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Development of a chemiluminescence enzyme-linked immunosorbent assay for the simultaneous detection of imidaclothiz and thiacloprid in agricultural samples

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A novel and sensitive enhanced chemiluminescence enzyme-linked immunosorbent assay (ECL-ELISA) for the simultaneous analysis of imidaclothiz and thiacloprid is described. The concentrations of coating antibodies and enzyme tracers were optimised by three different ECL-ELISA formats. Under the optimised conditions, when anti-imidaclothiz polyclonal and anti-thiacloprid polyclonal antibodies were immobilised in a single well, single-enzyme tracer ECL-ELISA was used to screen the corresponding pesticide residue, and multi-enzyme tracer ECL-ELISA was used for the analysis of imidaclothiz and thiacloprid residue mixtures. The average recoveries of the two pesticides from spiked tomato, cabbage, and rice samples were in the range of 83.7–117%. Meanwhile, the results showed that the multi-enzyme tracer ECL-ELISA could be applied to the accurate analysis of different proportions of imidaclothiz and thiacloprid (1 : 4 to 4 : 1, m/m) when the gross residues ranged from 20 to 250 μg kg^{-1}. The results of the multi-enzyme tracer ECL-ELISA in the real tomato samples correlated well with those of high-performance liquid chromatography, with a correlation coefficient of 0.996. Therefore, this new strategy for developing immunoassays is suitable for the simultaneous quantitative detection of imidaclothiz and thiacloprid residues in agricultural samples.

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Introduction

Imidaclothiz and thiacloprid are a new class of neonicotinoid pesticides against both sucking and chewing insects and have been widely used in agricultural crops.[1,2] Because the use of imidaclothiz and thiacloprid as a synergistic insecticidal composition can improve the control effect and reduce the use of pesticides, they are used against sucking insects in many crops, such as rice, fruits, and vegetables, as well as lawns.[3] These substances are promising insecticides. Therefore, a suitable method for analysing imidaclothiz and thiacloprid residues is of great significance.

The application of a traditional method for the simultaneous detection of imidaclothiz and thiacloprid, namely, high-performance liquid chromatography, has been reported.[4] Although the method has high precision and sensitivity, it requires complex sample preparation and purification procedures. Such costly and time-consuming instrumental techniques are unsuitable for monitoring a large number of samples. Immunoassays provide a simple, rapid, sensitive, and cost-efficient screening method for the analysis of pesticide residues.

To date, a broad-selective immunoassay has been developed by combining multiple specific antibodies[5] or by using a bispecific monoclonal antibody[6] as well as by employing a broad-selective antibody that originates from a multi-hapten antigen[7] or a class-selective antibody derived from a generic hapten.[8–12] By using a multi-hapten antigen to obtain the broad-specificity antibody, this assay is technically a more difficult approach to obtaining optimal immunogens and is not a controllable way of ensuring equal hapten availability to the animal immune system. Using "general-structure" haptens is the most common approach to broad-specificity antibodies. However, one precondition of using these "general-structure" haptens is that the involved pesticides must have similar molecular structures. Moreover, it is difficult to synthesise these "general-structure" haptens, and the recognition "spectrum" of the antibodies could not previously be exactly confirmed.[7] These methods allow the qualitative analysis of mixed pesticides; however, none have been applied to the direct quantitative analysis of mixed analytes because of the vast differences in assay sensitivity among various pesticides. Thus, it would be more efficient and ideal to develop a method that can be used to directly detect two or more analytes with one standard curve instead of one assay for each individual target.

The present work aims to develop a novel multi-residue immunosorbent assay for the detection of individual and gross pesticide residues of imidaclothiz and thiacloprid using the

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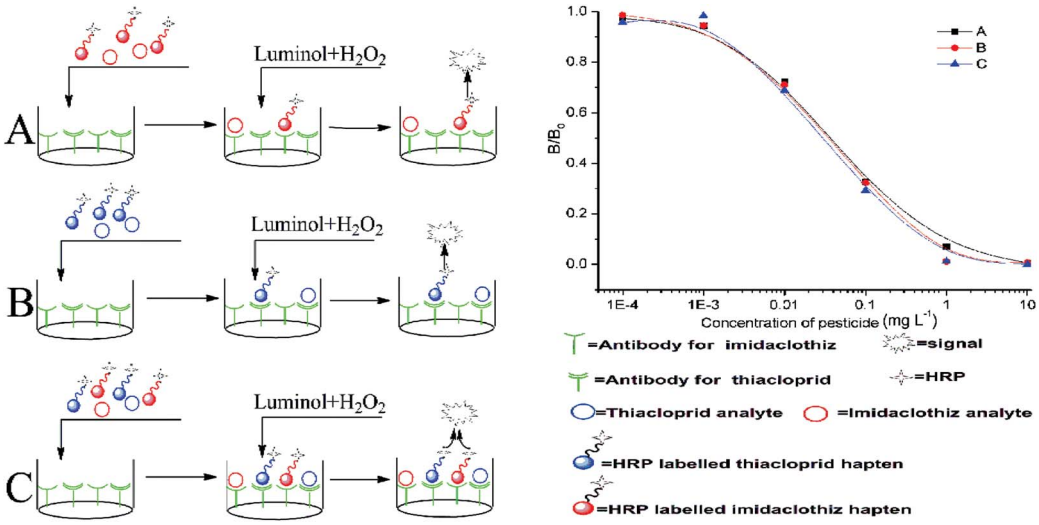

Fig. 1 Schematic of the proposed multi-residue immunosorbent assay. Anti-imidaclothiz and anti-thiacloprid antibodies were immobilised in a single well. (A) Imidaclothiz in single-enzyme tracer ECL-ELISA, (B) thiacloprid in single-enzyme tracer ECL-ELISA, and (C) imidaclothiz and thiacloprid in multi-enzyme tracer ECL-ELISA.

identical standard curve (Fig. 1) and evaluate the analytical reliability using tomato, cabbage, and rice as model samples as well as to suggest the potential utility of the simultaneous detection of imidaclothiz and thiacloprid residues in agricultural samples. The use of an assay with one standard curve for multi-residues provides a new strategy for the direct quantitative analysis of the total quantity of mixed pesticides.

Experimental section

Chemicals and instruments

Imidaclothiz and thiacloprid were obtained from Jiangsu Pesticide Research Institute (Jiangsu, China). Imidaclothiz wettable powder (WP, 10%) was obtained from Nantong Jiangshan Agrochemical and Chemicals Co., Ltd. (Jiangsu, China). Thiacloprid suspension concentrate (SC, 48%) was obtained from Tianjin Xingguang Agrochemical and Chemicals Co., Ltd. (Tianjin, China). Bovine serum albumin (BSA), ovalbumin (OVA), horseradish peroxidase (HRP), Freund's complete and incomplete adjuvants, polyoxyethylene sorbitan monolaurate (Tween-20), luminol, and *p*-iodophenol were purchased from Sigma Chemical Co. (Shanghai, China). All other chemicals and organic solvents were of reagent grade. Chemiluminescence and absorbance measurements were conducted with a SoftMax Pro 5.4.1 Microplate Luminometer (Molecular Devices, USA). Transparent 96-well microtiter plates (Roskilde, Denmark) were used for the colourimetric assay and white polystyrene microtiter wells (Costar, USA) for chemiluminescent detection. Imidaclothiz and thiacloprid were detected using an Agilent 1200 HPLC chromatograph (Agilent, USA). Hapten densities of the HRP-conjugates were analysed by using an Ultraflextreme MALDI-TOF-MS (Bruker, USA).

Buffers and solutions

The buffers and solutions used include carbonate-buffered saline (CBS, 0.05 mol L^{-1}, pH 9.6), phosphate-buffered saline (PBS, 0.01 mol L^{-1}, pH 7.4), phosphate-buffered saline containing 0.05% Tween-20 (PBST), and substrate solution (1.0 mmol L^{-1} luminol, 0.025 mmol L^{-1} *p*-iodophenol, 1.7 mmol L^{-1} H_2O_2 in 0.1 mol L^{-1} Tris–HCl buffer, pH 8.6).

Hapten synthesis

Thiacloprid hapten (Th) was synthesised according to Liu *et al.*,[13] and imidaclothiz hapten (Im) was synthesised according to Fang *et al.*[14]

Preparation of protein conjugates

Protein conjugates were synthesised by the activated ester method.[15] Haptens were conjugated to BSA for use as immunogens and the carrier HRP as enzyme tracers. The conjugates were dialysed in PBS at 4 °C in darkness for 72 h and stored at −20 °C. The conjugation was confirmed by UV-Vis spectroscopy. The number of hapten molecules per molecule of protein (hapten density) of the HRP-conjugate was analysed by using MALDI-TOF-MS and the hapten density of the immunogen was estimated directly by the molar absorbance.[16]

$$\text{Hapten density} = (\varepsilon_{\text{conjugation}} - \varepsilon_{\text{protein}})/\varepsilon_{\text{hapten}}$$

Immunisation and antibody preparation

Each immunogen was used to immunise two male New Zealand white rabbits according to a previously described method.[15] The rabbits had free access to drinking water and a commercial standard laboratory diet (CZZ, Nanjing, China) and were housed according to the EEC 609/86 Directives regulating the welfare of experimental animals. The first injection was 2 mg of immunogen 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 the back of each rabbit. An additional 3 mg of the immunogen, emulsified with

Freund's incomplete adjuvant, was injected as a booster shot after 3 weeks. The booster shoots were given four times in two-week intervals. Ten days after the fourth injection, the immunised rabbit was bled. The antiserum was purified using the salting-out method with caprylic acid–ammonium sulfate[17] and stored at −20 °C after freeze-drying.

Direct competitive enhanced chemiluminescence enzyme-linked immunosorbent assay (ECL-ELISA)

Direct competitive format A ECL-ELISA. Micro-well plates were coated with 100 μL per well of anti-thiacloprid antibody or anti-imidaclothiz antibody in CBS. The plates were washed five times with PBST, and unbound active sites were blocked with 200 μL of 2.5% (w/v) skim milk per well for 1 h at 37 °C. After the plates were washed, 50 μL per well of pesticide standard and 50 μL per well of Th–HRP or Im–HRP in PBS were added, and the plates were incubated for 1 h. The plates were then washed, and peroxidase activity was determined by adding 150 μL per well of a freshly prepared substrate solution. Chemiluminescence emission was measured immediately after the addition of the substrate (5 min, 1 s per well).

Direct competitive format B ECL-ELISA. The antibody for imidaclothiz and the antibody for thiacloprid were simultaneously coated in a single well. The result was then subjected to the same preparation as format A ECL-ELISA.

Direct competitive format C ECL-ELISA. The antibodies for thiacloprid and imidaclothiz were simultaneously coated in one micro-well, and a solution of multi-enzyme tracers was used for multi-analytes. The result was then subjected to the same preparation as format A ECL-ELISA.

Sample analysis

Sample collection. Tomato, cabbage, and rice samples without imidaclothiz and thiacloprid were obtained from a local supermarket.

Analysis of spiked samples. Mixture standards were prepared by mixing imidaclothiz and thiacloprid standards in different proportions (1 : 4, 1 : 1, and 4 : 1, m/m) in methanol and stored at 4 °C in dark vials.

Tomato, cabbage, and rice samples (10.0 g) were ground, spiked with mixture standards at 20, 40, and 250 μg kg^{-1}. The samples were thoroughly mixed and then allowed to stand at room temperature overnight. The samples were mixed with 10 mL methanol and submitted to ultrasonic extraction for 30 min. After being centrifuged at 4000 × *g* for 10 min, the supernatant was diluted ten times with PBS and analysed by ECL-ELISA. Each sample was treated and measured in triplicate. The recoveries and relative standard deviation (RSD) were calculated.

Analysis of imidaclothiz and thiacloprid residues on real tomato samples. Imidaclothiz WP (10%) and thiacloprid SC (48%), diluted with water, were sprayed onto tomato samples. Tomato samples (0.5 kg) were then picked randomly at 2 h and 1, 2, 3, 5, 7, 10, and 14 days and simultaneously analysed with ECL-ELISA and HPLC.[18,19]

Data analysis

Standards and samples were run in triplicate wells, and the mean chemiluminescence intensity values were calculated. Standard curves were obtained by plotting the chemiluminescence intensity against the logarithm of analyte concentration and fitted to a four-parameter logistic equation.

$$y = \{(A - D)/[1 + (x/C)^B]\} + D$$

where A is the asymptotic maximum (chemiluminescence intensity in the absence of analyte, RLU_{max}), B is the curve slope at the inflection point, C is the x value at the inflection point (corresponding to the analyte concentration that reduces RLU_{max} to 50%), and D is the asymptotic minimum (background signal).

Results and discussion

Conjugation identification

The effectiveness of the conjugation reaction was monitored by UV spectroscopy.[20] As shown in Fig. 2, obvious differences were observed between the spectra of the hapten, conjugates, and the corresponding carrier protein. The peak shape of the conjugates showed that the carrier protein and hapten had been coupled successfully. The hapten density of the immunogen was 13.5 : 1 for imidaclothiz and 10 : 1 for thiacloprid by the molar absorbance, and the hapten density of hapten–HRP was 2.5 : 1 for imidaclothiz and 1.6 : 1 for thiacloprid by using MALDI-TOF-MS.

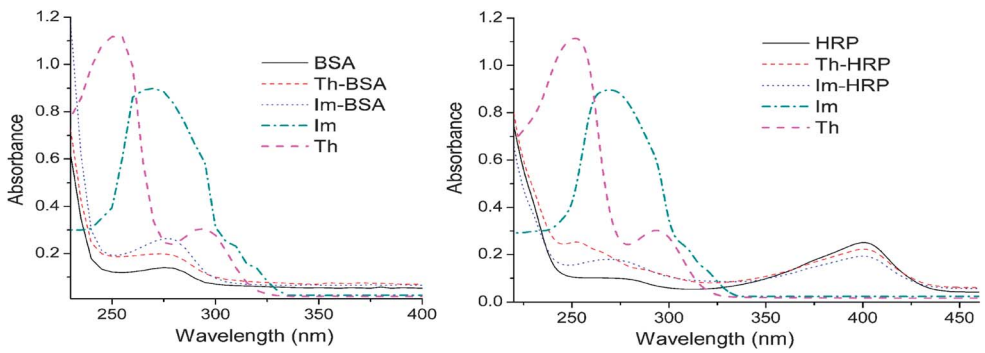

Fig. 2 Ultraviolet absorption spectra of hapten, BSA, HRP, immunogen, and hapten–HRP.

Antibody characterization

The affinity constant is a numerical constant used to describe the bonding affinity of antigen–antibody at equilibrium.[21] The affinity constants of the anti-imidaclothiz antibody and anti-thiacloprid antibody were tested by using direct non-competitive ELISA. According to the results, the affinity constants of the anti-thiacloprid antibody (1.6×10^7 L mol^{-1}) and anti-imidaclothiz antibody (3×10^7 L mol^{-1}) were very similar. So it could be concluded that the similar affinity constant was a prerequisite for the development of such an assay format.

Feasibility of the simultaneous detection of imidaclothiz and thiacloprid

The antibodies for imidaclothiz and thiacloprid were coated in different wells in an ELISA plate. When Im–HRP was added to the coated wells, only those wells coated with the antibody for imidaclothiz had colour development; there was no colour in wells coated with the antibody for thiacloprid. Additionally, there was no colour in wells coated with the antibody for imidaclothiz when Th–HRP was added. It was concluded that the presence of Im–HRP (or Th–HRP) did not affect the reaction between Th–HRP (or Im–HRP) and the anti-thiacloprid (or anti-imidaclothiz) antibody.

Optimisation of working concentration

To develop an ECL-ELISA for detecting both imidaclothiz and thiacloprid pesticide residues using the same standard curve, the concentrations of coating antibodies and enzyme tracers were optimised.

Choice of coating antibodies. To select the most suitable combination of antibodies for imidaclothiz and thiacloprid, the two antibodies were separately screened against the single-enzyme tracer using a direct competitive format A ECL-ELISA. The polyclonal antibody for imidaclothiz (1.25 and 2.5 μg per well) and that for thiacloprid (0.31, 0.62, and 1.25 μg per well) were preliminarily selected based on their similar sensitivities (IC_{50}) and were mixed in six different combinations (I: 2.5 + 1.25, II: 2.5 + 0.62, III: 2.5 + 0.31, IV: 1.25 + 1.25, V: 1.25 + 0.62, and VI: 1.25 + 0.31).

The antibody combinations were coated in one well for further optimisation using format B ECL-ELISA. The IC_{50} and limit of detection (IC_{20}) of imidaclothiz and thiacloprid are shown in Table 1. The antibody combinations (III, IV, and V) were separately coated, and the IC_{20} values for imidaclothiz were 5.9, 22.3, and 7.7 μg L^{-1}, and the IC_{20} values for thiacloprid were 5.0, 14.8, and 10.7 μg L^{-1}, respectively. When the antibody combinations (III, IV, and V) were coated, the corresponding IC_{20} value for imidaclothiz and thiacloprid was closer than that of other combinations (I, II, and VI). When the antibody combinations (III and V) were coated, the IC_{50} values for imidaclothiz and thiacloprid differed substantially, but IC_{50} values for combination IV were almost identical. Hence, combination IV was used for the coating antibodies in the assay.

Table 1 Choice of antibody combinations

Combination	Coating antibody concentration[a] (μg per well)	Im–HRP IC_{50} (μg L^{-1})	Im–HRP IC_{20} (μg L^{-1})	Th–HRP IC_{50} (μg L^{-1})	Th–HRP IC_{20} (μg L^{-1})
I	2.5 + 1.25	59.2	6.6	194.1	14.8
II	2.5 + 0.62	46.5	4.9	148.3	10.7
III	2.5 + 0.31	53.7	5.9	106.2	5.0
IV	1.25 + 1.25	123.1	22.3	166.4	14.8
V	1.25 + 0.62	59.4	7.7	135.7	10.7
VI	1.25 + 0.31	74.1	10.9	403.5	52.5

[a] Coating antibody concentration (anti-imidaclothiz polyclonal antibody + anti-thiacloprid polyclonal antibody).

Table 2 Choice of enzyme tracers with combination IV as the coating antibodies

Enzyme tracer (ng per well)		IC_{50} (μg L^{-1})	IC_{20} (μg L^{-1})	Slope	R^2 of linear equation
Im–HRP	25	18.9	2.2	−0.320	0.970
	50	35.5	3.6	−0.309	0.989
	75	48.6	4.4	−0.289	0.997
	100	74.8	7.9	−0.307	0.989
	125	91.6	15.7	−0.392	0.993
	150	150.0	25.8	−0.388	0.998
Th–HRP	12.5	40.0	5.0	−0.333	0.991
	25	30.9	3.5	−0.311	0.992
	50	89.5	16.8	−0.324	0.996
	75	125.3	20.7	−0.385	0.949
	100	192.5	19.5	−0.302	0.971
	125	232.2	35.7	−0.369	0.985

Choice of enzyme tracers. At the optimal concentration of coating antibodies, the enzyme tracer dilution was optimised using the format B ECL-ELISA to obtain the same standard curve. The IC_{50}, IC_{20}, and curve slope were important parameters to study the parallelism of the standard curves.

As shown in Table 2, the assay using 50 ng per well of Im–HRP afforded an imidaclothiz standard curve with a slope of −0.309 and an IC_{50} value of 35.5 μg L^{-1}, and when Th–HRP (25 ng per well) was used in the assay, the thiacloprid curve parameters were −0.311 and 30.9 μg L^{-1} for the slope and the IC_{50}, respectively. The results showed that the imidaclothiz standard curve was generally similar to the thiacloprid curve, so Im–HRP (50 ng per well) and Th–HRP (25 ng per well) were used as enzyme tracers.

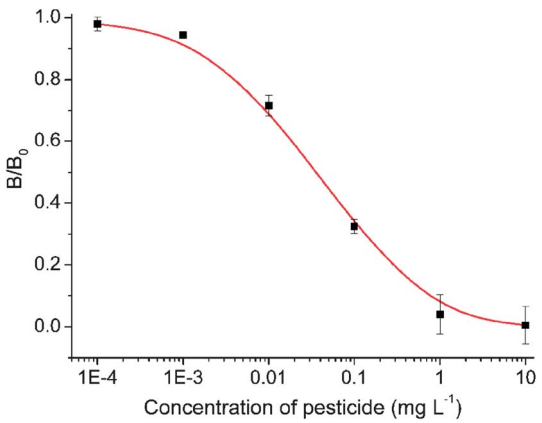

Fig. 3 Standard curve for imidaclothiz and thiacloprid.

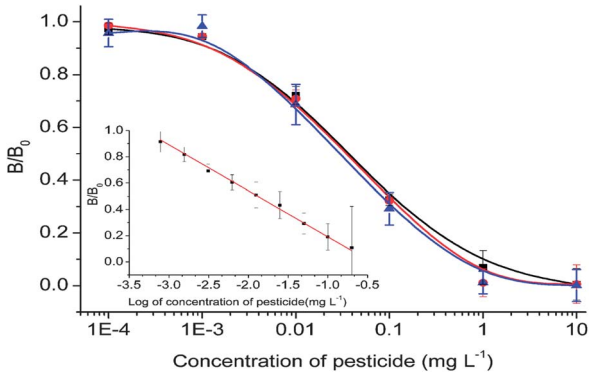

Fig. 4 Calibration curve for pesticides. (■) Single-enzyme tracer format with Im–HRP (50 ng per well) for screening imidaclothiz, (●) single-enzyme tracer format with Th–HRP (25 ng per well) for screening thiacloprid, and (▲) multi-enzyme tracer format with a mixture of Im–HRP (25 ng per well) and Th–HRP (12.5 ng per well) for screening imidaclothiz and thiacloprid.

Based on the similar linearity of imidaclothiz and thiacloprid, a fitting curve was constructed. The central section of the curve, with a nearly linear response, was accepted as the assay working range (Fig. 3). The working range for imidaclothiz and thiacloprid was 3.5–275 μg L^{-1}, and the limit of detection was 1.7 μg L^{-1}. The established maximum residue limit (MRL) of thiacloprid[22] is 0.02 mg L^{-1}, whereas no MRLs are provided for imidaclothiz in EC or other standard regulations. Thus, the developed single-enzyme tracer ECL-ELISA can satisfy the detection requirements for pesticide residues in the samples.

Multi-enzyme tracer ECL-ELISA

With the optimal concentrations of coating antibodies and enzyme tracers, the multi-enzyme tracer ECL-ELISA (format C) was developed for the simultaneous detection of imidaclothiz and thiacloprid.

The competitive curve of the mixture standard (1 : 1, m/m) was obviously different from that of the single-enzyme tracer format when Im–HRP (50 ng per well) and Th–HRP (25 ng per well) were mixed as multi-enzyme tracers, while the three curves were almost the same when the mixture of Im–HRP (25 ng per well) and Th–HRP (12.5 ng per well) was used as an enzyme tracer (Fig. 3). Therefore, the mixture of Im–HRP (25 ng per well) and Th–HRP (12.5 ng per well) was chosen as a multi-enzyme tracer. Based on the similar linear sections of their standard curves, a calibration curve was obtained (inset of Fig. 4). The linear working range defined as the concentration resulting in 20–80% inhibition was 3.6–275 μg L^{-1}, and the limit of detection was 1.8 μg L^{-1}.

Recovery of spiked samples

The spiked recoveries were used to evaluate the potential application of single-enzyme and multi-enzyme tracer ECL-ELISA to

Table 3 Recovery of spiked samples

Sample	Total spiked level (μg kg^{-1})	Theoretical (μg kg^{-1}) Imidaclothiz + thiacloprid	Found (μg kg^{-1}) ± RSD (%) ($n = 3$) Imidaclothiz	Thiacloprid	Gross
Tomato	250	50 + 200	44.6 ± 2.2	234.8 ± 6.2	272.8 ± 5.8
		125 + 125	130.5 ± 1.8	128.8 ± 3.3	260.5 ± 3.2
		200 + 50	202.0 ± 6.1	48.1 ± 5.3	263.1 ± 2.9
	40	8 + 32	7.8 ± 4.2	34.5 ± 3.8	43.0 ± 5.8
		20 + 20	18.6 ± 3.9	19.4 ± 1.0	41.3 ± 4.1
		32 + 8	32.8 ± 1.2	8.0 ± 5.8	39.2 ± 2.0
	20	4 + 16	3.4 ± 7.3	15.3 ± 2.8	19.1 ± 3.8
		10 + 10	8.9 ± 4.2	8.8 ± 3.6	17.2 ± 5.1
		16 + 4	14.6 ± 4.3	3.4 ± 6.3	16.7 ± 3.8
Cabbage	250	50 + 200	46.3 ± 5.8	211.5 ± 3.8	263.9 ± 2.0
		125 + 125	126.5 ± 4.2	127.5 ± 4.8	260.3 ± 2.0
		200 + 50	203.2 ± 2.2	52.3 ± 5.3	266.5 ± 3.0
	40	8 + 32	7.5 ± 7.4	33.9 ± 7.6	43.1 ± 6.6
		20 + 20	19.9 ± 7.0	19.5 ± 5.4	42 ± 7.9
		32 + 8	33.8 ± 4.1	7.9 ± 5.8	39.7 ± 4.1
	20	4 + 16	3.8 ± 6.1	15.6 ± 6.0	20.2 ± 3.8
		10 + 10	8.8 ± 8.7	9.5 ± 9.1	18.4 ± 6.2
		16 + 4	16.2 ± 8.6	3.5 ± 8.6	17.9 ± 5.8
Rice	250	50 + 200	48.0 ± 4.2	210.4 ± 2.1	262.2 ± 2.2
		125 + 125	123.6 ± 3.1	126.0 ± 2.7	260.6 ± 3.7
		200 + 50	203.1 ± 1.6	52.2 ± 2.4	262.2 ± 1.6
	40	8 + 32	7.5 ± 6.6	33.9 ± 5.5	43.8 ± 5.0
		20 + 20	21.3 ± 8.7	20.8 ± 6.9	42.3 ± 4.2
		32 + 8	32.6 ± 4.0	7.8 ± 4.1	41.4 ± 4.3
	20	4 + 16	3.93 ± 5.1	15.9 ± 4.6	21.4 ± 5.4
		10 + 10	9.6 ± 8.7	10.5 ± 8.0	18.7 ± 6.4
		16 + 4	16.8 ± 3.2	3.5 ± 6.6	18.8 ± 4.4

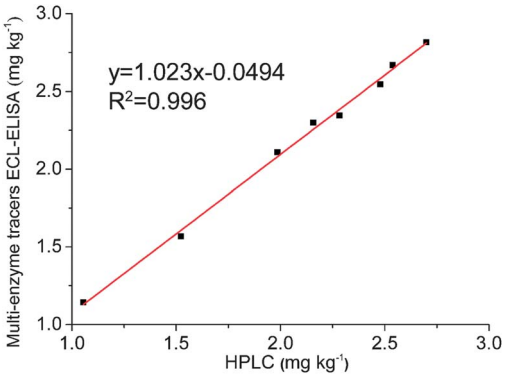

Fig. 5 Correlation between multi-enzyme tracer ECL-ELISA and HPLC results for real tomato samples.

detect imidaclothiz and thiacloprid residues in spiked samples. The recoveries and relative standard deviation (RSD) are presented in Table 3. The matrix interference of the samples can be minimised by 10-fold dilution with PBS. For single-pesticide residues of imidaclothiz and thiacloprid, the average recoveries were from 84.4% to 117%, and the RSD ranged from 1.0% to 7.3%. For mixtures of imidaclothiz and thiacloprid, the average recoveries were 83.7% to 109%, and the RSD ranged from 2.0% to 5.8%. Thus, the multi-enzyme tracer ECL-ELISA can be applied for the accurate analysis of different proportions of imidaclothiz and thiacloprid (1 : 4 to 4 : 1, m/m) when the gross residues range from 20 to 250 μg kg^{-1}. This finding indicated that the established ECL-ELISA was a potential screening tool for imidaclothiz and thiacloprid residue determination.

Validation of the assay with HPLC

The dissipation of imidaclothiz and thiacloprid applied to real tomato samples was monitored with the ECL-ELISA and HPLC methods (Fig. 5). The ECL-ELISA results agreed well with the HPLC results ($y = 1.023x - 0.0494$, $R^2 = 0.996$). These results suggested that the imidaclothiz and thiacloprid in the real samples could be simply, rapidly, and accurately detected by ECL-ELISA.

Conclusions

To the best of our knowledge, this paper reports for the first time the investigation of the direct detection of gross pesticide residues using identical standard curves. The screening method of coating antibodies and enzyme tracers was accomplished in a novel, simple fashion using three different ECL-ELISA formats. Several spiked agricultural samples were analysed by ELISA, and the accuracy and precision were well within the requirements for residue analysis. The multi-enzyme tracer ECL-ELISA results for the real tomato samples were confirmed by HPLC with a high correlation coefficient of 0.996.

The use of this method with one standard curve in the direct quantitative analysis of the total quantity of imidaclothiz and thiacloprid has been reported for the first time, with a substantial advantage in terms of reducing detection time and workload. The assay time and costs of ECL-ELISA for imidaclothiz and thiacloprid were half of those of previous immunoassays. In addition, the luminol substrate of ECL-ELISA is safer than the *o*-phenylenediamine substrate of previous ELISA methods.[13,14] This study provides a new strategy for the development of multi-residue immunoassays for the direct quantitative analysis of the total quantity of mixed analytes and has potential application for many other pesticides and toxins with similar inhibition curves.

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