min, the mixture was centrifuged for 10 min at 4000 g. The 1 mL of supernatant was diluted appropriately with PBST and then analyzed by ECL-EIA.

The real tomato samples were obtained as followed: Thiacloprid SC (48%), diluted with water, were sprayed onto tomato samples. Samples were then picked randomly and simultaneously analyzed with ECL-EIA and HPLC with Eclipse XDB-C18 (250mm×4.6mm I.D., 5μm) at the wavelength of 245 nm. The mobile phase was 70:30 (v:v) water (0.2% phosphoric acid):acetonitrile. The flow rate was 1.0 mL/min and the column temperature was 30 ºC.

2.7 ECL-EIA protocol

Thiacloprid standards or sample extract (50 μL per well) and antibody diluted in assay buffer (50 μL per well) was added to the coated wells, and incubation for 1 h. Following a further wash, 100 μL of goat anti-rabbit IgG-horseradish peroxidase diluted in PBST was added with incubation at 37 °C for 1 h. The plates were washed three times, and peroxidase activity was determined by adding 150 μL per well of a freshly prepared substrate solution. The chemiluminometric signal generated from the HRP-luminol-H_2O_2 system was measured immediately after the addition of the substrate (30 s) and the results were expressed in relative light units (RLU). The measurement was run three times in triplicate wells.

2.8 Preparation of ECL substrate and optimization of ECL-EIA conditions

The sensitivity of ECL-EIA depends on both assay procedure and the characteristics

of substrates. ECL emission intensity is sensitive to a variety of environmental factors such as solvent, solution pH and other species present in the system.[13] The effects of the concentrations of luminol, H_2O_2, PIP and ECL detection buffer solution were investigated to get an excellent substrate solution. These luminescence results are given in counts per second (cps).

On the basis of excellent ECL substrate，the effects of the working concentration, organic solvent, ionic intension and pH value were studied to improve the sensitivity of the immunoassay by single-factor experiments.

2.9 Determination of Cross-reactivity

Under the optimum conditions, cross-reactivity was studied using standard solutions of thiacloprid and its analogs.

3. Results and Discussions

3.1 Enhanced chemiluminescent substrate solution

In the HRP-luminol-H_2O_2 system, there are two factors (the influence of pH on the enzyme activity and the effect of pH on the generated ECL signal), which determine the overall response in this system.[20] The optimal pH value for HRP is 6-7, while the ECL reaction between luminal and H_2O_2 shows the maximum ECL intensity at pH 10-11.[20] So, the effect of medium pH on the immunoassay was studied based on standard S_0 (the thiacloprid concentration was 0 μg/L) and NSB (nonspecific binding). The results are presented in Table 1. The ECL signal of NSB in three buffers was no significant difference, while the ECL emission for standard S_0 in the Tris–HCl buffer

(pH 8.6) gave the biggest signal. So, Tris-HCl buffer was selected for the subsequent assays.

As the ECL reagent, the effect of the concentrations of luminol, H_2O_2 and PIP, which affect the ECL intensity, were investigated based on standard S_0, NSB and S/N (signal to noise ratio). The results are shown in Table 2.

The experiment results showed that the S/N value increased from 1 to 3 mmol/L and reached the maximum value at the luminol concentration of 3 mmol/L, and ECL signal for standard S_0 was bigger. The effect of H_2O_2 concentration on the ECL intensity was also optimized. The results showed that ECL signal with biggest S/N at the H_2O_2 concentration of 0.0625 mmol/L, while ECL signal was not enough. To ensure ECL signal, 5.0 mmol/L H_2O_2 was selected for the subsequent assays. Under optimum conditions, the ECL emission of luminal-H_2O_2 system was enhanced upon addition of PIP. The experimental results showed that when PIP concentration was 0.075 mmol/L, ECL signal with biggest S/N could be obtained. Thus, Tris-HCl buffer (pH 8.6) solution containing 3.0 mmol/L luminol, 5.0 mmol/L H_2O_2 and 0.075 mmol/L PIP was selected as the ECL detection solutions.

3.2 Development of ECL-EIA

To develop an ECL-EIA for detecting thiacloprid pesticide residues in environmental and food samples, experimental parameters (the concentrations of coating antigen and the polyclonal antibody, organic solvent, ionic strength, and pH) were studied based on excellent chemiluminescent substrate solution. The RLU_{max}/IC_{50} ratio was used as a primary criterion to evaluate ECL-EIA performances, the highest ratio indicating the

highest sensitivity.[21]

Taking all these factors into account, the optimal conditions for the ECL-EIA were found as follows: the coating antigen (1:8000) and the antibody (0.3 mg/L) produced the highest RLU_{max}/IC_{50} ratio; 5% methanol, pH 5.5, and an ionic strength of 0.4 mol/L were used for the ECL-EIA.

Under the optimum conditions, the competitive standard curve for thiacloprid is shown in Fig. 2. Based on the similar linear section of standard curve, a calibration curve was obtained (inset of Fig. 2). A limit of detection (LOD) and the sensitivity (IC_{50}) of the ECL-EIA were 0.092 ng/mL and 1.80 ng/mL, respectively. Compared to the MRLs of thiacloprid in the European Union, China and Japan,[4] the sensitivity of the ECL-EIA can meet the requirements of detection of thiacloprid. In addition, the sensitivity of the developed ECL-EIA had a significant improvement and was 5.5-fold higher than EIA. The ECL detection could offer an improved analytical performance in terms of detectability, due to the superior characteristics of the detection system, which is based on the enzymatic oxidation of luminol by hydrogen peroxide in the presence of peroxidases under mild alkaline conditions.[9,22] A further advantage obtained by using the ECL detection is the rapidity of the assay, since the light intensity of ECL can be measured immediately after the addition of the substrate (30 s), while the colorimetric assay requires a 20-30 min incubation step and stop step, prior to signal detection.[12,22]

3.3 Specificity

The cross-reactivity (CR) was estimated as the percentage obtained by calculating the

ratio of the IC_{50} value of thiacloprid to that of the given analogue (Table 3). The antibody showed negligible cross-reactivity with most of the analogues, except acetamiprid (0.72 % in the EIA and 0.43 % in the ECL-EIA), due to the same structure of the =N–CN moiety between acetamiprid and thiacloprid.[1] The results indicated the antibody was a high specificity to thiacloprid.

3.4 Analysis of spiked samples

The recoveries of thiacloprid from water, soil, pear, tomato, cabbage and apple samples are presented in Table 4. The recoveries were 79.7 %-119 %, and coefficients of variation were 4.2 %-11.2 %. These data are well within the requirements of residue analysis, and indicated that the established ECL-EIA was a potential screening tool for thiacloprid residue determination.

3.5 Correlation of ECL-EIA and HPLC

The real tomato samples were analyzed by the ECL-EIA and HPLC. And the results are presented in Fig. 3. A good correlation was obtained between the ECL-EIA (Y) and HPLC (X) results, with a linear regression equation of $Y = 0.9587 X + 0.0384$ ($R^2 = 0.993$, n=8). The result of correlation further demonstrated the reliability of the proposed ECL-EIA method.

4. Conclusions

In summary, an ultrasensitive ECL-EIA for detection of thiacloprid in environmental and food samples was successfully developed based on enhanced chemiluminescent substrate solution. The method using the HRP-luminol-H_2O_2 system to a significant

improvement in sensitivity compared with the already reported EIA. The spiked tests showed that the accuracy and precision of the ECL-EIA were well within the requirements of residue analysis for thiacloprid. It is noteworthy that in the study of blind samples, we conducted both ECL-EIA and HPLC to demonstrate the use of ECL-EIA as an advantageous analytical method in thiacloprid assessment. Therefore, the developed ECL-EIA presented here can be employed for the rapid and reliable analysis of thiacloprid in environmental and food samples.

Acknowledgments

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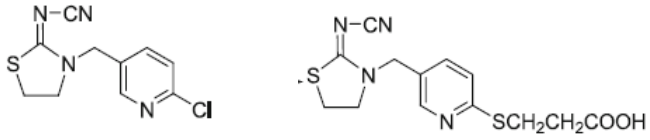


Thiacloprid Thiacloprid hapten

Fig. 1 Molecular structures for thiacloprid and thiacloprid hapten

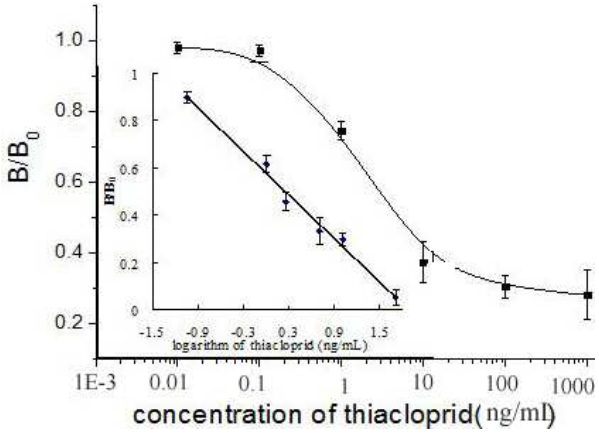


Fig.2 Calibration curve for thiacloprid by ECL-EIA

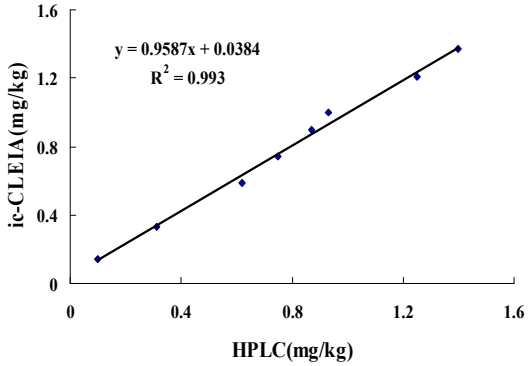


Fig. 3 Correlation between ECL-EIA and HPLC results for real tomato samples

Table 1 Effect of buffer pH on ECL intensity

Substrate buffer	NSB（cps）	S_0（cps）
Citrate-buffered saline (pH 6.0)	553	766
Phosphate-buffered saline (pH 7.4)	800	6590
Tris–HCl buffer (pH 8.6)	583	87541

Table 2 Effect of the concentrations of luminol, H_2O_2 and PIP on ECL intensity

Factors	Concentration (mmol/L)	NSB (cps)	S_0 (cps)	S/N
luminol	1	781	413645	530
	1.5	789	471891	598
	2.5	1098	574987	523
	3	889	558263	628
	3.5	865	536678	620
H_2O_2	0.625	672	462783	689
	1.25	784	513890	655
	2.5	921	567324	615
	5	945	586231	620
	10	1062	627893	591
PIP	0.075	873	378797	433
	0.15	1002	412783	412
	0.2	1289	419781	326
	0.3	1181	336631	285

Table 3 Cross-reactivity of thiacloprid and its analogs

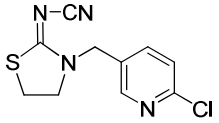
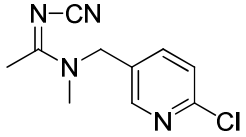
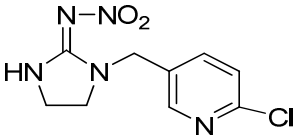
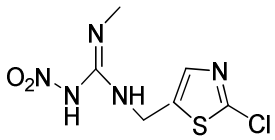
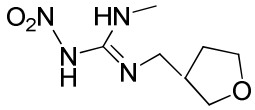
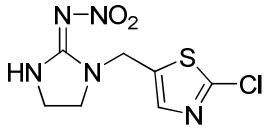
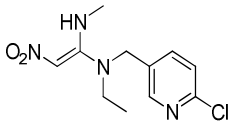
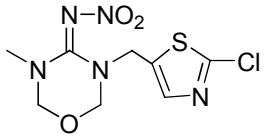
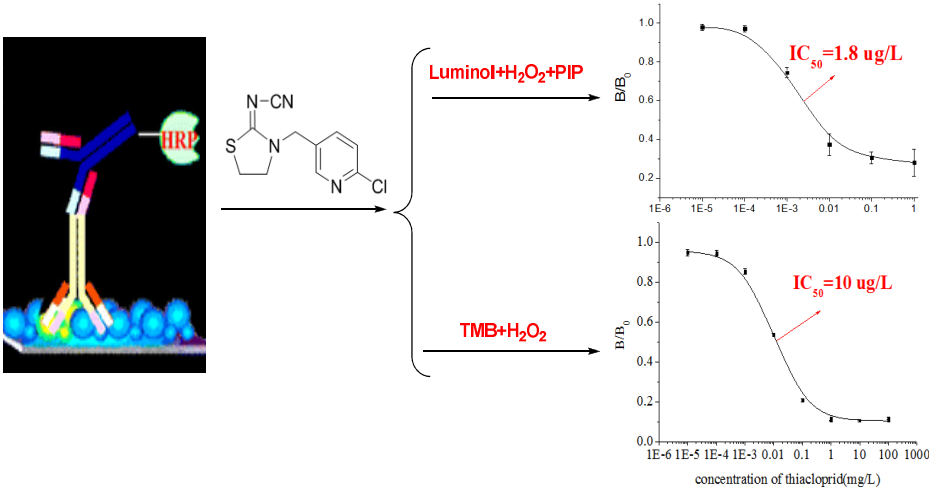
Compound	Structure	ECL-EIA		EIA	
		IC_{50} (ng/mL)	CR (%)	IC_{50} (ng/mL)	CR (%)
Thiacloprid		1.80	100	10.0	100
Acetamiprid		417	0.43	142	0.72
Imidacloprid		>1000	<0.01	437	0.23
Clothianidin		>1000	<0.01	852	0.12
Dinotefuran		>1000	<0.01	6281	0.02
Imidaclothiz		>1000	<0.01	>1000	<0.01
Nitenpyram		>1000	<0.01	>1000	<0.01
Thiamethoxam		>1000	<0.01	>1000	<0.01

Table 4 Results of recovery and coefficient of variation of the ECL-EIA for the detection of thiacloprid from spiked environmental and food samples

Sample	Spiked concentration (ng/mL, ng/g)	Mean recovery ± SD (%, n=3)	RSD (%)
Rice paddy water	10	95.3±6.5	6.8
	1	103±5.2	5.0
	0.5	87.8±9.8	11.2
Canal water	10	102±5.5	5.4
	1	80.7±3.9	4.8
	0.5	81.5±8.1	9.9
Pond water	10	113 ± 6.2	5.5
	1	86.9 ± 4.5	5.2
	0.5	83.6± 6.0	7.1
Rice paddy soil	50	110±7.4	6.7
	5	99.1±4.8	4.8
	2.5	108±7.7	7.1
Vegetable field soil	50	79.7±6.4	8.0
	5	119±5.8	4.9
	2.5	88. 3±3.7	4.2
Park soil	50	82.6±4.6	5.6
	5	89.7±5.3	5.9
	2.5	99.5±6.3	6.3
Pear	50	91.0±7.6	8.4
	5	117±6.8	5.8
	2.5	85.6±5.9	6.9
Tomato	50	90.0±7.1	7.9
	5	109±9.3	8.5
	2.5	87.3±5.1	5.8
cabbage	50	93.4±7.1	7.6
	5	98.6±5.8	5.9
	2.5	89.3±6.9	7.7
apple	50	88.5±6.3	7.1
	5	92.1±4.6	5.0
	2.5	106±5.6	5.3


HRP
N-CN
S
N
N
Cl
LuminoI+H2O2+PIP
TMB+H2O2
B/B0
IC50=1.8 ug/L
IC50=10 ug/L
1.0
0.8
0.6
0.4
0.2
0.0
1E-6 1E-5 1E-4 1E-3 0.01 0.1 1 10 100 1000
concentration of thiacloprid(mg/L)