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Enzyme-Linked Immunosorbent Assay for Simultaneous Detection of Two Fungicides Kresoxim-methyl and Trifloxystrobin in Oranges

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Abstract

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To assemble an indirect competitive enzyme-linked immunosorbent assay (ELISA) for estimating two strobilurin fungicides at the same time, the hapten was synthesised which contained the common active group, (E)-2-(2-bromophenyl)-2-(methoxyimino) acetic acid methyl ester (OEBr) in kresoxim-methyl and trifloxystrobin. The immunogen and coating antigen were respectively prepared through conjugating the above-mentioned hapten with BSA and OVA by the mixed anhydride and activated ester methods, and polyclonal antibodies were produced by immunised rabbits. An enzyme-linked immunosorbent assay was developed for simultaneous detection of kresoxim-methyl and trifloxystrobin. In ELISA, the antiserum showed high affinity and sensitivity to kresoxim-methyl and trifloxystrobin, and their IC_{50} value and detection limit (expressed as IC_{10}) were 14.7 and 0.0044 μg/ml, respectively, for kresoxim-methyl, and 22.9 and 0.014 μg/ml, respectively, for trifloxystrobin. The cross-reaction rate was below 0.1% for other strobilurin fungicides. Recovery study of ELISA from spiked samples of homogenised peeled oranges (final concentrations of 100, 10, and 1 μg/ml) resulted in recovery levels in the range of 82–104%.

Keywords: kresoxim-methyl; trifloxystrobin; polyclonal antibody; ELISA; recovery rate

Kresoxim-methyl, methyl(E)-methoxyimino[α-(*o*-tolyloxy)-*o*-tolyl]acetate, and trifloxystrobin, methyl(E)-methoxyimino-{(E)-α[1-(α,α,α-trifluoro-*m*-tolyl)ethy-lideneaminooxy]-*o*-tolyl}acetate, are two strobilurin fungicides that contain a β-methoxyacrylate derivative group and have a synthetic active structure similar to that of natural strobilurin A (obtained from different wood-rotting fungi) (Clough 1993; Sauter *et al*. 1999; Zhao *et al*. 2006). Fungal spore germination is highly vulnerable to strobilurin fungicides, kresoxim-methyl, and trifloxystrobin, which are typically broadly active against almost all fungi, particularly when they are applied early as a preventative measure. Additionally, these two fungicides are considered to be lower-risk fungicides than conventional fungicides due to their low mammalian toxicity and benign profile toward avian species, though they negatively affect aquatic species (Plaza *et al*. 2002; Lahlali *et al*. 2006).

Due to their broad-spectrum activity and outstanding environmental tolerability, these two fungicides have set high standards for controlling fungal diseases, thereby making a key contribution to storage strategies for agricultural products (Bartlett *et al*. 2002; Guan *et al*. 2002).

The proper monitoring of fungicide residues is an important component of food safety control in agricultural products. Indeed, many food authorities have established maximum residue limits (MRLs) of strobilurin fungicides for grains, fruits, and vegetables (Zhang 2003). In Europe and in the USA, MRLs for kresoxim-methyl range between 0.05 and 1.5 mg/kg

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in most fruits and vegetables (Mercader *et al*. 2014a), and for trifloxystrobin they range from 0.05 mg/kg to 30 mg/kg in authorised crops (Mercader *et al*. 2014b). The most stringent limits of trifloxystrobin and kresoxim-methyl concentrations in oranges are 0.02–2.0 and 0.05 mg/kg, respectively, in the European Union, and 0.05–10.0 and 0.03 mg/kg in Japan (Campillo *et al*. 2010).

The residues of these fungicides can be analysed by high performance liquid chromatography with diode array detection (HPLC-DAD), gas chromatographic method with micro-electron capture detection (GC-μECD), and gas chromatography-mass spectrometry (GC/MS), and the sample preparation involves liquid-liquid microextraction, matrix solid-phase dispersion (MSPD), and headspace solid-phase microextraction (HSSPME) (Liapis *et al*. 2006). The above methods have been used to measure the residues of kresoxim-methyl and trifloxystrobin in food products such as oranges, grapes, strawberries, and ketchup (Wang *et al*. 2009).

However, these methods are expensive, complicated, and not suitable for the analysis of a large number of samples for screening purposes. Considering the simplicity and cost-effectiveness for detecting the fungicide residues, immunoassays have recently gained increasing interest as an alternative method for large monitoring programs (Lequin 2005). The immunological method is an efficient screening technique for the monitoring of illegal and harmful chemicals in food and the environment, and these assays offer significant advantages, such as speed and high sample throughput (Shimomura *et al*. 2001). Consequently, immunoassay formats such as enzyme-linked immunosorbent assay (ELISA), fluorescence polarisation (FPIA), and time-resolved fluorescence (TR-FIA) offer easy-to-use, rapid, and sensitive methods that have been successfully utilised for the detection of fungicides. Enzyme-linked immunosorbent assay (ELISA) was the preferred assay for monitoring pesticide active ingredients and metabolites among the immunoassay formats. Mercader developed the ELISA methods for the detection of trifloxystrobin and kresoxim-methyl, and the results showed that the limit of detection was 0.3 and 0.1 ng/ml for kresoxim-methyl and trifloxystrobin, respectively (Mercader *et al*. 2008, 2014b). However, Mercader's method could not simultaneously detect two strobilurin fungicides. According to the analysis of chemical structure, there was a common active group, (E)-2-(2-bromo-phenyl)-2-(methoxyimino) acetic acid methyl ester (OEBr) in kresoxim-methyl and trifloxystrobin, which could be used as the hapten core.

In the present study a novel hapten was synthesised on the basis of OEBr, and an enzyme-linked immunosorbent assay was developed for simultaneous detection of kresoxim-methyl and trifloxystrobin. The spiked samples were analysed to verify the potential of a new ELISA method for the screening of orange contamination.

MATERIAL AND METHODS

Animal preparation. Three New Zealand white rabbits were obtained from the experimental Animal Research Institute of Hangzhou Normal University. Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China 2006), and were approved by the animal ethics committee of Hangzhou Normal University (Zhejiang, China).

Chemicals and reagents. Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete adjuvant (FCA), and Freund's incomplete adjuvant (FIA) were purchased from Sigma (St. Louis, USA); N-hydroxy succinimide (NHS), R250-coomassie brilliant blue, N,N'-dicyclohexylcarbodiimide (DCC), tributylamine, isobutyl chloroformate, pure ethyl alcohol; dichloromethane, methyl alcohol, N,N-dimethylformamide (DMF), orthoboric acid, borax, NaOH, and $NaHCO_3$ were purchased from the Mike Chemical Instrument Corporation Limited (Hangzhou, China); Tris base, acrylamide, N,N'-methylenebisacrylamide, ammonium persulfate (AP), N,N,N,N-tetramethylethylenediamine (TEMED), DL-dithiothreitol (DTT), and glycine were purchased from the Lanbao Biotech Company (Hangzhou, China). FCA and FIA were purchased from Sigma. Goat anti-rabbit IgG/HRP was purchased from the Boyao Biotech Company (Shanghai, China); TMB-component colour liquid and dimethyl sulfoxide were purchased from Aladdin (Shanghai, China); kresoxim-methyl, trifloxystrobin, pyraclostrobin, metominostrobin, dimoxystrobin, picoxystrobin, and azoxystrobin standards were purchased from the Anpel Scientific Instrument Company (Shanghai, China).

Main instruments and equipment. A UV-vis spectrophotometer, infrared spectrophotometer, and UV spectrophotometer were purchased from

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the Persee General Company (Beijing, China); an electrospray ionisation mass spectrometer (ESI-MS) and nuclear magnetic resonance analyser (NMR) were recorded using an impact HD spectrometer and a DRX-600 NMR spectrometer (BrukerAvance, Germany-Switzerland); absorbance in ELISA analysis was recorded with a microplate reader (Thermo Labsystems, Philadelphia, USA); a refrigerated centrifuge was purchased from the Xingke Scientific Instrument Corporation Limited (Hunan, China); disposable syringes (10 and 1 ml), disposable medical three-way valve, centrifuge tubes (1 and 50 ml), and a dialysis bag (molecular weight cut off 8000–14 000) were purchased from the Mike Chemical Instrument Corporation Limited (China).

Buffers and solutions. The following buffers were used: phosphate-buffered saline (PBS, pH 7.4) containing 138 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, and 2.7 mM KCl; washing buffer (PBST) – PBS that contained 0.05% (v/v) Tween 20; coating solution – carbonate-buffered saline (CBS, pH 9.6) containing 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ in 1 l water; blocking buffer – 0.01 M PBS that contained 5% (v/v) skim milk; substrate solution – TMB-component colour liquid; stopping solution – 2 M H_2SO_4.

Hapten synthesis. (E)-2-(2-bromo-phenyl)-2-(methoxyimino) acetic acid methyl ester (OEBr), having the active group of kresoxim-methyl and trifloxystrobin, and mercaptopropionic acid were used as synthetic raw materials to introduce a carboxyl group via a substitution reaction to prepare hapten (Cunha *et al*. 2007). The synthetic route is shown in Figure 1. Five millilitres of 1 equiv mercaptopropionic acid ethanol solution and 2 equiv NaOH were added into a beaker flask (50 ml) and stirred on a magnetic stirrer to accelerate the reaction until all components dissolved. After ethanol containing 1 equiv OEBr was added to the reaction solution, the mixture was heated and refluxed for 1.5 hour. The filtered transparent solution was removed from the organic solvent via rotary evaporation. The residue was dissolved with $NaHCO_3$ solution and transferred to a separatory funnel. The product was extracted twice with 5 ml *n*-hexane, and the organic phase was discarded. The concentrated hydrochloric acid was used to adjust the aqueous product pH to 3. The product was then extracted 3 times by liquid-liquid distribution with methylene chloride, and the organic phase was collected. The organic phase was dried over anhydrous Na_2SO_4 in a vacuum to obtain a yellow liquid, which was the hapten.

Hapten identification. A minimal amount of the resulting yellow oil was dissolved in methanol or deuterated DMSO for structural analysis via ESI-MS and NMR, respectively.

Immunogen and coating antigen preparation and identification. Both conjugates used in this study were prepared by activation of the free carboxylic group of the hapten and reaction with the amine groups of the carrier protein, which were usually used in the reaction of polypeptide ligation (Zhang *et al*. 2011). Two carrier proteins were used: BSA for the immunising conjugate and OVA for the coating conjugate.

Immunogen synthesis. Hapten (0.1 mmol) and 0.1 mmol NHS were dissolved in 1.0 ml DMF, and 1.0 ml DMF containing 0.1 mmol DCC was then dropwise added to the above solution with magnetic stirring for the synthesis of the immunogen. The mixture was stirred at room temperature for 1 h, and stirred at 4°C overnight. The supernatant was extracted after centrifugation on the following day. With magnetic stirring, the supernatant was dropwise added to 6.0 ml of borate buffer (pH 8.0) that contained 60 mg BSA over a period of 30 minutes. The initial hapten to BSA molar ratio in the mixture

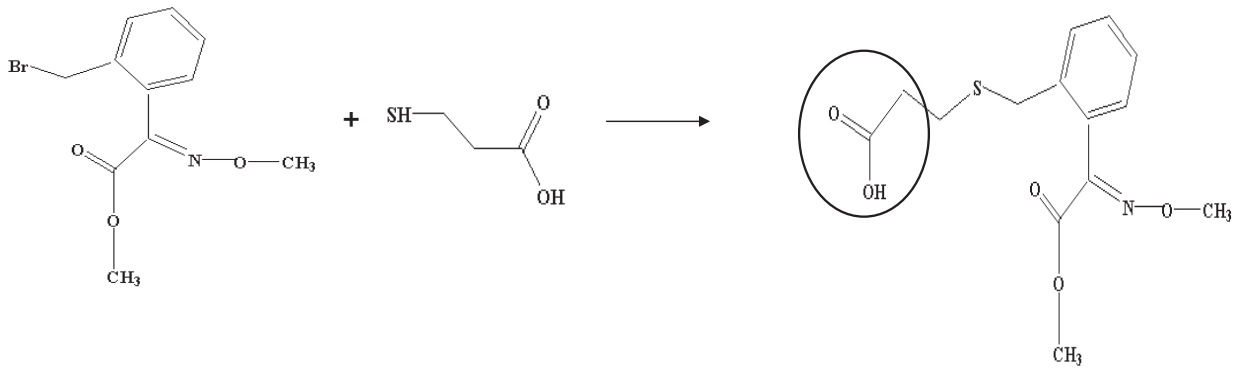

Figure 1. The synthesis of hapten. The OEBr reacted with mercaptopropionic acid to introduce a carboxyl group (in the circle) and synthesise hapten

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was 100 : 1. Then, the mixture was stirred at room temperature for 1 h and stirred at 4°C overnight. The solution was transferred to a dialysis bag maintained in PBS (pH 7.4) at 4°C, and the PBS was changed every 4 hours. Three days later, the product was preserved by freeze-drying.

Coating conjugate synthesis. Hapten (80 μmol) was dissolved in 1 ml DMF, and equivalent amounts of tributylamine and isobutyl chloroformate were added to the solution for the synthesis of coating antigen. After the above mixture reacted at room temperature for 1 h, 500 μl of borate buffer containing 15 mg/ml OVA was added to the reaction liquid with magnetic stirring for 2 h. The initial hapten to OVA molar ratio in the mixture was 20 : 1. After the reaction, the solution was transferred to a dialysis bag and dialysed with distilled water for 30 minutes. The dialysis bag was stored in PBS (pH 7.4) that was changed every 4 hours. Three days later, the product was preserved by freeze-drying. The degree of hapten to protein conjugation was measured spectrophotometrically. If conjugation occurred, the UV-vis spectrum of the conjugate was slightly different from that of the free protein. Therefore, the hapten to protein molar ratio was the average calculated from the absorbance values at 280 and 260 nm by assuming that the molar absorptions of the hapten and the protein were the same for the free and the conjugated forms. The IR spectroscopy was used to infer the existence of some groups or bonds in the molecule by the characteristic absorption peaks. The conjugation rates of the immunogen and coating antigen were analysed by TNBS colorimetry (Bai *et al*. 2001; Xiao *et al*. 2006). To this end, BSA and OVA were respectively diluted in distilled water to final concentrations of 0, 0.6, 0.7, 0.8, 0.9, and 1.0 mg/ml, 1 ml of each concentration of this solution was added to 1ml of CBS (pH 10). The standard curve was determined according to the absorbance value and protein concentrations. The slope indicated the absorbance value of BSA (or OVA) per unit concentration. The immunising conjugate and coating conjugate were respectively diluted in distilled water to final concentrations of 0, 0.6, 0.7, 0.8, 0.9, and 1.0 mg/ml, and 1 ml of each diluted solution was added to 1 ml of CBS (pH 10). The curve was prepared based on the absorbance value and protein concentration. The slope indicated the absorbance value of the sample per unit concentration:

(a) amino consumption = $(OD_{\text{protein with unit concentration}} - OD_{\text{sample with unit concentration}})/OD_{\text{protein with unit concentration}}$

(b) the combination rate of artificial antigen and carrier protein = amino consumption of artificial antigen × the number of amino acids contained in each molecule of carrier protein.

Preparation of vaccine and immunisation for rabbits. The immunogen and adjuvant were completely emulsified to prepare the vaccine. Two sterile syringes were connected via a sterile three-way valve, and the emulsion solution was mixed to develop a milky oil-in-water mixture by pushing the syringes. The above mixing process was accomplished on a sterile workbench. At first, 2 mg of lyophilised immunogen was dissolved in 0.5 ml sterile saline and emulsified with 0.5 ml FCA to obtain the vaccine with the final concentration of 2 mg/ml; in the later experiment, 2 mg of lyophilised immunogen was dissolved in 1 ml sterile saline and emulsified with 1 ml FIA to obtain the vaccine with the final concentration of 1 mg/ml; in the last immune experiment, 1 mg of lyophilised immunogen was dissolved in 1 ml sterile saline with the final concentration of 1 mg/ml. The mixture was packed and stored at 4°C. Three New Zealand white rabbits were immunised with subcutaneous and intramuscular injections of the vaccine. Seven days before the first immunisation, the rabbits were bled from the ear vein. The initial immunisation was performed by injecting 1 ml vaccine for each rabbit, in which the immunogen concentration was 2 mg/ml; then these rabbits were boosted six times at two-week intervals by injecting 1 ml vaccine for each rabbit, in which the immunogen concentration was 1 mg/ml (2 mg lyophilised immunogen was dissolved in 1 ml sterile saline and emulsified with 1 ml FIA) after the third booster, each rabbit was bled from the ear vein on the 7th day after each immunisation. The serum titres were determined by ELISA to monitor the quality of the antisera from the immunised rabbits. The last booster was administered by injecting 1 ml of vaccine for each rabbit, in which the immunogen concentration was 1 mg/ml (1 mg of lyophilised immunogen was dissolved in 1 ml sterile saline), when the titre reached a certain level. Seven days after last booster, the blood was collected from the carotid artery of each rabbit.

Separation of serum. After each blood sample was incubated in the centrifuge tube at 37°C for an hour, the tube was placed at 4°C until the serum precipitated. The blood was centrifuged at 4°C and 5000 r/min for 10 min to separate the serum.

Establishment of the enzyme-linked immunosorbent assay. The indirect competitive ELISA was established

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as follows: the first step was the determination of optimal working concentrations. The appropriate dilutions of the rabbit antiserum and coating conjugate to optimise the final diagnostic assay were titrated through the use of a checkerboard ELISA.

The second step was done like the instruction: each well of a microtitre plate was coated with 100 µl of coating conjugate at an optimal concentration. After an incubation period of two hours at 37°C, the plate was washed three times with PBST for 3 min and blocked with 200 µl of blocking solution per well, followed by an additional incubation at 37°C for 2 hours. After the plate was washed three times, competition was done with 50 µl per well of standard or sample solution in PBST with 10% methanol and 50 µl per well of antibody solution in PBS by incubation at 37°C for 30 minutes. The plate was washed three times with PBST for 3 min, and 50 µl of horseradish peroxidase-labelled goat anti-rabbit IgG solution that had been diluted 1000 times was added to each well to serve as the secondary antibody. The plate was incubated at 37°C for 30 minutes. After the plate was washed again three times for 3 min with PBST, 100 µl of TMB reagent was added to each well. Finally, the plate was incubated at 37°C for 10–15 min in the dark before 100 µl of stopping solution was added to stop the reaction. The absorbance value was read at 450 nm using an ELISA plate reader. PBS solution and negative pre-immune rabbit serum were read as blank and negative controls.

The third step was the construction of the standard curve. Hapten was used as the standard. Different concentrations of hapten were mixed with antibody to get the final concentrations of hapten, 0.001, 0.01, 0.1, 1, 10, 100 µg/ml. In the standard curve, the *x*-axis stands for the logarithm of hapten concentration (µg/ml); the *y*-axis stands for B/B_0, where B is the absorbance value of the well with different concentrations of hapten and B_0 is the absorbance value of the well without hapten. Microsoft Excel (Seattle, USA) was used to draw the standard curve according to the known data.

Specificity of the assay. Specific detection was used to determine the response capacity of kresoxim-methyl or trifloxystrobin to the antibody. The best working concentrations of antigen and antibody were selected as the reaction conditions. Based on the established method for cross reactivity, pyraclostrobin, metominostrobin, dimoxystrobin, picoxystrobin, and azoxystrobin, which are also strobilurin fungicides, were used in the experiment. Concentrations of 200, 20, 2, 0.2, 0.02, and 0.002 µg/ml strobilurin fungicides were prepared in PBST that contained 10% methanol, and the specificity was determined with ELISA. The individual IC_{50} values, half maximal inhibitory concentrations, were obtained from the calibration curves by using a particular analyte. Antibody affinity was estimated as cross-reactivity values (CR), which were calculated as percentage values from the quotient between the IC_{50} for hapten and the IC_{50} for the fungicides:

$$CR\ (\%) = (IC_{50}\ \text{of hapten})/(IC_{50}\ \text{of tested fungicide}) \times 100$$

Sensitivity determination. Solutions containing 200, 20, 2, 0.2, 0.02, or 0.002 µg/ml of hapten, kresoxim-methyl and trifloxystrobin were respectively prepared in a PBST solution containing 10% methanol. Sensitivity was estimated as the concentration of the analyte at the inflection point of the fitted curve, typically corresponding to a 50% inhibition (50%), the limit of detection was estimated as the concentration of the analyte that provided a 10% inhibition. The value of the inhibition rate was $(B_0 - B)/B_0 \times 100\%$, where B – absorbance of the well with different analyte concentrations, and B_0 – absorbance of the well without analyte.

Sample treatment. Samples of oranges were obtained from the local market. The oranges were peeled and homogenised before the detection. 10 g of the homogenised samples was weighed and transferred to 50 ml plastic centrifuge tubes containing 10 ml acetonitrile with 1% acetate, then the solutions were vortexed for 3 minutes. The samples were then placed into a refrigerator at 4°C for 30 minutes. Next, 4 g of CH_3CH_2COONa and 1 g of $MgSO_4$ were added, and the solution was vortexed for another minute to separate the water and acetonitrile. The tube was then centrifuged for 5 min at 6000 r/min at room temperature. 1 ml aliquot of the supernatant was pipetted into a 1.5 ml centrifuge tube containing 150 mg $MgSO_4$, 20 mg PSA, and 5 mg C18. The tube was shaken vigorously by hand for 30 s and then vortexed, second centrifugation, taking an aliquot of the supernatant for evaporation, and evaporation to dryness. The dry residue in the tube was fully dissolved in 1 ml of methanol and stored at –20°C. Before the ELISA, 1 ml of methanol including the sample was mixed with 9 ml of PBST, and the mixture solvent should be adjusted to pH 7.4 with NaOH to ensure the accuracy of the results (Zhang *et al*. 2009).

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Analyses of residue recovery rate in spiked samples. The absence of kresoxim-methyl and trifloxystrobin was verified in selected orange samples in the examination by the established ELISA method, which was used to determine the recovery rate. Kresoxim-methyl or trifloxystrobin was added into samples of homogenised peeled oranges, respectively, to reach the final concentration of 100, 10, and 1 g/ml. These spiked samples were pre-treated as described above in Section 3.8 (vortexed with acetonitrile, the extract was purified and evaporated, the residue was re-dissolved), and the obtained solutions were analysed by the established ELISA according to Section 3.5.

RESULTS

Hapten identification. In the experiment, the ESI-MS was $C_{14}H_{17}O_5NS$, Mr = 311 when the abundance was 100, *m/z* 334.00 $[M+Na]^+$ ^1H NMR (400 MHz, DMSO-d_6): δ = 2.603 (m,2H,CH_2-), δ = 2.656 (m,2H,CH_2-), δ = 3.369 (m,2H,CH_2-), δ = 3.4 (m,3H,CH_3O-), δ = 3.908 (s,3H,CH_3COO-), δ = 7.08 (m,1H,CH-), δ = 7.233 (m,1H,CH-), δ = 7.303 (m,2H,2 × CH-), δ = 12.716 (s,1H,COOH-). The aim of the hapten synthesis was to preserve the main structure of the target analyte (OEBr, the active group of kresoxim-methyl and trifloxystrobin), which would allow the development of a specific immunoassay for the analyte. This hapten retained the main geometric and electronic properties of the analyte and could be used to produce the immunogen and coating antigen.

Identification of immunogen and coating antigen. The UV-vis spectrum of the hapten coupled with BSA or OVA (Figure 2) suggested that the UV absorption of BSA, hapten, Hapten-BSA and OVA, hapten, and Hapten-OVA all differed. BSA and OVA were observed significant for a wave trough at 230–240 nm, but it did not apply to hapten. Moreover, the wave troughs of Hapten-BSA and Hapten-OVA slightly sharpened, which indicated that the combined BSA and OVA retained the properties of natural protein. The absorption maximum shifted, which may have been due to the conjugation of the amino groups of hapten to the carrier protein.

Hapten showed marked IR absorption peaks at 2000 cm^{-1} and in the range of 1720–1706 cm^{-1}, which are characteristic of the -OH group of carboxylic acid; thus, the structure contained a -COOH group. The peak at approximately 1750 cm^{-1} was attributed to a C=O group, which was due to the carbonyl group conjugating to the benzene ring to reduce the absorption frequencies. The significant absorption peak at 1600–1450 cm^{-1} was attributed to the benzene group and C=N group of hapten. A non-vibration absorption peak was observed at 1250 cm^{-1} and was attributed to the CH_2 group of the SCH_2 group. No peaks were observed below 2000 cm^{-1} for BSA and OVA. The absorption was partly masked in the range of 3500~2000 cm^{-1}, and the absorption characteristics below 2000 cm^{-1} were retained for Hapten-BSA and Hapten-OVA, which indicated the successful conjugation of the protein and hapten. These UV absorption spectrum and infrared spectroscopy data preliminarily indicated that the artificial antigens had been synthesised.

Determination of combined ratio. Most of the free amino groups of the carrier protein derived from lysine, and each molecule of BSA and OVA contained 56 and 20 lysine units, respectively. Too many or too few hapten molecules attached to the carrier protein molecule could lead to a weak immune

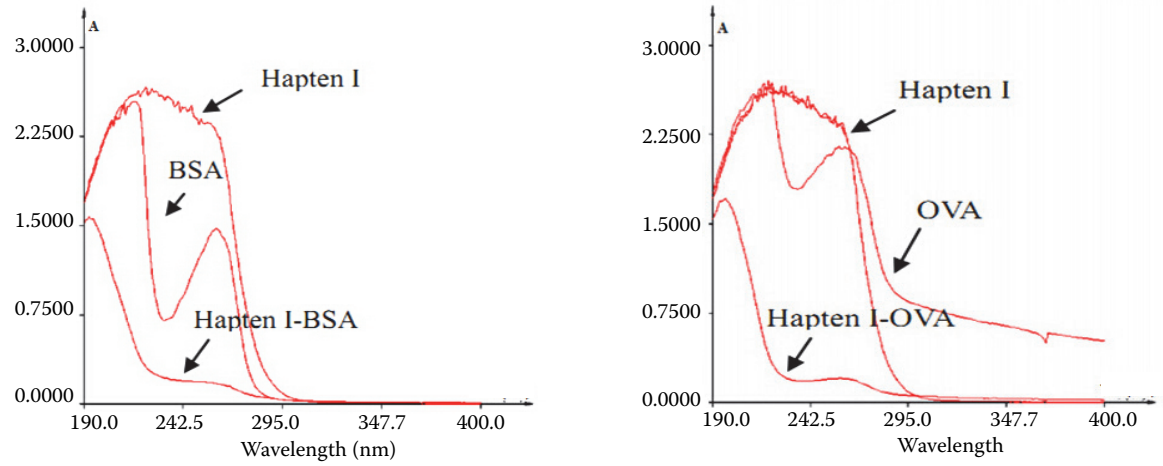

Figure 2. UV absorption spectrum of the hapten coupling with bovine serum albumin (BSA) or ovalbumin (OVA)

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response (Sinkule *et al*. 1991). Each molecule of BSA should optimally be connected to 5–20 hapten molecules, each molecule of OVA should optimally be connected to 5–15 hapten molecules.

The standard curve of the combined ratio was $y = 1.646x - 0.1542$, $R^2 = 0.9976$ for BSA, and $y = 1.712x - 0.1431$, $R^2 = 0.9916$ for OVA. This finding indicated that the absorbance values of BSA and OVA were 1.646 and 1.712 per unit concentration, respectively. According to the above method, the calculated curve for the immunising conjugate was $y = 1.269x - 0.3082$, $R^2 = 0.9568$. The absorbance value with per unit concentration was 1.269, and the combined ratio was $[(1.646–1.269)/1.646] \times 56 \approx 12.8$. The calculated curve for the coating conjugate was $y = 0.954x - 0.2132$, $R^2 = 0.9778$. The absorbance value per unit concentration was 1.344, and the combined ratio was $[(1.712–0.954)/1.712] \times 20 \approx 8.8$. The combined ratios of the immunising conjugate and coating conjugate indicated a good coupling effect in this experiment.

Determination of polyclonal antibody titre. In this experiment, three rabbits remained in good health during immunisation, which indicated that the immunisation dose, immune intervals, and immune method were appropriate. The titre result showed that the OD value of the negative serum was 0.205. The antiserum titre of the first rabbit reached 64 000, and those of the 2nd and 3rd rabbits were 32 000. In general, the higher the titre, the higher the concentration of antibody, so the antiserum of the first rabbit was used as antibodies to do the following experiment.

Development of indirect competitive ELISA detection method. The ELISA method consists of many detection steps, and each step can be affected by different factors, such as the working concentrations of antigen and antibody, coating time, temperature, or solvent (concentrations or pH). Because an excessive organic solvent could improve the solvation of small molecule fungicides but inhibit the antiserum activity, hapten and a small molecule fungicide standard were dissolved in 10% methanol according to

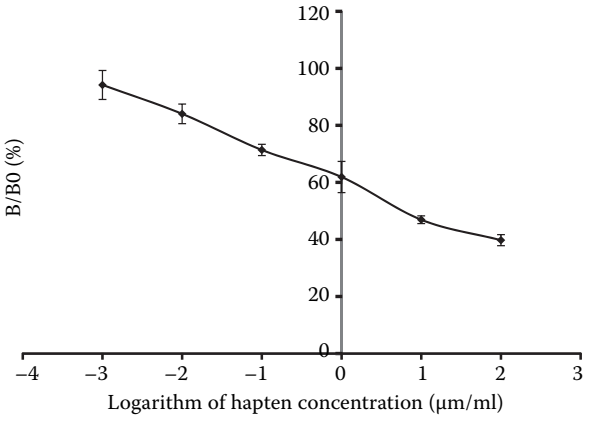
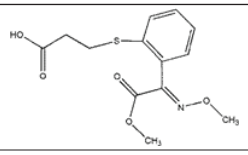
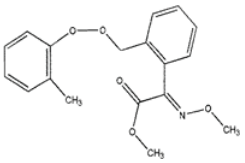
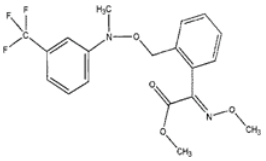
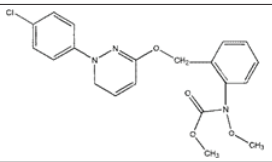
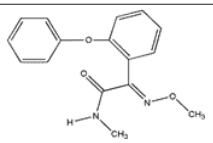
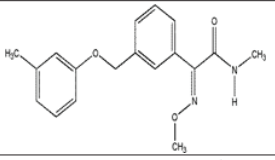
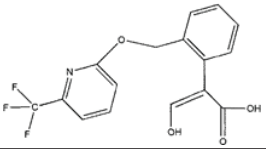
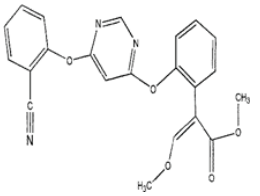


Figure 3. Standard curve for the indirect competitive hapten ELISA, $y = -11.232x + 60.742$, $R^2 = 0.9949$

Table 1. Cross-reaction to different antigens for antibody

Compound	Structure	IC_{50} (μg/ml)	IC_{10} (μg/ml)
Hapten		9.5	0.005
Kresoxim-methyl		15.3	0.0074
Trifloxystrobin		19.6	0.011
Pyraclostrobin		–	–
Metomino-strobin		–	–
Dimoxystrobin		–	–
Picoxystrobin		–	–
Azoxystrobin		–	–

– not detected; IC_{10} – minimum detection limit; IC_{50} – half maximal inhibitory concentration

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Table 2. Recovery rate and precision

Fungicide	Addition level (μg/ml)					
	100		10		1	
	recovery %	RSD (%, *n* = 3)	recovery%	RSD (%, *n* = 3)	recovery%	RSD (%, *n* = 3)
Kresoxim-methyl	104.35	11.1	95.57	10.8	82.73	8.8
Trifloxystrobin	92.43	7.4	91.75	10.2	91.10	10.9

RSD – relative standard deviation

the results of different reports (Yi *et al*. 2007). The coating antigen was dissolved in carbonate buffer at pH 9.6 because at this pH value the coating antigen can conjugate to microplate maximal.

According to the analysis, when the OD_{450} was approximately equal to 1, the optimal working concentration was when the coating conjugate concentration was 10 μg/ml and the antibody was diluted 2000-fold with PBS. The standard curve is represented in Figure 3, which shows that the regression equation for hapten is $y = 10.679x + 39.5$, and $R^2 = 0.9988$. The IC_{50} was 9.04 μg/ml, and IC_{10} was 0.0025 μg/ml for synthesised hapten.

Cross-reactivity (CR) studies were performed to identify other pesticides that could be recognised by the selected antibody. For kresoxim-methyl and trifloxystrobin, the CR was 6.14 and 3.95%, respectively. The CR was less than 0.1% for pyraclostrobin, metominostrobin, dimoxystrobin, picoxystrobin, and azoxystrobin, which did not contain any methoxyimino methyl acetate group. The detection data showed that the obtained polyclonal antibody was specific to the identical part of the structure of both kresoxim-methyl and trifloxystrobin, which contained the hapten structure (OEBr) (Table 1). After hapten was linked to the carrier protein, the protein in the resulting conjugate did not cover up the part of the hapten structure opposite to the binding carboxyl group. This part of the hapten molecule remained fully exposed to space and served as an antigenic determinant. The immunised animals could then produce the corresponding antibody to the identical part of the structure of both fungicides.

Analyses of residue recovery rate in spiked samples. Kresoxim-methyl or trifloxystrobin residues were extracted from the spiked samples and examined by the established ELISA method to obtain recovery values, and the results are shown in Table 2. The recovery rates ranged from 82% to 104% for kresoxim-methyl, and approximately 91% for trifloxystrobin, which can meet the requirement of the product test.

DISCUSSION

In this study, a common antigen including the OEBr was designed and synthesised, which led to the production of a polyclonal antibody. According to the established enzyme-linked immunosorbent assay, when the hapten was replaced with other strobilurin fungicides, the results showed that the rabbit polyclonal antibody could have a good reaction with the fungicide including the active group, OEBr, and was suitable to the simultaneous detection of kresoxim-methyl and trifloxystrobin. In specificity testing, there was no cross-reactivity between the prepared antibody and other strobilurin fungicides, which indicated that the developed ELISA method had good specificity for kresoxim-methyl and trifloxystrobin.

The residues of kresoxim-methyl and trifloxystrobin could also be analysed with different spectrometry apparatuses. In the references, the minimum detectable mass fraction and recovery rate were 0.02 mg/kg and 81.8–99.15% by HPLC, respectively (Chen *et al*. 2011; Wan *et al*. 2011), 0.005 mg/kg and 80.2–103% by GC (Zhang *et al*. 2008), and 0.001–0.02 mg/kg and 76–106% by LC-ESI-MS-MS (Anna *et al*. 2004). In our experiment, IC_{50} values were 14.7 and 22.9 μg/ml, and the limits of detection were 0.0044 and 0.014 g/ml, respectively, for kresoxim-methyl and trifloxystrobin, which were below the common maximum residue limit, 0.05 g/ml (Mercader *et al*. 2014a). According to the determined results for spiked orange samples, the recovery rates of the new ELISA assay were 82.73–104.35% for kresoxim-methyl and 91.1–92.43% for trifloxystrobin, which were the values consistent with those of the above spectrometry examination.

In Mercader's study, the ELISA method was developed for the detection of trifloxystrobin and kresoxim-methyl, and the results were obtained for strawberry, tomato, and cucumber samples spiked with trifloxystrobin, which showed the limit of detection below 0.1–0.3 ng/ml and better than in our study,

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and excellent recovery was at 0.01 g/kg (Mercader *et al*. 2008, 2014b). The difference in detection limit could come from different antibodies in the two above-mentioned ELISA assays. A polyclonal antibody was used in our ELISA method, but a monoclonal antibody was used in Mercader's study. It is inferred that monoclonal antibodies had better specificity than polyclonal antibodies (Urusov *et al*. 2010), and the sensitivity of polyclonal antibody may not be better than that of monoclonal antibody. But a polyclonal antibody was easily prepared and at a lower cost, which was advantageous to be applied in detection if it could meet the sensitivity of detection. Moreover, the polyclonal antibody produced in our experiment can be used to detect two different strobilurin fungicides, kresoxim-methyl, and trifloxystrobin, at one time, which would be more convenient for screening the residues of kresoxim-methyl and trifloxystrobin in practice.

CONCLUSION

An ELISA method was developed based on a kind of polyclonal antibody against two strobilurin fungicides, kresoxim-methyl, and trifloxystrobin, which could meet the requirements of trace analysis. Through more continuous optimisation of experimental conditions, it can be speculated that the method would have a better prospect for the rapid detection of kresoxim-methyl and trifloxystrobin residues.

References

Anna S., Luciana B., Mirella B. (2004): Application of liquid chromatography with electrospray tandem mass spectrometry to the determination of a new generation of pesticides in processed fruits and vegetables. Journal of Chromatography A, 1036: 161–169.

Bai J.S., Bu F.R. (2001): Polyethylene glycol coupling product of human hemoglobin synthesis and characterization. Chinese Journal of Blood Transfusion, 14: 150–152.

Bartlett D., Clough J., Godwin J. (2002): The strobilurin fungicides. Pest Management Science, 58: 649–662.

Liapis E.D.B.K.S. (2006): Validation of a multi-residue method for the determination of pesticide residues in apples by gas chromatography. International Journal of Environmental and Analytical Chemistry, 86: 63–68.

Campillo N., Viñas P., Aguinaga N., Férez G., Hernández-Córdoba M. (2010): Stir bar sorptive extraction coupled to liquid chromatography for the analysis of strobilurin fungicides in fruit samples. Journal of Chromatography A, 1217: 4529–4534.

Chen C.T., Cheng M.J., Li O.Y. (2011): Analysis of kresoxim-methyl·hexaconazole in 40 % SC by HPLC. Pesticide Science and Administration, 32: 42–45.

Clough J. (1993): The strobilurins, oudemansins, and myxothiazols, fungicidal derivatives of β-methoxyacrylic acid. Natural Product Reports, 10: 565–57.

Cunha S., Lehotay S., Mastovska K., José O.F., Maria B. (2007): Evaluation of the QuEChERS sample preparation approach for the analysis of pesticide residues in olives. Journal of Separation Science, 30: 620–632.

Guan- A.Y., Hu N.D. (2002): The strobilurin fungicides. World Pesticides, 24: 16–19.

Lahlali R., Serrhini M., Friel D. (2006): *In vitro* effects of water activity, temperature and solutes on the growth rate of *P. italicum* Wehmer and *P. digitatum* Sacc. Journal of Applied Microbiology, 101: 628–636.

Lequin R. (2005): Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). Clinical Chemistry, 51: 2415–2418.

Mercader J.V., Celia S.P., Consuelo A., Antonio A.S., Antonio A.F. (2008): Hapten synthesis and monoclonal antibody-based immunoassay development for detection of the fungicide trifloxystrobin. Journal of Agricultural and Food Chemistry, 56: 2581–2588.

Mercader J.V., Rosario L.M., Esteve-Turrillas F.A., Consuelo A., Antonio A.S., Antonio A.F. (2014a): Sensitive monoclonal antibody-based immunoassays for kresoxim-methyl analysis in QuEChERS-based food extracts. Journal of Agricultural and Food Chemistry, 62: 2816–2821.

Mercader J.V., López-Moreno R., Esteve-Turrillas F.A., Abad-Somovilla A., Abad-Fuentes A. (2014b): Immunoassays for trifloxystrobin analysis. Part II. Assay development and application to residue determination in food. Food Chemistry, 162: 41–46.

Plaza P., Usall J., Teixido N. (2003): Effect of water activity and temperature on germination and growth of *Penicillium digitatum*, *P. italicum* and *Geotrichum candidum*. Journal of Applied Microbiology, 94: 549–554.

Sauter H., Steglich W., Anke T. (1999): Strobilurins: evolution of a new class of active substances. Angewandte Chemie International Edition, 38: 1328–1349

Shimomura M., Zhang W., Sakino M., Lee K.H., Ikebukuro K. (2001): Simple and rapid detection method using surface plasmon resonance for dioxins, polychlorinated biphenylx and atrazine. Analytica Chimica Acta, 434: 223–230.

Sinkule J.A., Rosen S.T., Radosevich J.A. (1991): Monoclonal antibody 44-3A6 doxorubicin immunoconjugates: comparative in vitro anti-tumor efficacy of different conjugation methods. Tumour Biology, 12: 198–206.

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Urusov A.E., Zherdev A.V., Dzantiev B.B. (2010): Immunochemical methods of mycotoxin analysis (review). Applied Biochemistry a Microbiology, 46: 253–2660.

Wan H.J., Ye S.F., Tan B. (2011): Analysis of trifloxystrobin by HPLC. Agrochemicals, 2011: 820–821.

Wang Y.F., Ge B.K., Gao J.H., Chang C.Y., Chen Q.Y. (2009): Determination of 8 strobilurins in fruits and vegetables by liquid chromatography-mass spectrometer. Food Research and Development, 30: 130–133.

Xiao Z.L., Sun Y.M., Zhang M.M. (2006): Synthesis of Methamidophos derivative and preparation of artificial antigens. Food Science, 27: 377–380.

Yi G., Shuai M. (2007): Effect of incubative time for the accuracy of ELISA. Journal of Laboratory and Clinical Medicine, 4: 353–355.

Zhang G. (2003): Strobilurin fungicides applications, development status and prospects. Pesticide Science and Administration, 24: 30–34.

Zhang J., Wang C.N.B., Wei C.J. (2009): Development of ELISA kit for deltamethrin residue. Journal of Beijing University of Agriculture, 24: 27–30.

Zhang H., Ye B.B., Wang X.Y., Chen C., Wu L. (2008): Determination of kresoxim-methyl and trifioxystrobin residues in tomato and cucumber by gas chromatography. Chemistry, 6: 465–468.

Zhang H., Wen W., Song G.P. (2011): Preparation of folic acid artificial antigen and identification of antiserum. Chinese Agricultural Science Bulletin, 27: 78–81.

Zhao L., Ma L.Y. (2010): The biological properties of strobilurin fungicides. Modern Pesticides, 5: 9–12.

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