



Competitive and noncompetitive phage immunoassays for the determination of benzothiostrubin



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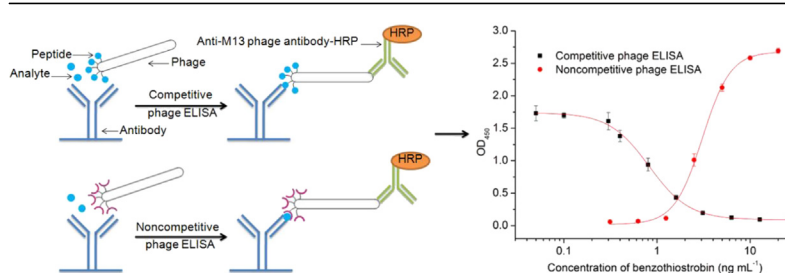
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HIGHLIGHTS

- Benzothiostrubin peptidomimetics have been isolated.
- Anti-benzothiostrubin immunocomplex phage-displayed peptides have been obtained.
- Competitive and noncompetitive phage ELISAs have been developed.
- The IC_{50} and SC_{50} of the phage ELISAs are 0.94 and 2.27 $ng\ mL^{-1}$, respectively.
- The phage ELISAs have better performance than conventional ELISA.

GRAPHICAL ABSTRACT



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ABSTRACT

Twenty-three phage-displayed peptides that specifically bind to an anti-benzothiostrubin monoclonal antibody (mAb) in the absence or presence of benzothiostrubin were isolated from a cyclic 8-residue peptide phage library. Competitive and noncompetitive phage enzyme linked immunosorbent assays (ELISAs) for benzothiostrubin were developed by using a clone C3-3 specific to the benzothiostrubin-free mAb and a clone N6-18 specific to the benzothiostrubin immunocomplex, respectively. Under the optimal conditions, the half maximal inhibition concentration (IC_{50}) of the competitive phage ELISA and the concentration of analyte producing 50% saturation of the signal (SC_{50}) of the noncompetitive phage ELISA for benzothiostrubin were 0.94 and 2.27 $ng\ mL^{-1}$, respectively. The noncompetitive phage ELISA showed higher selectivity compared to the competitive. Recoveries of the competitive and the noncompetitive phage ELISAs for benzothiostrubin in cucumber, tomato, pear and rice samples were 67.6–119.6% and 70.4–125.0%, respectively. The amounts of benzothiostrubin in the containing incurred residues samples detected by the two types of phage ELISAs were significantly correlated with that detected by high-performance liquid chromatography (HPLC).

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1. Introduction

Immunoassays are practical analytical tools for detecting pesticides because of their well-known advantages of simplicity, low cost and large parallel-processing capacity [1,2]. Pesticides are low-molecular-weight chemicals that are buried in the antibody

binding pocket after binding, so they are usually detected using a competitive format. In this format, a competitor (a competing hapten conjugated with a carrier protein, enzyme, fluorophore, etc.) that competes with the analyte for binding to the antibody is a necessary reagent [3]. Generally, the sensitivity of the immunoassay can be significantly improved by using heterologous competing haptens, but the chemical synthesis of a series of competing haptens depending on the analyte may be difficult, expensive and potentially hazardous [4,5]. Besides, some immunoassays have cross-reactivity with analogues of the target because of the analogues can also bind to the antibody [6,7]. These limitations hinder the widespread use and application of immunoassay in detection of pesticide residues.

The phage display technique offers attractive materials to develop alternate immunoassays that may enhance assay performance by increasing sensitivity and selectivity [8]. Phage-displayed peptide libraries have been a powerful tool for isolating peptides that specifically bind to analyte-free antibodies (peptidomimetics) or analyte-bound antibodies (anti-immunocomplex peptides) [9]. Analyte peptidomimetics can be used as surrogate competing haptens to accelerate the development of heterologous immunoassays. Some investigators have successfully obtained analyte peptidomimetics that can be used as a competing hapten for low-molecular-weight compounds from phage display peptide libraries, and the analyte peptidomimetics show better sensitivity than chemical synthesis of homologous and heterologous competing haptens [3,9–18]. Anti-immunocomplex phage peptides can specifically recognize an analyte-bound antibody to enhance the affinity and selectivity of the primary antibody because of the formation of a ternary complex, which translates into an improved noncompetitive immunoassay for a low-molecular-weight compound [8]. Although only eight low-molecular-weight compounds (brominated diphenyl ether 47, clofazone, malachite green, leucomalachite green, gibberellin, 2,2',4,4'-tetrabromodiphenyl ether, phenoxybenzoic acid, molinate and atrazine) have been successfully detected by noncompetitive immunoassays based on anti-immunocomplex phage peptides, each presented better performance than the corresponding conventional competitive immunoassays [8,9,19–24]. Additionally, real-time polymerase chain reaction and loop-mediated isothermal amplification have been successfully used to detect low-molecular-weight chemicals by using phage-displayed peptides because of the unique characteristic that phage-displayed peptides can be connected to nucleic acids (single stranded DNA) [3,25,26].

Benzothiostrubin, 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 [27,28], and patent applications for preparation and use of benzothiostrubin have submitted in China, the United States, Europe, and worldwide (Pub. No.: CN 102302012 B, CN 101379967 A, CN 101268780 B, US 2010/0292285 A1, WO 2007/073637 A1, WO 2009/135407 A1). In our previous study, a homologous competitive enzyme-linked immunosorbent assay (ELISA) was developed for the detection of benzothiostrubin. The half maximal inhibition concentration (IC_{50}) and the limit of detection (LOD, IC_{10}) were 7.55 and 0.428 ng mL⁻¹, respectively. The assay had cross-reactivity (CR) of 0.34% with pyraclostrobin [29].

In this study, the phage-displayed peptides that specifically recognize anti-benzothiostrubin monoclonal antibody (mAb) 4E₈ in the absence or presence of benzothiostrubin were isolated from a cyclic 8-amino-acid random peptide library. Both competitive and noncompetitive phage ELISAs for benzothiostrubin based on the isolated phage-displayed peptides were developed and compared with the conventional ELISA reported earlier [29]. The developed phage ELISAs were validated by high-performance liquid

chromatography (HPLC) in the analysis of the samples containing incurred residues.

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade unless specified otherwise. Benzothiostrubin (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), tetramethylbenzidine (TMB), isopropyl-β-D-thiogalactoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal), and polyoxyethylene sorbitan monolaurate (Tween-20) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Mouse anti-M13 monoclonal antibody-horse radish peroxidase (HRP) conjugate was purchased from GE Healthcare (Piscataway, NJ). *Escherichia coli* ER2738 was purchased from New England Biolabs (Ipswich, MA). The mAb 4E₈ and the cyclic 8-amino-acid random peptide library (the transducing units (TU) and titer of the library were 5.52×10^9 pfu μg⁻¹ and 3.4×10^{13} pfu mL⁻¹, respectively) were developed previously [15,29].

2.2. Biopanning

Three wells of each microtiter plate were coated with purified 4E₈ mAb (10 μg mL⁻¹) in 100 μL of phosphate-buffered saline (PBS) by overnight incubation at 4 °C. Nonspecific binding was blocked by incubation with 300 μL of PBS containing 3% bovine serum albumin (BSA) for 1.5 h at 37 °C. To eliminate nonspecific binding of phage to BSA, another plate coated with 100 μL of 3% BSA in PBS was used for preabsorption. The phage library (10¹¹ phage) diluted with PBS was first added to the preabsorption plate and incubated at room temperature for 1 h. Then, the supernatant was transferred to the plate coated with 4E₈ mAb and incubated with shaking at room temperature for 1 h. The wells were washed 10 times with PBS containing 0.1% (v/v) Tween 20 (PBST), and 100 μL of benzothiostrubin (10 μg mL⁻¹ in PBS) was added to each well with shaking for 1 h to compete the binding phage from the coating antibody (peptidomimetics panning). The phage library that had been used for peptidomimetic panning was transferred into other wells of the mAb-coated plate that had been preincubated with 10 μg mL⁻¹ benzothiostrubin and washed five times with PBST, followed by incubation at room temperature for 1 h. The bound phage was eluted with 100 μL of 0.1 mol L⁻¹ glycine-HCl (pH 2.2) per well for 15 min and neutralized with 13 μL of 1 mol L⁻¹ Tris-HCl (pH 9.1) (anti-immunocomplex phage peptides panning). The elution solutions were then collected and used to infect *E. coli* ER2738 for amplification and titration. The amplified phages were used for a subsequent round of panning. In the second and third rounds of panning, the concentration of coating antibody was reduced to 5 and 2.5 μg mL⁻¹, while the concentration of benzothiostrubin was reduced to 1 and 0.1 μg mL⁻¹, respectively.

After three rounds of panning, 180 μL of ER2738 cell culture (mid-log phase, OD₆₀₀ = 0.5 AU) was mixed with 10 μL of diluted phage eluates. The infected cells were transferred to culture tubes containing 5 mL 45 °C top agar and poured on an LB/IPTG/Xgal plate. The plates were incubated overnight at 37 °C. A total of 48 clones for peptidomimetics or anti-immunocomplex phage peptides were picked, transferred to diluted ER2738 culture and grown at 37 °C with shaking for 4.5 h. Cells were pelleted by centrifugation at 10000 rpm for 10 min and the supernatants were collected for phage ELISAs. The positive clones as described above were further amplified and used for phage DNA isolation as introduced in the

plasmid mini kit instruction manual (Omega Bio-Tek, Inc., GA, USA). The product of phage DNA was submitted for DNA sequencing using the primer 96gIII (CCCTCATAGTTAGCGTAACG) (GenScript Nanjing Co. Ltd., Jiangsu, China).

2.3. Phage ELISAs

Competitive and noncompetitive phage ELISAs were set up to screen phage capable of binding the benzothiostrubin-free mAb and benzothiostrubin immunocomplex. The microtiter plates were coated with 4E₈ mAb at a concentration of 10 µg mL⁻¹ by incubation for 2 h at 37 °C, and blocked for 1.5 h at 37 °C with 1% BSA in PBS. Fifty microliters of phage supernatant of each clone was mixed with 50 µL of 0.2 µg mL⁻¹ benzothiostrubin in 10% methanol-PBS or 10% methanol-PBS. The mixtures were added to the wells and incubated at room temperature for 1 h. After the wells were washed six times with 0.1% PBST, 100 µL of anti-M13 phage antibody conjugated with HRP (1:5000 dilution in PBS) was added. After 1 h incubation and washing six times, the amount of bound enzyme was determined by adding 100 µL of peroxidase substrate (25 mL of 0.1 M citrate acetate buffer (pH 5.5), 0.4 mL of 6 mg mL⁻¹ TMB in dimethyl sulfoxide (DMSO), and 0.1 mL of 1% H₂O₂). The absorbance at 450 nm was determined after the reaction was stopped by adding 50 µL of 2 M H₂SO₄ per well (Fig. 1).

2.4. Optimization of the competitive and noncompetitive phage ELISAs

The optimal concentrations of phage and antibody were determined by the checkerboard titration method for the competitive and noncompetitive phage ELISAs [8,30]. The experimental parameters, including the ionic strength, pH value and organic solvent, were sequentially studied to determine the optimal conditions to achieve the maximum sensitivity. Serial concentrations of benzothiostrubin were detected in PBS solutions containing different concentrations of NaCl (from 0.035 to 3.2 mol L⁻¹) and methanol (from 5 to 40%, v/v) as well as solutions with various pH values (from 4.5 to 9.5) by using the competitive and noncompetitive phage ELISAs. The parameters of the competitive phage ELISA were evaluated using the A_{max}/IC₅₀ and IC₅₀, and A_{max}/SC₅₀ and SC₅₀ were used to evaluate the parameters of the noncompetitive phage ELISA.

2.5. Selectivity of the phage ELISA

A series of concentrations (0.01–10 µg mL⁻¹) of the analogues were tested using the competitive and noncompetitive phage

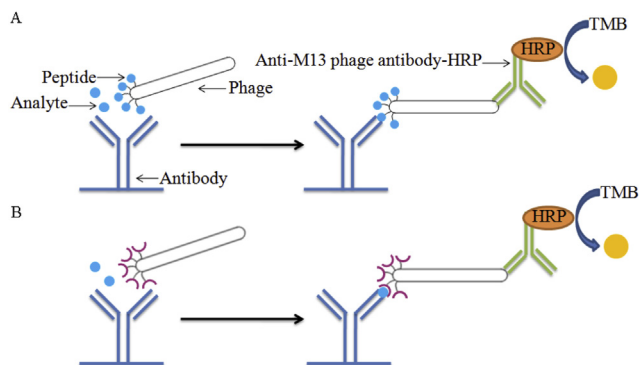


Fig. 1. Schematic diagram of competitive phage ELISA (A) and noncompetitive phage ELISA (B). The 96-well ELISA plate was coated with 1.0 µg well⁻¹ benzothiostrubin antibody. Then 50 µL of phage and 50 µL of PBS with or without benzothiostrubin were added to the wells. A 1:5000 dilution of M13 phage antibody–HRP was used for the detection of bound phage.

ELISAs. For the competitive phage ELISA, the cross-reactivity (CR) was calculated based on the IC₅₀ values using the following formula: CR = [IC₅₀ (benzothiostrubin)/IC₅₀ (analogue)] × 100%. For the noncompetitive phage ELISA, the CR was calculated based on the SC₅₀ values by the formula: CR = [SC₅₀ (benzothiostrubin)/SC₅₀ (analogue)] × 100%. The CR of benzothiostrubin was defined as 100%.

2.6. Analysis of spiked agricultural samples

Four different agricultural samples (cucumber, tomato, pear and rice) were chosen to evaluate the performance of the phage ELISAs. Cucumber, tomato, pear and rice were purchased from local markets. All samples were cut into pieces and homogenized. The homogenized samples were spiked with known concentrations of benzothiostrubin in methanol (the final concentrations were 0.1, 0.2 and 0.3 mg kg⁻¹). The spiked samples were thoroughly mixed and allowed to stand at room temperature overnight.

All samples (10 g) were extracted twice by a vortex mixer in 10 mL of PBS containing 50% methanol for 1 min and centrifuged for 5 min at 4000 rpm. The supernatant was transferred into a 25-mL volumetric flask and adjusted to 25 mL with PBS. After appropriate dilution, the solutions were analysed via the competitive and noncompetitive phage ELISAs. Each pesticide was spiked and analysed in triplicate.

2.7. Practical application of the immunoassays

Tomato and rice were collected from farms where benzothiostrubin had been used in Nanjing, China. The concentrations of benzothiostrubin in the samples were simultaneously analyzed by competitive phage ELISA, noncompetitive phage ELISA and HPLC to evaluate the correlation between the methods. For ELISAs, the extraction and analysis of the samples were performed as described above. For HPLC, 10 g of a homogenized tomato or rice sample was weighed in a 250-mL flask, and 10 mL distilled water and 50 mL acetonitrile were added. The sample was vigorously shaken for 1 h at room temperature. The extracts were filtered into a 100 mL cylinder with plug containing 5 g sodium chloride, and the mixture was homogenized and let stand for 30 min. A half of the supernatant was transferred into a 150 mL Florence flask, and concentrated to dryness by a rotatory evaporator at 45 °C. The concentrate of tomato was dissolved in 3 mL acetone/n-hexane (5/95, v/v) and loaded onto a Florisil SPE column, that was equilibrated with 5 mL n-hexane. The SPE column was eluted with 5 mL acetone/n-hexane (20/80, v/v). The eluting solution was collected and dried using a vacuum rotary evaporator at 45 °C. The concentrate of rice was dissolved in 10 mL acetone/n-hexane (20/80, v/v) and loaded onto an Alumina-N SPE column, that was equilibrated with 5 mL n-hexane. The effluent liquid was collected and dried using a vacuum rotary evaporator at 45 °C. The residues of tomato and rice were dissolved with 2 mL acetonitrile and detected by HPLC (Agilent 1260) with an SB-C₁₈ column (250 mm × 4.6 mm i.d.) using acetonitrile/water (65/35, v/v) as the mobile phase at a flow rate of 0.8 mL min⁻¹ at 30 °C. The detection wavelength was 230 nm and the injection volume was 20 µL. The concentrations of benzothiostrubin in the analysed samples were calculated by using the calibration curve and regression equation.

3. Results and discussion

3.1. Isolation of phage-displayed peptides

After three rounds of panning, a majority of clones showed significant differences in signal with or without benzothiostrubin in

the competitive or noncompetitive phage ELISA (Fig. S1, see electronic supplementary material). Phage DNA from the positive clones was isolated, and the nucleotide sequence of each was determined. Twenty different sequences (designated as C2-3, C3-3, C4-1, C5-2, C7-1, C8-1, C10-1, C12-1, C13-1, C16-1, C17-1, C18-1, C23-1, C24-1, C25-1, C27-1, C36-1, C39-1, C40-1, C45-2) were identified binding to benzothiothiobin-free mAb (Table 1). The clones C2-3 and C18-1 shared the consensus motif of TPXGSL, C3-3 and C23-1 had the consensus sequence GLAXFM, while PXGAWXH was common to C17-1 and C24-1. No evident consensus motif was observed among the other clones. Three different sequences (designated as N1-17, N2-4 and N6-18) were detected binding to the benzothiothiobin immunocomplex (Table 2). The clones N1-17 and N6-18 shared the consensus motif of PXIWPXXW, where X represents different amino acids. These results indicated that the motifs of TPXGSL, GLAXFM and PXGAWXH contributed significantly to binding to the benzothiothiobin-free mAb, and the motif of PXIWPXXW contributed significantly to the benzothiothiobin immunocomplex recognition.

3.2. Selection of phage-displayed peptide

The positive clones that specifically bind to mAb 4B₈ were employed to develop competitive assays for benzothiothiobin. The concentrations of phage and antibody are shown in Table S1, and the IC₅₀ values of benzothiothiobin were in the range 0.83–6.28 ng mL⁻¹ (Table S2). The clone C3-3 was superior to the other phage-displayed peptides in terms of sensitivity. Each positive clone that was specifically for the benzothiothiobin immunocomplex was used in a noncompetitive assay for benzothiothiobin. Serial dilutions of phage particles were added to the wells of the plate coated with four different concentrations of mAb 4B₈ (10, 5, 2.5, and 1.25 μg mL⁻¹) in the absence or presence of benzothiothiobin (0 or 100 ng mL⁻¹). The maximal signal difference was observed at antibody concentration of 10 μg mL⁻¹ and phage concentrations of 1.25 × 10⁹ pfu mL⁻¹ for N1-17 and N2-4, and 7.8 × 10⁷ pfu mL⁻¹ for N6-18 (Figs. S2 to S4). The assay setup with clone N6-18 performed with the highest sensitivity (SC₅₀ = 3.40 ng mL⁻¹), followed by clones N1-17 and N2-4 (SC₅₀ values of 5.77 and 3.44 ng mL⁻¹, respectively) (Fig. S5).

3.3. Optimization of the phage ELISAs

Competitive and noncompetitive phage ELISAs are based on antigen–antibody interactions, and the ionic strength and pH value are additional factors influencing the equilibrium constant [31]. Therefore, the sensitivities of the assays are often improved by optimizing the ionic strength and pH value of the working solution. The pH 7.4, 0.14 M NaCl PBS buffer was selected for the competitive

Table 1
Peptide sequences isolated with the benzothiothiobin-free mAb.

Clone name	Sequence ^a	Clone name	Sequence
C2-3	PATPLGSL (3)	C39-1	PWYVYPQGS (1)
C18-1	KGTPMGSL (1)	C4-1	GTPYGSLLK (1)
C3-3	SGLAEFMS (3)	C5-2	EGPLRSIN (2)
C23-1	TGLAPFMK (1)	C8-1	LTHADLDY (1)
C7-1	LAGADPHV (1)	C10-1	QTAFGMLP (1)
C17-1	PIGAWYHI (1)	C12-1	IYHEGHSM (2)
C24-1	PQGAWHHL (1)	C16-1	PNTWIAHA (1)
C13-1	PSTYLPGA (1)	C25-1	LPQHLLAS (1)
C27-3	PWYYPGFG (3)	C36-1	MLGPRDNE (1)
C45-2	PWPWATPL (2)	C40-1	IPNMMGRS (1)

^a A total of 29 clones were sequenced. The numbers of isolates bearing the same sequence are indicated in parentheses.

Table 2
Peptide sequences isolated with the benzothiothiobin immune complex.

Clone name	Sequence ^a
N1-17	PNIWPESW (17)
N2-4	LHSKHTYE (4)
N6-18	PDIWPTAW (18)

^a A total of 39 clones were sequenced. The numbers of isolates bearing the same sequence are indicated in parentheses.

phage ELISA because it exhibited the highest A_{max}/IC₅₀ and lower IC₅₀ (Tables S3 and S4). When the PBS buffer contained 0.2 mol L⁻¹ NaCl at pH 7, the noncompetitive phage ELISA exhibited the highest A_{max}/SC₅₀ and lower SC₅₀ (Figs. S6 and S7).

Organic solvent can improve the solubility of the analytical, but high concentration of organic solvent often affects antigen–antibody binding. In addition, organic solvents are generally used to extract analyte from the samples, and the sample extract is directly diluted with buffer for removing the matrix interference. Therefore, the effect of organic solvent must be studied in the optimization of an immunoassay. Methanol is commonly selected as an organic co-solvent in immunoassays because of its weaker effect on antigen–antibody binding. When the final concentration of methanol was less than or equal to 2.5% (50 μL of phage supernatant in PBS was mixed with 50 μL of analyte in 5% methanol-PBS), the effects of methanol on the phage ELISAs were negligible (Figs. S8 and S9). In conclusion, the optimum parameters were 2.5% methanol and 0.14 M NaCl at pH 7.4 for the competitive phage ELISA, 2.5% methanol and 0.2 M NaCl at pH 7 for the noncompetitive phage ELISA.

3.4. Sensitivity of the phage ELISAs

Under the optimum conditions, the standard curves of benzothiothiobin were obtained using the relationship between the OD₄₅₀ value and the concentration of benzothiothiobin shown in Fig. 2. The IC₅₀ values, IC₁₀ values and liner range (IC₁₀ to IC₉₀) of the competitive phage ELISA were 0.94, 0.22 and 0.22–3.94 ng mL⁻¹, while the SC₅₀ values, SC₁₀ values and liner range (SC₁₀ to SC₉₀) of the noncompetitive phage ELISA were 2.27, 1.11 and 1.11–4.62 ng mL⁻¹, respectively. The competitive phage ELISA showed higher sensitivity and wider liner range compared to the

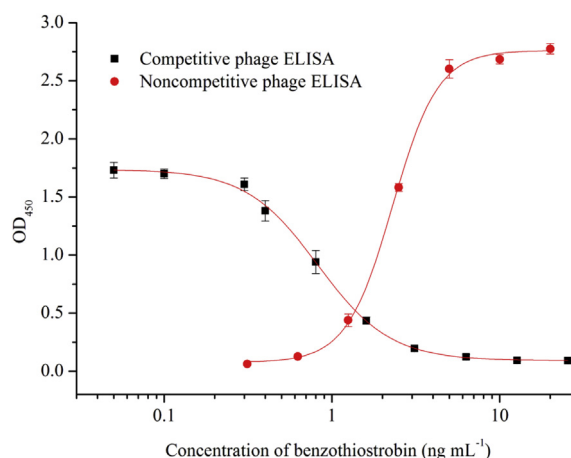


Fig. 2. Standard curves for benzothiothiobin by competitive and noncompetitive phage ELISAs. Serial dilutions of benzothiothiobin standard were mixed with phage-displayed peptides in optimized buffer, then 100 μL of the mixtures were added to the antibody-coated wells. Each point represents the mean value of three replicates.

noncompetitive. However, the liner range and sensitivity were tunable by adjusting the amount of phage peptide or anti-M13 phage antibody–HRP [8]. In our previous study, a conventional competitive ELISA with an IC_{50} of 7.55 ng mL^{-1} and IC_{10} of 0.43 ng mL^{-1} was developed by using the same mAb 4B₈ [29]. As compared with the IC_{50} value of the conventional competitive ELISA, the sensitivity of the competitive and noncompetitive phage ELISAs were improved more than 8-fold and 3-fold, respectively. For the HPLC, a good linearity was acquired for benzothiostrubin within the range of $50\text{--}10000 \text{ ng mL}^{-1}$, and the equation of the linear regression was $y = 120.0x - 0.702$ ($R^2 = 1$). The LOD (signal-to-noise (S/N) ratio of 3) and limit of quantification (LOQ, S/N ratio of 10) of the HPLC were 1.30 and 4.33 ng mL^{-1} , respectively. Therefore, the phage and conventional ELISAs showed higher sensitivity compared to the HPLC.

3.5. Selectivity of the phage ELISAs

Table 3 shows the CR results of the competitive and noncompetitive phage ELISA for the analogues of benzothiostrubin. The

noncompetitive phage ELISA exhibited no CR (less than 0.03%) for the analogues when the concentration of tested compounds was $10 \text{ }\mu\text{g mL}^{-1}$. The competitive phage ELISA showed 0.34% CR for pyraclostrobin, which conformed to the conventional competitive ELISA in our previous study [29]. In this study, the phage-displayed peptide N6-18 could specifically recognize the benzothiostrubin immunocomplex to form a ternary complex which promoted an increased selectivity for the analyte. Therefore, the noncompetitive format showed higher selectivity than the competitive format. This is consistent with previously reported results [22].

3.6. Analysis of spiked samples

Due to the excellent sensitivity and selectivity of an immunoassay, matrix interference can be simply eliminated by diluting with buffer. When the extracts of the cucumber, tomato, pear and rice were diluted equal or more than 8-fold (the total dilution was equal or more than 40-fold, containing 2-fold by mixing with phage in the phage ELISA procedure and 2.5-fold during extraction), the standard curves of the competitive and noncompetitive

Table 3
 IC_{50} and SC_{50} values and cross-reactivity of a set of analogues structurally related to benzothiostrubin by competitive and noncompetitive phage ELISAs.

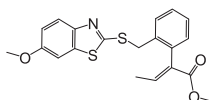
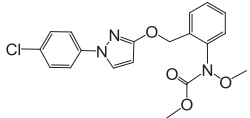
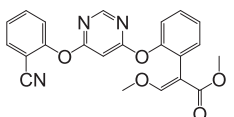
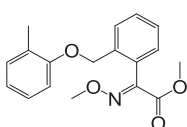
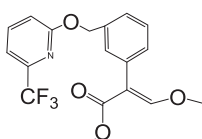
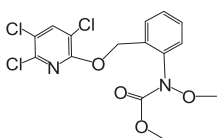
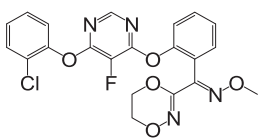
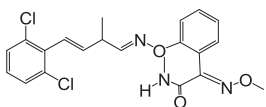
Compound	Chemical structure	Competitive		Noncompetitive	
		IC_{50} (ng mL^{-1})	CR (%)	SC_{50} (ng mL^{-1})	CR (%)
Benzothiostrubin		0.94	100	2.27	100
Pyraclostrobin		275.3	0.34	>10000	<0.03
Azoxystrobin		>10000	<0.01	>10000	<0.03
Kresoxim-methyl		>10000	<0.01	>10000	<0.03
Picoxystrobin		>10000	<0.01	>10000	<0.03
Chloropiperidine Ester		>10000	<0.01	>10000	<0.03
Fluoxastrobin		>10000	<0.01	>10000	<0.03
Fenaminstrobin		>10000	<0.01	>10000	<0.03

Table 4Average recoveries of samples spiked with benzothiostrubin by competitive and noncompetitive phage ELISAs ($n = 3$).

Sample	Spiked (ng g^{-1})	Competitive			Noncompetitive		
		Measured \pm SD (ng g^{-1})	Average recovery (%)	RSD (%)	Measured \pm SD (ng g^{-1})	Average recovery (%)	RSD (%)
Cucumber	100	71.0 \pm 9.1	71.0	12.8	118.6 \pm 9.6	118.6	8.1
	200	200.1 \pm 5.6	100.0	2.8	221.6 \pm 11.6	110.8	5.2
	300	206.2 \pm 4.5	68.7	2.2	232.7 \pm 26.8	77.6	11.5
Tomato	100	98.0 \pm 6.4	98.0	6.6	115.7 \pm 12.5	115.7	10.8
	200	239.3 \pm 18.4	119.6	7.7	235.1 \pm 4.2	117.5	1.8
	300	339.9 \pm 11.2	113.3	3.3	240.0 \pm 12.5	80.0	5.2
Pear	100	74.1 \pm 5.9	74.1	8.0	125.0 \pm 5.5	125.0	4.4
	200	237.3 \pm 12.4	118.7	5.2	217.2 \pm 14.1	108.6	6.5
	300	307.0 \pm 12.3	102.3	3.9	211.3 \pm 9.7	70.4	4.6
Rice	100	67.6 \pm 9.3	67.6	13.7	89.5 \pm 7.7	89.5	8.6
	200	187.6 \pm 21.7	93.8	11.6	193.5 \pm 13.9	96.7	7.2
	300	341.7 \pm 13.9	113.9	4.1	225.4 \pm 8.7	75.1	3.9

Table 5

Comparison of benzothiostrubin residues between the phage ELISAs and HPLC in authentic samples.

Matrix	Method	Sample (ng g^{-1})							P value ^a
		1	2	3	4	5	6	7	
Tomato	HPLC	120	339	659	2070	76.0	50.3	1070	–
	Competitive	102	347	673	1890	59.9	43.1	758	0.172
	Noncompetitive	124	353	598	2140	81.3	61.4	1130	0.398
Rice	HPLC	246	41.1	398	74.9	1380	578	114	–
	Competitive	174	38.9	415	84.2	1420	692	96.8	0.575
	Noncompetitive	219	53.8	430	62.1	1280	562	88.1	0.258

^a Significant difference assessment between the HPLC and the phage ELISAs by using Student's t test.

immunoassays prepared during the matrix dilution were similar to those in the matrix-free buffer (Figs. S10 and S11). Therefore, the spiked samples were diluted at least 40-fold to remove the matrix interferences before the phage ELISA analysis. As shown in Table 4, the mean recoveries and the relative standard deviations (RSDs) were 67.6–119.6% and 2.2–13.7% for the competitive phage ELISA, and 70.4–125.0% and 1.8–11.5 for the noncompetitive phage ELISA, respectively.

Fig. S12 shows the representative HPLC chromatograms of the standard benzothiostrubin sample, blank sample and spiked sample. These chromatograms indicated that the matrix interferences of tomato and rice were removed by the sample treatments. The accuracy and precision of the HPLC method was evaluated by measuring the spiked tomato and rice samples (the final concentrations were 100, 200 and 300 ng g^{-1}), the average recoveries

ranged from 80.8% to 100.5%, and the RSDs were less than or equal to 5.5% (Table S5).

3.7. Analysis of samples containing incurred residues

Table 5 shows the results from the immunoassays and HPLC for benzothiostrubin-positive tomato and rice samples in Nanjing, China. The concentrations of benzothiostrubin in tomato samples as determined using the competitive phage ELISA, noncompetitive phage ELISA and HPLC were in the ranges 43.1–1890 ng g^{-1} , 61.4–2150 ng g^{-1} and 50.3–2070 ng g^{-1} , respectively. The concentrations in rice determined using the same methods as above were in the ranges 38.9–1420 ng g^{-1} , 53.8–1280 ng g^{-1} and 41.1–1380 ng g^{-1} , respectively. The P values were greater than 0.05, implied that the data between the HPLC and phage ELISAs were not

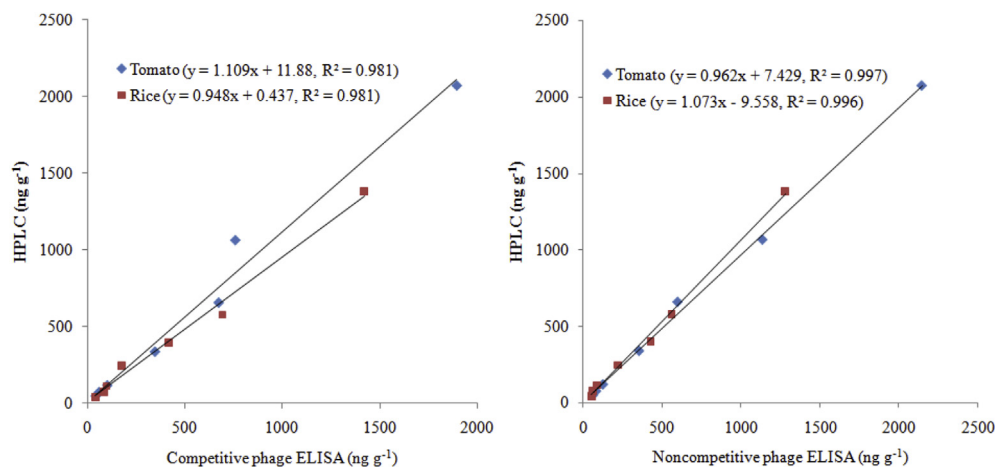


Fig. 3. Correlations between the phage ELISAs and HPLC for the samples containing incurred residues. Fourteen tomato and rice samples obtained from fields treated with benzothiostrubin were detected by competitive phage ELISA, noncompetitive phage ELISA and HPLC. The equation of the line and correlation coefficient obtained from linear regression of the combined phage ELISAs and HPLC data for benzothiostrubin in tomato or rice samples is shown.

significantly different. Besides, the phage ELISAs and HPLC techniques yielded comparable results and good correlations (Fig. 3). Therefore, the competitive and noncompetitive phage ELISAs presented in this study were reliable and accurate.

4. Conclusions

In the current study, twenty-three phage-displayed peptides were isolated from the cyclic 8-residue peptide phage library for simultaneous development of competitive and noncompetitive format immunoassays. The IC_{50} values of the competitive phage ELISA and the SC_{50} values of the noncompetitive phage ELISA were 0.94 and 2.27 ng mL⁻¹ respectively, which were improved more than 8-fold and 3-fold compared to the conventional competitive ELISA with an IC_{50} of 7.55 ng mL⁻¹. The competitive phage ELISA presented higher sensitivity and wider liner range compared to the noncompetitive. The noncompetitive format showed higher selectivity than both the phage-peptide and conventional competitive formats. The two formats of phage ELISAs present similar accuracies and precisions in the analysis of agricultural samples. The accuracy and precision of the assays were validated by HPLC, and results were comparable with correlations $> R^2 = 0.98$. Therefore, the phage ELISAs presented in this study are suitable as convenient quantitative tools for the rapid screening of benzo-thiostrobin in agricultural products. These results demonstrate that the presented phage-displayed peptide technology is a convenient and efficient method for the developments of heterologous competitive and noncompetitive format immunoassays for small molecules to enhance the assay performance.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.aca.2015.07.056>.

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