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Development of Immunoassays for Detecting Clothianidin Residue in Agricultural Products

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Supporting Information

ABSTRACT: Two enzyme-linked immunosorbent assays (ELISAs) based on polyclonal antibodies (PcAbs) for clothianidin are described: colorimetric detection format (ELISA) and pattern of chemiluminescent assay (CLEIA). Clothianidin hapten was synthesized and conjugated to bovine serum albumin (BSA) and ovalbumin (OVA) to produce immunogen and coating antigen. Anticlothianidin PcAbs were obtained from immunized New Zealand white rabbits. Under optimal conditions, the half-maximal inhibition concentration (IC_{50}) and the limit of detection (LOD, IC_{20}) of clothianidin were 0.046 and 0.0028 mg/L for the ELISA and 0.015 and 0.0014 mg/L for the CLEIA, respectively. There were no obvious cross-reactivities of the antibodies with its analogues except for dinotefuran. Recoveries of 76.4−116.4% for the immunoassays were achieved from spiked samples. The results of immunoassays for the spiked and authentic samples were largely consistent with gas chromatography. Therefore, the proposed immunoassays would be convenient and satisfactory analytical methods for the monitoring of clothianidin in agricultural products.

KEYWORDS: *clothianidin, hapten, polyclonal antibodies, enzyme-linked immunosorbent assay, chemiluminescent enzyme immunoassay*

INTRODUCTION

Clothianidin [(*E*)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine] belongs to a new class of neonicotinoid insecticides, which has been widely applied to rice, leafy vegetables, tomato, and tea to control noxious insects, with excellent systemic action.[1,2] The carry-over of clothianidin to agricultural products can occur and increase human exposure. The most effective control measure depends on a rigorous program of monitoring the food-producing chain using sensitive and reliable analytical methods to minimize health risks.

Currently, several instrument-based detection methods for clothianidin have been developed, such as gas chromatography (GC)[3] and high-performance liquid chromatography (HPLC).[4−6] They are characterized by low limits of detection and high precision and sensitivity, but these methods are difficult to meet the high-throughput, rapid, screening of large numbers of agricultural samples.[7]

Immunoassays have emerged as fast, simple, and economic detection methods.[8] Generally, the signal of absorption, chemiluminescence, or electricity is detected in enzyme immunoassays. As a basic immunoassay, the conventional colorimetric assay (ELISA) has been researched and applied widely. ELISA has been demonstrated to be not only rapid and specific but also suitable as the reference method for the quantitative determination of compounds. In recent years, chemiluminescent enzyme immunoassay (CLEIA) has gained increasing attention in different fields because of its high sensitivity, good specificity, wide range of applications, lower consumption of immunoreagents, and absolute nontoxicity of reagents when compared with the colorimetric detection.[9−11] Furthermore, owing to the glow type emission kinetics of the enhanced chemiluminescence substrate, the light emission can be steady in 2−3 min after substrate addition, thus providing a rapid detection of analytical signal. These advantages of chemiluminescent techniques make CLEIA a useful detection method for toxicological analysis.

Several antibody-based studies for neonicotinoid insecticide have been developed.[12−16] Recently a paper developed an ELISA for clothianidin based on monoclonal antibodies, but there was a lack of application of the method to real contaminated samples.[17] This study was designed to systematically develop an ELISA and a CLEIA based on polyclonal antibodies for the detection of clothianidin in agricultural products. In this paper, the approach to prepare the hapten is more straightforward and the cross-reactivity with dinotefuran has significantly decreased compared with that previously reported. In addition, the immunoassays have been applied to authentic samples and confirmed by GC-ECD.

MATERIALS AND METHODS

Reagents. Clothianidin (97.6% purity) and the pesticide standards used for cross-reactivity studies were supplied by Jiangsu Pesticide Research Institute (Jiangsu, China). Bovine serum albumin (BSA), ovalbumin (OVA), and goat anti-rabbit immunoglobulin horseradish peroxidase conjugate (GAR−HRP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3-Mercaptopropionic acid (3-MPA), 3,3′,5,5′-tetramethylbenzidine (TMB), luminol, H_2O_2, *p*-iodophenol, polyoxyethylene sorbitan monolaurate (Tween-20), tris-

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(hydroxymethyl)aminoethane (Tris), and other chemical reagents were all purchased from Aladdin (Shanghai, China). The polyclonal antibodies were obtained from immunized New Zealand white rabbits. All animal studies were performed in accordance with the EEC 609/86 directives regulating the welfare of experimental animals.

Buffers and Solutions. Phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.4), carbonate-buffered saline (CBS, 0.05 mol/L, pH 9.6), and phosphate-buffered saline containing 0.05% Tween-20 (PBST) were used. The TMB solution contained 0.4 mmol/L TMB and 3 mmol/L H_2O_2 in citrate buffer (pH 5.0). The enhanced chemiluminescence solution contained 1 mmol/L luminol, 0.025 mmol/L *p*-iodophenol, and 1.5 mmol/L H_2O_2 in Tris-HCl buffer (0.1 mol/L, pH 8.5).

Instruments and Equipment. Electrospray ionization−mass spectrometry (ESI-MS) data were obtained with a LC-MS^{QDECA} (Finnigan, San Jose, CA, USA). Nuclear magnetic resonance (NMR) spectra were recorded on a DRX 500 spectrometer (Bruker, Karlsruhe, Germany). Ultraviolet−visible (UV−vis) spectra were obtained on a UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan). Microtiter plates used were 96-well transparent microplates (Nunc, Roskilde, Denmark) and 96-well white microplates (Corning, MA, USA) with high binding capacity (Maxisorp and Costar), respectively. Washing steps were carried out using a Wellwash Plus (Thermo, MA, USA). Absorbance and chemiluminescence emission were detected using a SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA). The ELISA and CLEIA were confirmed with an Aglient 7890A gas chromatograph (Aglient, Wilmington, DE, USA).

Hapten Synthesis. The hapten synthetic route is illustrated in Figure 1. A mixture of 1.02 g (4.0 mmol) of clothianidin, 0.42 g (4.0 mmol) of 3-MPA, and 0.45 g (8.0 mmol) of potassium hydroxide in 20 mL of ethanol was stirred at 80 °C for 2 h. Following filtration and concentration, the residue was dissolved in water (50 mL). The solution was adjusted to pH 2 using 1 mol/L HCl and extracted with ethyl acetate (3 × 30 mL). The extract was washed with water (3 × 30 mL), dried over anhydrous sodium sulfate, and concentrated. The concentrated residue was recrystallized using methanol to yield a white solid. The product was characterized by ESI-MS and NMR: ESI-MS, *m*/*z*, 320 $[M + H]^+$ and 342 $[M + Na]^+$; ^{1}H NMR (400 MHz, d_6-DMSO) δ 2.69 (t, J = 6.9 Hz, 2H, CH_2COO), 2.79−2.80 (d, J = 4.3 Hz, 3H, CH_3), 3.32 (t, J = 6.9 Hz, 2H, CH_2S), 4.50−4.51 (d, J = 4.0 Hz, 2H, CH_2NH), 7.60 (s, 1H, thiazol-4-H), 7.92 (s, 1H, $NHCH_3$), 9.13 (s, 1H, $NHCH_2$), and 12.44 (s, 1H, COOH).

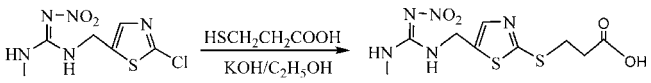


Figure 1. Synthetic route to the clothianidin hapten.

Preparation of Hapten−Protein Conjugates. The hapten was conjugated with BSA using the carbodiimide method to produce immunogen[18] and conjugated with OVA by the mixed anhydride method to acquire a coating antigen.[19] The conjugates were dialyzed in PBS over 72 h at 4 °C and stored at −20 °C. UV−vis spectroscopy data were used to confirm the formation of the conjugates, and the hapten densities (the number of hapten molecules per molecule of protein) of the conjugates were estimated directly by the mole absorbance at 280 nm:[20]

$$\text{hapten density} = (\varepsilon_{\text{conjugate}} - \varepsilon_{\text{protien}})/\varepsilon_{\text{hapten}}$$

Immunization and Antibody Preparation. The PcAbs were raised against immunogen in male New Zealand white rabbits. The immunization schedule was conducted using the procedure described previously.[21] The first injection was of immunogen (2 mg) diluted in physiological saline and emulsified with an equal volume of Freund's complete adjuvant. The emulsion was then intradermally injected at multiple sites on each rabbit's back. A further 3 mg of immunogen with Freund's incomplete adjuvant was injected as a booster shot after 3 weeks. The booster shoots were given four times at 2 week intervals. The rabbits were bled after the last injection. The antiserum was centrifuged and purified by precipitation with caprylic acid−ammonium sulfate[22] and then stored at −20 °C after freeze-drying.

Immunoassay Procedure. Microplates were coated with the coating antigen (100 μL/well, in CBS) overnight at 4 °C. The plates were washed five times with PBST and blocked by incubation with 5% skim milk in PBS (200 μL/well) for 0.5 h at 37 °C. After another washing step, either the sample or standard in PBST containing methanol (50 μL/well) was added followed by addition of the diluted polyclonal antibody (50 μL/well, in PBST) together for 1 h at 37 °C. Following a further wash, the diluted GAR/HRP (100 μL/well, 1:3000 in PBST) was dispensed into each well and incubated for 1 h at 37 °C. Then the plates were washed again.

For the ELISA, the performance was carried out on 96-well transparent microplates. The HRP activity of trace bound to the plate was measured by adding the TMB solution (100 μL/well). The reaction was stopped with 2 mol/L sulfuric acid (50 μL/well) after 15 min at 37 °C of incubation, and the absorbance was measured at a wavelength of 450 nm.

For the CLEIA, the immunoreaction of CLEIA was performed on 96-well white microplates. The peroxidase activity was revealed by adding 150 μL/well of a freshly prepared enhanced chemiluminescence solution. Chemiluminescence emission, which becomes stable within 2−3 min, was measured using a 1 s integration time at 425 nm.

Immunoassay Optimization. Experimental parameters (organic solvent, ionic strength, and pH) were studied to improve the sensitivity of the immunoassays. The evaluations of the immunoassays were based on the IC_{50}, the maximum of absorbance (A_{max}), the maximum of relative light units (RLU_{max}), and the coefficient of correlation (R^2) of their linear equation. Competitive curves were created in PBST solutions containing series concentrations of methanol (from 5 to 40%, v/v), Na^+ (from 0.1 to 0.6 mol/L), and pH values (ranging from pH 4.5 to 9.5) to evaluate the effects of the solvent, ionic strength, and pH, respectively.

Cross-Reactivity. Cross-reactivity (CR) was studied using the standard solution of the clothianidin and some of its analogues. The CR values were calculated as follows:

$$\text{CR \%} = (\text{IC}_{50}\text{ of clothianidin}/\text{IC}_{50}\text{ of analogue}) \times 100$$

Recovery. The accuracy and precision of immunoassays were evaluated by recovery of spiked samples. Blank samples (including 10.0 g of each river water, soil, rice, cabbage, or tomato sample) were spiked with clothianidin at 0.005, 0.01, 0.05, or 0.5 mg/kg and stored overnight. Then the river water samples were directly analyzed by ELISA and CLEIA to estimate the recoveries. Other samples were extracted twice by sonication in 20 mL of methanol for 10 min and centrifuged for 10 min at 4000 rpm. The supernatant was filtered through anhydrous sodium sulfate and concentrated. After dilution at an appropriate multiple, the solutions were analyzed by the immunoassays.

Each analysis was performed in triplicate. Samples without clothianidin were used as blanks in all case. The recoveries and relative standard deviations (RSDs) were calculated.

Matrix Effects on Immunoassays. Soil, rice, cabbage, and tomato were employed to study matrix effects. The concentrated samples were diluted (2-, 4-, 8-, and 16-fold) with PBST containing methanol (20% for ELISA and 10% for CLEIA). The matrix effects were determined by comparing clothianidin standard curves prepared in matrix extract and standards curves prepared in matrix-free PBST solution.

Method Validation. Authentic samples (including paddy water, soil, rice, cabbage, and tomato) were collected from farms where clothianidin had been used in Nanjing, China. The immunoassays were utilized to investigate the clothianidin residues in those samples. In addition, the samples were extracted by vigorous shaking for 1 h with acetonitrile. After the organic phase was anhydrated and concentrated, the samples were diluted with 2 mL of acetone and further confirmed by GC-ECD.[3] The GC column was a DB-17 fused silica capillary column (30 m × 320 μm × 0.25 μm), and nitrogen was used as the carrier gas. The column temperature was initially held at 180 °C for 1 min and then raised to 220 °C at 15 °C/min, which was held for 3 min. The measured results were compared with the results from the ELISA and CLEIA.

RESULTS AND DISCUSSION

Identification of Conjugations. UV−vis spectra showed qualitative differences between the hapten, carrier protein, and conjugates, especially at 280 nm, indicating these conjugates were coupled successfully. The molar ratios (hapten/protein) were estimated as 15:1 and 8:1 for immunogen and coating antigen, respectively.

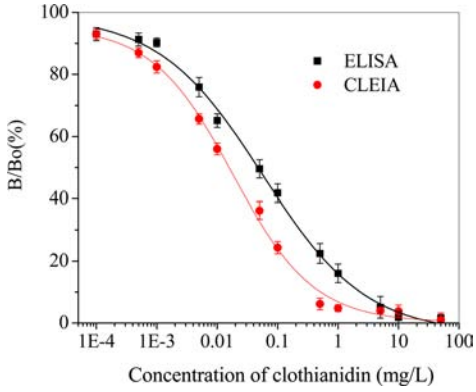


Figure 2. Standard curves for clothianidin by ELISA and CLEIA.

Operation Concentrations. By the ELISA procedure, the 0.25 μg/mL coating antigen and 0.3 μg/mL antibody were selected as ELISA operation concentration, when the A_{max} value reached 1.0. By CLEIA procedure, the operation concentrations of the coating antigen and antibody were 0.12 and 0.1 μg/mL based on the RLU_{max} and IC_{50}.

Optimization of Immunoassays. There are many parameters that influence the binding of the antibody to the analytes. Organic solvent, ionic strength, and pH were investigated to optimize the immunoassays. The highest A_{max}/IC_{50} and RLU_{max}/IC_{50} have been used to evaluate the parameters of ELISA and CLEIA, respectively. For solvent optimization, methanol was selected to improve the solubility of analytes. With the methanol concentrations being 20 and 10%, ELISA and CLEIA showed the highest A_{max}/IC_{50} and RLU_{max}/IC_{50}. The change of Na^+ concentration from 0.1 to 0.6 mol/L influenced the immunoassays dramatically. It could observed that the ELISA showed the highest A_{max}/IC_{50} at 0.4 mol/L Na^+, whereas the CLEIA displayed the highest RLU_{max}/IC_{50} at 0.5 mol/L Na^+. The IC_{50} values of two assays did not change markedly with pH

Table 1. Cross-Reactivity of Clothianidin toward Other Analogues

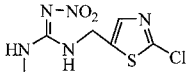
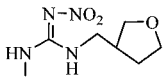
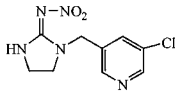
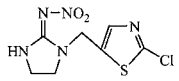
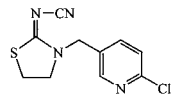
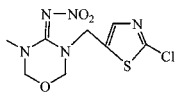
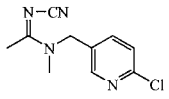
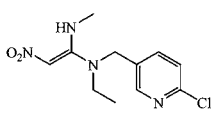
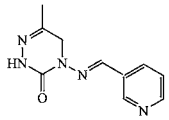
Compound	Structure	ELISA		CLEIA	
		IC_{50}(mg/L)	CR (%)	IC_{50}(mg/L)	CR (%)
Clothianidin		0.046	100	0.015	100
Dinotefuran		0.39	11.8	0.16	9.4
Imidacloprid		5.98	0.8	3.30	0.4
Imidaclothiz		7.02	0.6	4.01	0.4
Thiacloprid		>100	<0.05	>100	<0.02
Thiamethoxam		>100	<0.05	>100	<0.02
Acetamiprid		>100	<0.05	>100	<0.02
Nitenpyram		>100	<0.05	>100	<0.02
Pymetrozine		>100	<0.05	>100	<0.02

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Table 2. Recovery of Clothianidin in Spiked Samples

sample	spiked concentration (mg/kg)	ELISA mean recovery ± SD (%)	ELISA RSD (%)	CLEIA mean recovery ± SD (%)	CLEIA RSD (%)
river water	0.005	ND[a]	ND	82.7 ± 3.1	3.7
	0.01	89.2 ± 5.3	5.9	92.5 ± 4.4	4.7
	0.05	96.5 ± 1.7	1.8	95.3 ± 2.9	3.0
	0.5	102.2 ± 2.7	2.6	98.7 ± 3.1	3.1
soil	0.005	ND	ND	95.0 ± 5.6	5.9
	0.01	98.4 ± 4.3	4.4	89.5 ± 5.5	6.1
	0.05	94.5 ± 4.2	4.4	95.7 ± 2.8	2.9
	0.5	107.5 ± 7.4	6.9	100.5 ± 4.2	4.2
rice	0.005	ND	ND	107.4 ± 7.5	7.0
	0.01	99.6 ± 4.9	4.9	96.2 ± 6.0	6.2
	0.05	104.3 ± 2.4	2.3	101.2 ± 5.9	5.8
	0.5	106.4 ± 5.8	5.4	97.4 ± 4.2	4.3
cabbage	0.005	ND	ND	76.4 ± 7.2	9.4
	0.01	116.4 ± 8.5	7.3	89.4 ± 3.5	3.9
	0.05	105.4 ± 4.6	4.4	84.5 ± 4.2	5.0
	0.5	90.1 ± 4.6	5.1	92.4 ± 5.7	6.2
tomato	0.005	ND	ND	92.6 ± 6.0	6.5
	0.01	103.1 ± 7.8	7.6	90.5 ± 4.0	4.4
	0.05	89.1 ± 3.2	3.6	103.5 ± 5.6	5.4
	0.5	93.0 ± 6.4	6.9	101.7 ± 3.4	3.3

[a]ND, negative detecting (<LOD).

Table 3. Comparison of Clothianidin Residues between the Immunoassays and GC in Authentic Samples (n = 3)

sample	ELISA mean (mg/kg)	ELISA RSD (%)	CLEIA mean (mg/kg)	CLEIA RSD (%)	GC mean (mg/kg)	GC RSD (%)
paddy water	0.0502	2.8	0.0496	3.2	0.0491	2.7
soil	0.275	2.7	0.290	6.2	0.283	1.8
rice	0.0683	1.6	0.0709	2.8	0.0674	3.4
cabbage	0.0330	7.1	0.0286	3.3	0.0332	3.2
tomato	ND	ND	ND	ND	ND	ND

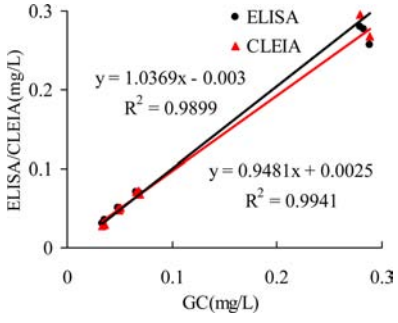

Figure 3. Correlation between the immunoassays and GC for the authentic samples.

values from 4.5 to 9.5. The ELISA was more sensitive at pH 7.5, and the RLU_{max}/IC_{50} of CLEIA reached a maximum at pH 6.5.

On the basis of these results, optimum parameters were 20% methanol, 0.4 mol/L Na^+, and pH 7.5 for ELISA. At the same time, 10% methanol, 0.5 mol/L Na^+, and pH 6.5 were chosen as optimum for the subsequent CLEIA.

Sensitivities. The standard curves for clothianidin analyzed by ELISA and CLEIA were constructed under optimal conditions. The graph between percent binding (% B/B_0) and the logarithm of concentration of clothianidin (mg/L) was plotted (Figure 2). The ELISA was shown to have an IC_{50} of 0.046 mg/L, a LOD of 0.0028 mg/L, and a linear range (IC_{20}–IC_{80}) of 0.0028–0.77 mg/L. The CLEIA showed higher sensitivity, with an IC_{50} value of 0.015 mg/L, a LOD value of 0.0014 mg/L, and a linear range of 0.0014–0.15 mg/L.

Compared to the maximum residue limit (MRL) of 0.05 mg/kg of clothianidin in the United States,[3] the sensitivity of immunoassays can meet the requirements of detection of clothianidin. The LOD values of HPLC and GC were 0.004 and 0.01 mg/L, respectively.[2,3] Immunoassays were more sensitive than instrument-based detection methods.

Specificity. The CRs for the analogues of clothianidin were tested (Table 1). The antibodies showed negligible cross-reactivity with most of analogues (CR % < 0.8%), except dinotefuran (11.8% in ELISA and 9.4% in CLEIA). The structure of clothianidin compared with that of dinotefuran has a similar *N*-methyl-*N*′-nitroguanidine moiety. Therefore, it could be assumed that the structure was identified by the antibody as characteristic of clothianidin. These suggested that the results obtained from the two immunoassays show false positive only when dinotefuran was present. It is necessary to distinguish clothianidin and dinotefuran with instrumental methods if the analytical data show a false-positive result.

Matrix Effects. Results of matrix effects showed that different sample matrices had different effects on the sensitivity of the optimized immunoassays, whereas the matrix effects of the same sample were similar for both methods. Matrix effects of soil, cabbage, and tomato samples were reduced to acceptable levels when samples were diluted at 4-fold. The rice sample matrix showed the greatest impact. As for the 8-fold-diluted rice sample, the interference had no effects on the sensitivity of the immunoassays. Considering that too much dilution would eventually reduce the limit of quantitation of samples, 4-fold dilution (soil, cabbage, and tomato) and 8-fold dilution (rice) were finally selected for subsequent immunoassay tests.

Analysis of Spiked Samples. The recoveries and the RSDs of the spiked samples are shown in Table 2. Acceptable recoveries of 89.1–116.4% for ELISA and 76.4–107.4% for CLEIA were obtained. All RSDs were below 10%, and the precision obtained for all samples met the requirement for a residue method. The recovery results were confirmed by GC for the same spiked samples. Good correlations between the results of GC and immunoassays were obtained ($y = 0.9089x + 0.0023$, $R^2 = 0.9983$) with the ELISA and ($y = 0.9901x + 0.0018$, $R^2 = 0.9937$) with the CLEIA.

Analysis of Authentic Samples. As shown in Table 3, the real samples of paddy water, soil, rice, and cabbage tested clothianidin-positive by immunoassays (from 0.0330 to 0.275 mg/L by the ELISA and from 0.0286 to 0.290 mg/L by the CLEIA), which is largely consistent with that by the GC (from 0.0332 to 0.283 mg/L). The tomato sample was negative both by immunoassays and by GC analysis. Also, no false-negative result was detected. The squared coefficients of correlation (R^2) were 0.9899 and 0.9941 for authentic samples when correlating the results of immunoassays (ELISA and CLEIA) and GC (Figure 3). Thus, the proposed immunoassays were reliable and accurate.

Comparison of Two Immunoassays. The sensitivity of an immunoassay strongly depends on the sensitivity of the detection method. The CLEIA based on the luminol/peroxide/enhancer system for HRP offers the possibility of improving the sensitivity of immunoassays with an IC_{50} of 0.015 mg/L compared to that of 0.046 mg/L by the conventional colorimetric detection of ELISA using the same antibody and HRP conjugate. It can be also observed that the CLEIA provided a lower LOD by comparison to the ELISA (0.0014 and 0.0028 mg/L, respectively). Due to the higher sensitivity of CLEIA, the spiked samples with 0.005 mg/kg could be accurately detected. Therefore, the established CLEIA was a more practical method than ELISA for clothianidin. In addition, the chemiluminescence signal of detection can be performed immediately after 3 min of substrate addition, whereas the ELISA requires 20−30 min of incubation and stop steps. Thus, CLEIA shortens the overall analytical procedure and testing time compared with ELISA. Moreover, CLEIA had the advantages of requiring less coating antigen and antibodies and higher specificity, therefore making CLEIA a more inexpensive and accurate testing method.

These results suggested that the proposed ELISA and CLEIA could be two feasible quantitative/screening methods for clothianidin in agricultural products due to their high sensitivity, rapidity, lower expenses, and high sample throughput.

■ ASSOCIATED CONTENT

Supporting Information

Table of effect of organic solvent, ionic strength, and pH value on immunoassay (Table 1S); figures of ultraviolet absorption spectra of hapten, BSA, OVA, immunogen, and coating antigen (Figure 1S), matrix effect of sample (soil, rice, cabbage, and tomato) on the sensitivity of the immunoassays (Figure 2S), and correlation between ELISA (a), CLEIA (b), and GC for the spiked samples (Figure 3S). This material is available free of charge via the Internet at http://pubs.acs.org.

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