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Development of a sensitive indirect competitive enzyme-linked immunosorbent assay based on the monoclonal antibody for the detection of benzothiostrobin residue†

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An indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) based on monoclonal antibodies (MAbs) for benzothiostrobin has been developed. The hapten of benzothiostrobin was synthesized and conjugated to bovine serum albumin and ovalbumin to generate an immunogen and a coating antigen. The immunogen was used to immunize BALB/c mice, resulting in anti-benzothiostrobin MAb. Under optimal conditions, the half maximal inhibition concentration (IC_{50}) and the limit of detection (IC_{10}) of the developed ic-ELISA were 7.55 and 0.428 μg L^{-1}, respectively. The cross-reactivity (CR) was less than 0.05% for the tested structural analogues and regarded as negligible except for pyraclostrobin, which exhibited a CR of 0.34%. The recoveries of benzothiostrobin ranged from 80.43 to 113.83% in environmental and agricultural samples, respectively, which conformed to the requirements for residue detection. The amount of benzothiostrobin detected by ic-ELISA in the samples was significantly (R^2 = 0.9894, y = 1.0867x + 0.0318) correlated with that detected by high-performance liquid chromatography (HPLC). The current study indicates that the established ic-ELISA is a potentially useful tool for detecting benzothiostrobin in environmental and agricultural samples.

1. Introduction

Strobilurin fungicide belongs to a new class of fungicides that exhibits broad-spectrum, efficient and environmentally friendly features. This new class of fungicides has attracted considerable attention from researchers. Benzothiostrobin, which is a fungicide developed by the Central China Normal University, is a novel strobilurin fungicide that exhibits highly efficient control of most fungal diseases in crops,[1] and the patents of preparing and using benzothiostrobin have been applied in China, United States, Europe, and worldwide (Pub. no.: CN 102302012 B, CN 101379967 A, CN 101268780 B, US 2010/0292285 A1, 09741682.0, WO 2007/073637 A1, WO 2009/135407 A1). To the best of our knowledge, benzothiostrobin was transferred to Jiangsu Seven Continent Green Chemical Co., Ltd. in 2010. Therefore, benzothiostrobin, at least in China, has potential prospective applications. In order to keep its potential residual risk under control, the development of a rapid, sensitive, and economical method for detecting benzothiostrobin in agricultural produce and the environment as a technical support is imperative and important.

High-performance liquid chromatography (HPLC) has been used successfully for the detection of benzothiostrobin and other pesticides,[2] which is characterized by low limits of detection (LOD) as well as high precision and sensitivity. However, the analytical methods are costly, time-consuming, and not suitable for the analysis of large numbers of samples.[3,4] During the past decade, many enzyme-linked immunosorbent assays (ELISAs) have been published for the analysis of residues for a broad variety of pesticides in different types of food samples,[5–11] and several immunochemical methodologies have been approved to analyze chemical residues in food, feed and environmental samples. In comparison, ELISAs provide a fast, sensitive, cost-effective, and selective method for the detection of pesticide residues. Several immunoassays for strobilurin fungicides have been reported.[12–18] However, studies on antibodies and the development of immunoassays for benzothiostrobin residue have not been published.

In this paper, an indirect competitive ELISA (ic-ELISA) method was developed based on monoclonal antibodies for the detection of benzothiostrobin. Due to this method's high sensitivity and accuracy, ic-ELISA provides an alternate method for the detection of benzothiostrobin in environmental and agricultural samples.

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2. Materials and methods

2.1. Materials and instruments

Benzothiostrobin (99.02%) was obtained from the Central China Normal University (Wuhan, China). The pesticide standards used for cross-reactivity studies were purchased from Dr Ehrenstorfer GmbH (Germany). Bovine serum albumin (BSA), ovalbumin (OVA), complete and incomplete Freund's adjuvants, *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC), *o*-phenylenediamine (OPD), and polyoxyethylene sorbitan monolaurate (Tween-20) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG was purchased from DingGuo ChangSheng Biotechnology Co. (Beijing, China). All the other reagents were of analytical grade. The BALB/c mice were purchased from the Center of Comparative Medicine of Yangzhou University (Yangzhou, China). All the animal studies were performed in accordance with institutional guidelines.

Mass spectral (MS) data were obtained with an HPLC-QTOF instrument (Bruker, Germany). Nuclear magnetic resonance (NMR) spectra were recorded on a DRX500 spectrometer (Bruker, Germany). Ultraviolet-visible (UV-Vis) spectra were obtained on a UV-2550 spectrophotometer (Shimadzu, Japan). The 96-well transparent microplates were purchased from Nunc (Roskilde, Denmark). The absorbances were read with an I-mark microtiter plate reader (Bio-RAD, USA) at 490 nm, and the ELISA plates were washed with a Wellwash Plus (Thermo, USA). Benzothiostrobin was detected using Agilent 1200 HPLC (Agilent, USA). The antibody was freeze-dried using a vacuum freeze-drying machine (Thermo Savant, USA). The protein A column was purchased from the Pall Corporation (Pall, USA).

2.2. Buffers and solutions

Phosphate-buffered saline (PBS, 0.01 mol L^{-1}, pH 7.4), carbonate-buffered saline (CBS, 0.05 mol L^{-1}, pH 9.6), and phosphate-buffered saline containing 0.05% Tween-20 (PBST) were used. The tetramethylbenzidine (TMB) solution contained 0.4 mmol L^{-1} TMB and 3 mmol L^{-1} H_2O_2 in a citrate buffer (pH 5.0).

2.3. Hapten synthesis

The synthesis route of the hapten is shown in Fig. 1. Here, 8.02 g of benzothiostrobin (0.02 mol) was added to 10 mL of 10 mol L^{-1} LiOH (0.1 mol), and the mixture was stirred at room temperature for 4 h. Then, 30 mL of water was added to the mixture followed by extraction with ethyl acetate. The aqueous phase was adjusted to pH 3.0 with 10% HCl and extracted with ethyl acetate, and the organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to obtain a yellow oil, which was purified on a silica gel column eluted using 150 mL of methanol–dichloromethane (1 : 50 v/v) and 200 mL of methanol–dichloromethane (1 : 30 v/v).[19] The second fraction was collected and concentrated to obtain hapten, which was characterized by ESI-MS and NMR: ESI-MS, *m*/*z*: 346 [M + H^+] and 368 [M + Na^+]; ^{1}H NMR (400 MHz, DMSO) δ: 3.79 (s, 2H, CH_2COO), 3.86 (s, 3H, OCH_3), 4.67 (s, 2H, SCH_2), 7.01 (dd, 1H, ArH), 7.32–7.22 (m, 3H, ArH), 7.47 (d, 1H, ArH), 7.52 (m, 1H, ArH), 7.88 (m, 1H, ArH), 12.52 (s, 1H, COOH).

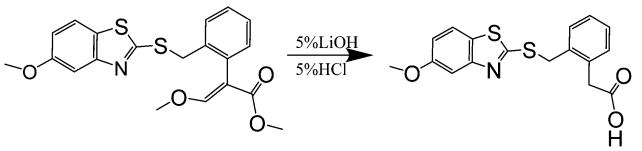


Fig. 1 Synthetic route of the benzothiostrobin hapten.

2.4. Preparation of hapten–protein conjugates

The hapten was conjugated with BSA *via* the carbodiimide method to produce an immunogen and conjugated with OVA *via* the mixed anhydride method to produce a coating antigen.[20] The conjugates were dialyzed in PBS over 72 h at 4 °C and stored at −20 °C. The formations of conjugates were confirmed by UV-Vis spectroscopy, and the hapten densities (the number of hapten molecules per molecule of protein) of the conjugates were estimated according to the following formula:

$$KA_x = \varepsilon A_x/(\rho_x/M_x),$$

$$KB_x = \varepsilon B_x/(\rho_x/M_x)$$

$$C_a/C_b = (\varepsilon C_a \times KB_b - \varepsilon C_b \times KB_a)/(\varepsilon C_b \times KA_a - \varepsilon C_a \times KA_b)$$

where A, B, and C represent the hapten, protein and conjugation, respectively; *x* represents a and b; εA_a, εB_a and εC_a are their ultraviolet absorbances at the characteristic absorption wavelength of hapten and εA_b, εB_b and εC_b are their ultraviolet absorbances at the characteristic absorption wavelength of the protein; ρ is the concentration; and *M* is the molar mass.[21,22]

2.5. Immunization and monoclonal antibody preparation

Six-week-old female BALB/c mice were immunized with immunogen *via* an intraperitoneal injection according to the method described by Kishiro *et al.*[23] The dosage of the first immunogen for each mouse was 100 μg (7.93 mg mL^{-1}) dissolved in physiological saline and emulsified with an equal volume of Freund's complete adjuvant. After 3 weeks, four subsequent injections were given at 2-week intervals using immunogen emulsified with incomplete Freund's adjuvant. The antisera were obtained from the tail vein of the mouse one week after the immunization, and the sera were tested by ic-ELISA to determine their ability to conjugate with benzothiostrobin. The mouse that exhibited the strongest response was used for the peritoneal cavity injections of 200 μg of immunogen in PBS 1 week after the last immunization. Three days after the booster injection, cell fusion was performed according to Nowinski *et al.*[24] Mouse spleen lymphocytes were fused with SP2/0 myeloma cells in a 5 : 1 ratio. The fused cells were cultured in a hypoxanthine–aminopterin–thymidine (HAT) medium at 37 °C in an atmosphere of 5% CO_2. Half of the media in the wells were replaced by fresh HAT media every third day. Fourteen days after cell fusion, the HAT media were changed to HT media. Culture supernatants were screened for their ability to

recognize benzothiostrobin, and hybridoma cells in the ELISA positive wells were cloned using the limiting dilution method. Stable antibody-producing clones were expanded. Ascites obtained from BALB/c mice were purified using a protein A column and stored at −20 °C after freeze-drying. The subtyping of MAb was identified by "mouse monoclonal antibody isotyping reagents" (Sigma).

2.6. Immunoassay procedure

ELISA was performed on 96-well polystyrene microplates. The coating antigen (benzothiostrobin–OVA) was diluted with CBS, pipetted into the wells (100 μL per well), and incubated overnight at 4 °C. The plate was washed 5 times with PBST and blocked by adding 1% OVA/PBS (200 μL per well), followed by incubation for 50 min at 37 °C. After 5 repeated washings with PBST, either the sample or the standard in the PBS containing methanol (50 μL per well) was added, followed by the addition of the diluted antibody (50 μL per well, in PBS) and incubated for 1 h at 37 °C. After an additional wash, the diluted goat anti-mouse IgG-HRP (1 : 10 000 in PBS, 100 μL per well) was dispensed into each well and incubated for 1 h at 37 °C. Then, the plates were washed again, and the substrate solution was added (100 μL per well). The reaction was terminated with sulfuric acid (2 mol L^{-1}, 50 μL per well) after 15 min of incubation. The absorbance was measured at 490 nm.

2.7. Assay optimization

The optimal concentrations of coating antigen and antibody were confirmed using the two-dimensional checkerboard method.[25] The experimental parameters including the organic solvent, ionic strength, and buffer pH value were sequentially studied to improve the sensitivity of the immunoassay. Competitive curves were run in PBS solutions containing different concentrations of methanol (from 5 to 30%, v/v) and Na^+ (0.1, 0.2, 0.3, 0.4 and 0.5 mol L^{-1}) as well as with various pH values (5.5, 6.5, 7.5 and 8.5), and the evaluations of the parameters were based on the A_{max}/IC_{50}, IC_{50} and the squared coefficients of correlation (R^2) of the linear equation.[26]

A standard curve for benzothiostrobin was obtained under the optimum conditions by plotting the percent binding (% B/B_0) as a function of the concentration of benzothiostrobin (C). The % B/B_0 was calculated using the following formula:

$$\% \; B/B_0 = [(A_x - A_{min})/(A_{max} - A_{min})] \times 100$$

where A_x is the absorbance of the sample, A_{max} is the absorbance in the absence of the analyte, and A_{min} is the absorbance of the background.

2.8. Cross-reactions

The cross-reactivity (CR) of ic-ELISA was studied by detecting the analogues of benzothiostrobin.[27] The CR values were calculated with the following formula:

$$CR\% = (IC_{50} \text{ of benzothiostrobin}/IC_{50} \text{ of analogue}) \times 100$$

2.9. Analysis of spiked samples

To evaluate the accuracy and precision of the immunoassays, the recoveries of the spiked samples were studied by ELISA. Ten grams of the blank samples (including river water, soil, rice, pear, or tomato sample) were spiked with benzothiostrobin at 0.01, 0.05, or 0.5 mg kg^{-1} and stored overnight. The river water samples were mixed with the same volume of PBS containing 10% methanol and analyzed by ELISA. Other samples were extracted twice by sonication in 20 mL of methanol for 10 min and centrifuged for 10 min at 4000 rpm.[28] After dilution at appropriate multiple steps, the solutions were analyzed *via* the immunoassays. The spiked recoveries were used to confirm the accuracy of ic-ELISA.

The matrix interferences of the samples were analyzed by testing 2 to 20-fold dilutions with PBS containing methanol.[29] The matrix interference was determined by comparing the benzothiostrobin standard curve prepared in matrix-free PBS with calibration curves prepared with a series of diluted matrix extracts.

2.10. Practical application of the immunoassays

Soil and pear (1, 3, 7, 14 days after sprayed) were collected from farms where benzothiostrobin had been used in Nanjing, China. The concentrations of benzothiostrobin in the samples were simultaneously analyzed by ELISA and HPLC to evaluate the correlation between the two methods. For ELISA, the extraction and analysis of the samples were performed as described above. For HPLC, the samples were extracted twice by sonicating in 20 mL of acetonitrile for 10 min and centrifuging for 10 min at 4000 rpm. The supernatant was filtered through anhydrous sodium sulfate and concentrated. Then, the concentrates were diluted with 2 mL of acetonitrile and detected by HPLC with SB-C_{18} column (250 mm × 4.6 mm i.d.) at 230 nm, and acetonitrile–0.05% trifluoroacetic acid (65 : 35, v/v) was used as the mobile phase at a flow rate of 0.8 mL min^{-1}.[2]

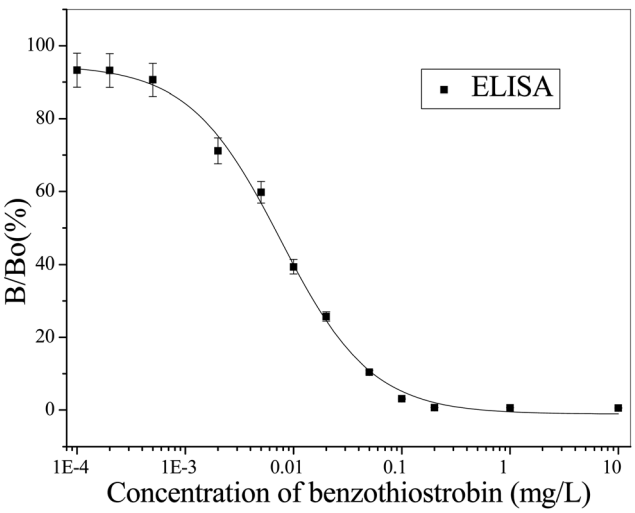


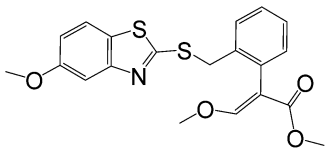
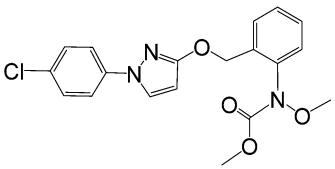
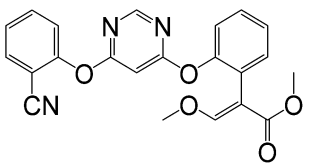
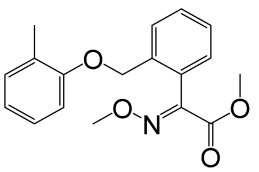
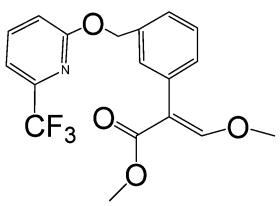
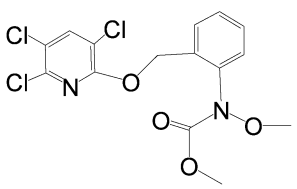
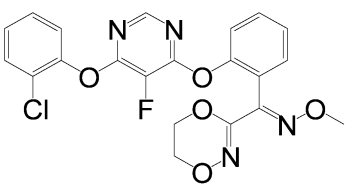
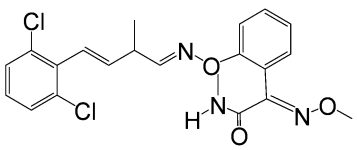
Fig. 2 Standard curve for benzothiostrobin by ELISA.

3. Results and discussion

3.1. Identification of artificial antigens and coupling ratio

Under alkaline conditions, the ethylene bond of the reactant dissociated. With the identification by ESI-MS and NMR, the structure of the obtained hapten was similar to benzothiostrobin, and the hapten contained a carboxyl group that could be conjugated to a carrier protein. In addition, the next study confirmed that the obtained hapten could be used to prepare specific anti-benzothiostrobin antibodies. The UV-Vis spectra

Table 1 Cross-reactivity of a set of analogs related to benzothiostrobin by ELISA

Compound	Structure	IC_{50} (mg L^{-1})	CR (%)
Benzothiostrobin		0.00755	100
Pyraclostrobin		2.25	0.34
Azoxystrobin		>100	<0.05
Kresoxim-methyl		>100	<0.05
Picoxystrobin		>100	<0.05
Chloropiperidine ester		>100	<0.05
Fluoxastrobin		>100	<0.05
Fenaminstrobin		>100	<0.05

indicated qualitative differences between the hapten, carrier protein and conjugates, especially at 280 nm, and confirmed that the carrier protein and hapten had been successfully coupled. The molar ratios (hapten/protein) were 17 : 1 and 8 : 1 for the immunogen and coating antigen, respectively.

3.2. Subtyping of MAb

Two cell lines that can stably produce MAbs against benzothiostrobin were obtained and named $4E_8$ and $2B_4$. By determining the immunoglobulin subclass, the results indicated that $4E_8$ and $2B_4$ were of the lgG_{2a} and lgM subclasses with a kappa light chain, respectively. In this study, $4E_8$ exhibited higher sensitivity than $2B_4$ and was used in further studies.

3.3. Optimization of immunoassays

The optimal concentrations of the coating antigen and antibody were 500 and 35 μg L^{-1}, respectively. For solvent optimization, methanol was used to improve the solubility of the analyte and evaluate its effect on the ELISA method of benzothiostrobin. With the methanol concentrations being 5%, the ic-ELISA method showed the highest A_{max}/IC_{50} and lowest IC_{50}. The ionic strength and pH of the buffer also affected the sensitivity of ic-ELISA; when the PBS buffer contained 0.3 mol L^{-1} Na^+ at pH 6.5, the assay exhibited the lowest IC_{50} and highest sensitivity. Therefore, the optimum parameters included 5% methanol and 0.3 mol L^{-1} Na^+ at pH 6.5, resulting in ELISA with the lowest IC_{50} value and maximum A_{max}/IC_{50}.

3.4. Sensitivities

Under the optimum conditions, the calibration curve was obtained using the relationship between the percent binding (% B/B_0) and the concentration of benzothiostrobin shown in Fig. 2. The assay indicated that IC_{50} was 7.55 μg L^{-1} and LOD (IC_{10}) was 0.428 μg L^{-1}. The linear range (IC_{10}–IC_{90}) was 0.428–54 μg L^{-1}. In a published article, the linear range was 0.1–10 mg L^{-1} for benzothiostrobin by HPLC analysis.[2] Comparing the two methods, ELISA was more sensitive than HPLC.

3.5. Specificity

Table 1 shows the CRs result for the analogues of benzothiostrobin. The highest cross-reactivity was observed for pyraclostrobin (0.34%). The antibody exhibited negligible cross-reactivity (CR% < 0.05%) for the other analogues. Therefore, the assays exhibited strong specificity for benzothiostrobin.

3.6. Matrix effects

The results of the matrix effects study indicated that different sample matrices had different effects on the sensitivity of the immunoassays. The matrices of the rice and tomato samples had a substantial impact, and their effects were reduced to acceptable levels by a 10-fold dilution. The matrices of the pear and soil samples exhibited a small impact. For a 5-fold dilution of the pear and soil samples, the interference had no effect on the sensitivity of the immunoassays. Because too much dilution would eventually reduce the limit of quantification of the samples, a 5-fold dilution (soil and pear) and 10-fold dilution (tomato and rice) were selected for subsequent immunoassays.

3.7. Analysis of spiked samples

As shown in Table 2, the immunoassay method had acceptable recoveries from 83.4 to 115.6%, and the relative standard deviations (RSDs) ranged from 1.8 to 6.8%. The test results were confirmed by HPLC, and good correlations between the HPLC results and the immunoassay results were obtained. These results indicated that the precision of ic-ELISA met the requirement of detecting residual benzothiostrobin in the samples.

3.8. Analysis of authentic samples

Table 3 shows the results from the established ELISA for benzothiostrobin-positive soil and pear samples in Nanjing, China. According to the results (from 70 to 1490 μg L^{-1}), the residue in the soil and pear samples progressively declined over time. The results of the immunoassays and HPLC for authentic samples exhibited good correlations ($R^2 = 0.9894$, $y = 1.0867x + 0.0318$)

Table 2 Recovery of benzothiostrobin in spiked samples ($n = 3$)

Sample	Spiked concentration (mg L^{-1} or mg kg^{-1})	Average recovery ± SD (%)	RSD%
River water	0.01	85.3 ± 2.2	2.6
	0.05	93.5 ± 1.7	1.8
	0.5	100.2 ± 3.8	3.8
Soil	0.01	89.7 ± 1.2	1.3
	0.05	89.4 ± 3.6	4.0
	0.5	95.5 ± 3.2	3.4
Rice	0.01	113.1 ± 6.3	5.6
	0.05	107.4 ± 2.8	2.6
	0.5	85.3 ± 3.5	4.1
Pear	0.01	93.3 ± 3.2	3.4
	0.05	95.6 ± 1.5	1.6
	0.5	100.1 ± 2.4	2.4
Tomato	0.01	115.6 ± 7.9	6.8
	0.05	109.8 ± 5.5	5.0
	0.5	83.4 ± 3.2	3.8

Table 3 Comparison of benzothiostrobin residues between the ELISA and HPLC in authentic samples ($n = 3$)

		ELISA		HPLC	
Sample		Mean (mg kg^{-1})	RSD (%)	Mean (mg kg^{-1})	RSD (%)
Soil	1 day	1.49	3.1	1.37	2.5
	3 days	1.12	2.9	1.01	2.6
	7 days	0.78	3.3	0.67	2.7
	14 days	0.64	3.2	0.57	2.6
Pear	1 day	0.81	5.1	0.70	4.2
	3 days	0.63	6.1	0.55	4.5
	7 days	0.45	4.8	0.31	5.0
	14 days	0.07	5.4	0.11	4.1

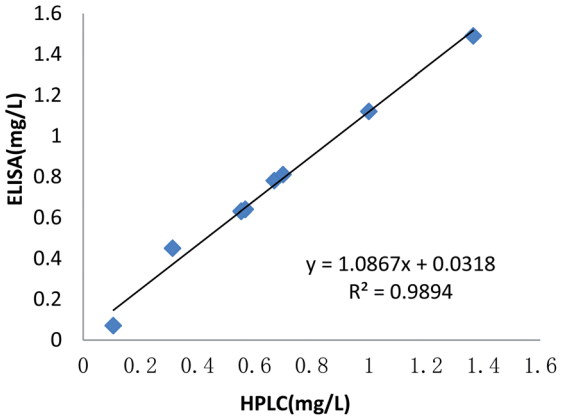


Fig. 3 Correlation between ELISA and HPLC for authentic samples.

(Fig. 3). Therefore, the developed immunoassay was reliable and accurate.

4. Conclusions

We reported an ic-ELISA method for detecting benzothiostrobin in environmental and agricultural samples. The quantitative data indicated that the specificity and accuracy of ic-ELISA were ideal and in good agreement with the HPLC measurements. These results indicate that the established ic-ELISA is a potentially useful tool for detecting benzothiostrobin in environmental and agricultural samples. Based on this study, more detection methods, such as chemiluminescence immunoassay, fluorescence immunoassay, and immunochromatographic assay, could be developed for detecting benzothiostrobin.

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