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Research Article

Development of an ELISA Method Detecting Strobilurin Fungicide through the Immune Magnetic Beads Collecting Samples

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Abstract: Trifloxystrobin, one of the strobilurin fungicides, is active as inhibitor of low toxicity and easy degradation against a wide range of fungal plant pathogens. In the experiment, Hydroxy from magnetic microsphere and amino of anti-Trifloxystrobin antibody could condensate to form Immune Magnetic Beads (IMB), which could specially capture trifloxystrobin in the food matrix and rapidly separated it through the magnetic field. An ELISA method (IMB-ELISA) was developed to detect the captured fungicide through examining the best reactive concentration, sensitivity, specificity, matrix effect and recovery rate. The half maximal inhibitory concentration (IC50) value obtained by the IMB-ELISA method was 2.28 μg/mL (R2 = 0.9837) against 12.57 μg/mL of conventional ELISA method (R2 = 0.9747). The recovery rates were 90.12-103.65% and the Coefficients of Variation (CVs) were in the range of 4.5-11.2%. In three kind of citrus samples, there was only low cross-reactivity in IMB-ELISA method by detecting matrix effects. Based on above experiment, the IMB-ELISA method was an efficient and sensitive method for Trifloxystrobin detection.

Keywords: ELISA, immunomagnetic beads, polyclonal antibody, trifloxystrobin

INTRODUCTION

Trifloxystrobin, methyl (*E*)-methoxyimino-{(*E*)-α[1-(α, α, α -trifluoro-*m*-tolyl) ethylideneaminooxy]-*o*-tolyl}acetate is a kind of strobilurin fungicide, which has the advantage of high-efficiency, permeation, outstanding environmental tolerability, long pesticide persistence length and broad-spectrum activity etc (Bartlett *et al*., 2002). The principle of this kind of fungicide is controlling the ATP product of the fungus as a respiratory inhibitor. Trifloxystrobin contains a β-methoxyacrylate derivative group, which could block electron transfer through the cytochrome b and cytochrome c and inhibits mitochondrial respiration in the target fungus (Bartlett *et al*., 2001; Bartlett *et al*., 2002). Simultaneously, it can lengthen the distance from Rieske Iron-Sulfur Protein (RISP) to cytochrome c to slow down or even stop the electron transfer (Bartlett *et al*., 2001). Due to its broad-spectrum activity and outstanding environmental tolerability, the fungicide had set high standards for controlling fungal diseases, thereby making a key contribution to storage strategies for postharvest fruits.

Nowdays, the monitoring of trifloxystrobin residues is an important topic for fruit safety. The most stringent limits of trifloxystrobin concentrations in citrus are 0.02-2 mg/kg in the European Union and 0.05-10 mg/kg in Japan (WTO/TBT-SPS, 2004). Method to detect trifloxystrobin residues mainly includes Headspace Solid-Phase Micro-Extraction (HS-SPME) (González-Rodríguez *et al*., 2011), High Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD) (Campillo *et al*., 2010), QuEChERS method containing a Gas Chromatography with a Nitrogen-Phosphorus Detector (GC-NPD) and ion trap mass spectrometry (GC-IT-MS) (Zhu *et al*., 2013), etc. But these methods are complicated, expensive and not suitable for analysis of a large number of samples for detection purpose.

The immune magnetic beads separation Specific purpose product, an emerging technology by the magnetic field was more and more paid attention to since Norwegian scientist Ugelstad (1979) prepared the polystyrene microspheres in a uniform particle size distribution. Magnetic beads less than 100 nm have larger specific surface area, high stability and high diffusivity. The intensity of magnetization could decline to zero and become a super paramagnetism when the particle size of the magnetic beads to the criticality. It has a high sensitivity to the magnetic field (Sandeep Kumar, 2013). Specifically coupled with macromolecule polymers, magnetic beads could form complexes by immobilized affinity adsorption, ion-exchange adsorption, hydrophobic effect with immune ligands. Under the magnetic force, magnetic beads can rapidly and sensitively separate or enrich the target materials, such as pathogenic microorganisms (Pappert *et al*., 2010; Jeníková *et al*., 2000), viruses (Patel and

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Blackburn, 1991), toxin (Singh, 2000) and chemicals (Byzova *et al*., 2010) through coupling them. Combining ELISA, PCR and other modern detection technology, the target materials obtained by immunomagnetic separation could be effectively implement precise analysis. In recent years, the immune magnetic beads were widely used in lots of areas such as food microorganism and toxins examination, cells separation, biochemistry and molecular genetics.

The Enzyme-Linked Immunosorbent Assay (ELISA) was used in food examination because of its high analytical capacity and simplicity for standardization (Mercader *et al*., 2014). Depending on the immobilized reagent and on the enzymatic detection step, the antigen detection of ELISA could be divided into two kinds, the indirect and indirect competition method. The indirect method is to connect antigens with coated wells firstly, then specific antibody is added to form the complexes after covering the enzyme labeled antibody and the concentration of antigens is calculated depending on the absorption value. Indirect competition method is to connect antibody with coated wells firstly, then the antigens and enzyme-labeled antigen would become a competition relationship to cover the enzyme labeled antibody which provide the concentration of antigens.

In this study, an ELISA detection method, a kind of quick and convenient detection technique, was established based on immune magnetic beads with Indirect Competitive Enzyme-Linked Immunosorbent Assay (IC-ELISA), which has more advantage in sample processing, equipment requirements, operation difficulty.

MATERIALS AND METHODS

Chemicals and instruments: Trifloxystrobin standard material (99.0% purity) and pyraclostrobin standard material (99.0% purity) was purchased from ANPEL Scientific Instrument (Shanghai, China). The anti-Trifloxystrobin antibody and hapten AR-OVA were prepared by our laboratory. 1-(3-Dimethylaminopropyl) -3-ethylcarbodiimide hydrochloride (EDC) was provided from Ziyi reagent Co., Ltd. (Shanghai, China). Rabbit Anti-mouse IgG-HRP (horseradish peroxidase) was obtained from Boster Biological Co., Ltd. (Wuhan, China). 3, 3', 5, 5'- Tetramethylbenzidine Liquid Substrate (TMB stabilizer) was purchased from Aladdin Industrial Co., Ltd (Shanghai, China). Monodisperse magnetic ferroferric oxide nanoparticles with carboxyl as the surface functional group (50 nm, 10 mg/mL) were provided by Huier Nano Technology Co., Ltd. (Hennan, China). Neodymium iron boron (NdFeB) magnet and Yili skimmed milk powder (fat content ≤1.5 g/100 g) were purchased from local supermarket (Hangzhou, China). Ethanol, hydrogen peroxide (30%) and other reagents were of chemical grade supplied by Gaojing Fine Chemical Industrial Co., Ltd. (Hangzhou, China). The immunomagnetic assay was spectrophotometrically read with an automatic microplate reader MultisKAN (MK3) of Thermo (USA). The blender used in this study was purchased from Shanghai (FLUKO Germany). The centrifuge (14RD) was purchased from Shanghai (China). The plastic centrifuge tubes (50 mL and 1.5 mL) were supplied by Mike Chemical Instrument Co., Ltd. (Hangzhou, China). Analytical balances capable of weighing from 0.1 mg to 0.01 g were obtained from Mettler-Toledo (Greifensee, Switzerland). Three kind of citrus samples were purchased from the local supermarket, including Sugar orange, Ponkan citrus, Honghe Papeda citrus.

Buffers and solutions: The following buffers were used: phosphate buffered saline (PBS pH7.4) containing 137 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl and 1.5 mM KH_2PO_4. Washing buffer (PBST): PBS with addition of 0.05% (v/v) Tween20. Stopping solution was 2 M H_2SO_4. Carbonate Buffered Saline (CBS pH 7.4) containing 15 mM Na_2CO_3, 35 mM $NaHCO_3$. Blocking buffer was 5% skimmed milk powder. 2-Morpholino-Ethanesulfonic acid Solution (MES): MES (300 mM) was suspended in 200 mL of water and the pH value was adjusted to 6 by using 0.5 M NaOH.

Conjugation of antibodies to MB and estimation: The flow chart of the IMB-ELISA assays is displayed in Fig. 1. Magnetic Beads (MB) were modified by antibodies as followed. 1 mL MB solution was dispersed through shocking 30 min on the vortex concussion instrument and the supernatant was removed after MB was completely adsorbed by NdFeB magnet. The MB was washed two times in a magnetic field with 1mL MES (15 mM pH6.0). The MB was resuspended in 100 μL MES with 100 μL EDC and the reaction was allowed to proceed at room temperature for 30 min. The MB was washed for two times with 1 mL MES (15 mM PH6.0). Finally, the MES including MB was mixed with serum antibody to get Immune Magnetic Beads (IMB) at room temperature overnight. IMB would be achieved through a magnetic field and washed two times with PBST before use.

Best reactive concentration of ELISA: IMB was diluted to 1/1000-1/12800 and IgG-HRP was 1/1000-1/4000. 100 μL/well of the diluted AR-OVA with CBS were added to a 96-well microtiter plate at 37°C for 2 h, then 200 μL/well of blocking buffer were added at 37°C for another 2 h. 100 μL/well of different dilution of IMB was added to a 96-well microtiter plate at 37°C for 30 min. The Rabbit Anti-mouse IgG-HRP 50 μL/well was mixed in the well to react for another 45 min. Then 100 μL/well of TMB stabilizer solution was added and the plate was incubated in a lucifugal

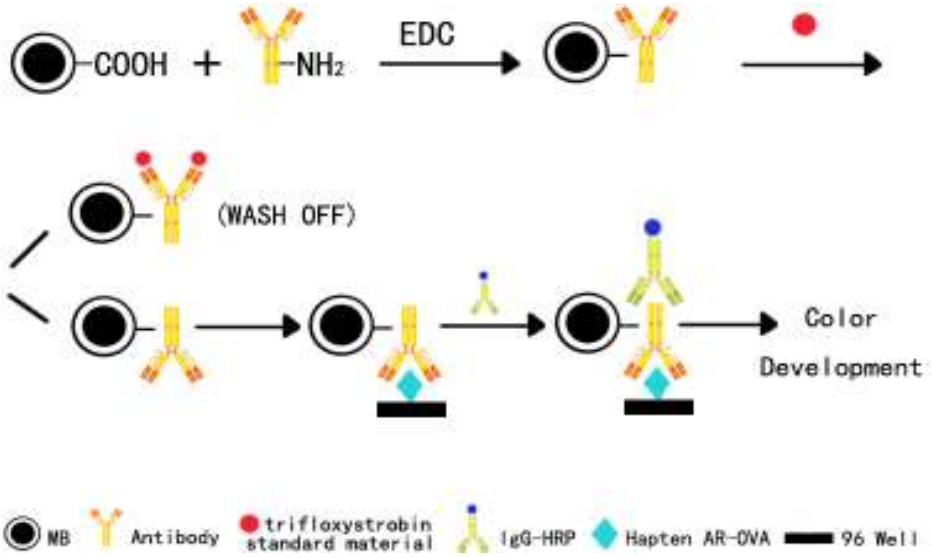

Fig. 1: The flow chart of the IMB-ELISA assays to detect trifloxystrobin

place for 15 min. Finally, the enzyme reaction was stopped with 100 μL/well stopping solution (2 M H_2SO_4) and the absorbance values of the plate were measured at 450 nm. The IMB was replaced with serum antibody as a controlled trial.

Sensitivity determination: The optimal concentration IMB (100 μL) was reacted with 6 different dilutions of trifloxystrobin standard materials (100 μL) at 37°C for 2 h, including 0.002, 0.02, 0.2, 2, 20, 200 μg/mL, respectively. Then IMB and IMB- trifloxystrobin were separated from reactive solution through magnetic field and the mixture was washed three times with PBST and resuspended in the PBST solution. The mixture was transferred to a 96-well microtiter plate containing the coated hapten AR-OVA for reaction at 37°C for 30 min. The Rabbit Anti-mouse IgG-HRP (100 μL/well) of optimal concentration was added to incubate for another 45 min. After 100 μL/well of TMB stabilizer solution was added for 15 min, the reaction was stopped with 100 μL/well stopping solution (2 M H_2SO_4) and the absorbance values of the plate were measured at 450 nm. The linear relationship between logarithmic concentrations of trifloxystrobin and B/B_0 values was developed. B_0 was the absorbance value without addition of trifloxystrobin and B was the absorbance value with trifloxystrobin. The IC50 value (half maximal (50%) Inhibitory Concentration (IC) of a substance) was calculated from standard calibration curve.

Specificity of the assay: Pyraclostrobin and trifloxystrobin were typical examples of strobilurin fungicide. They had the similar molecular structure (Fig. 2) and different active group. In the experiment,

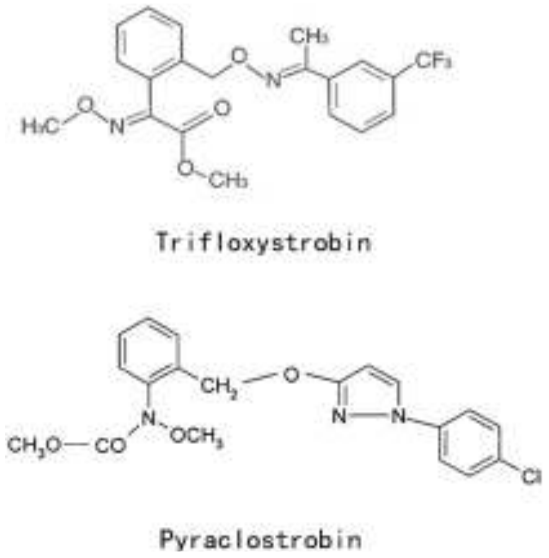

Fig. 2: The molecular structure of trifloxystrobin and pyraclostrobin. The structure of pyraclostrobin was similar to the trifloxystrobin which active group was methoxy imino acetic acid methyl ester

Pyraclostrobin was used as the competitor and determined as the immunomagnetic assay described above. The IC50 value of pyraclostrobin was obtained. The cross-reactivity was calculated:

$$CR = IC50\ (trifloxystrobin)/IC50\ (Pyraclostrobin) \times 100\%.$$

Matrix effects of samples: Three citrus samples, Sugar orange, Ponkan citrus and Honghe Papeda citrus, were respectively centrifuged and diluted to 1/5, 1/10 and 1/20 with PBS buffer, then mixed with 200, 20, 2, 0.2, 0.02, 0.002 μg/mL, respectively trifloxystrobin standard

materials (100 μL). The optimal concentration IMB (100 μL) was added to the above sample at 37°C for 2 h and collected through magnetic field, then washed three times with PBST and resuspended again. Finally, the mixture was transferred to a 96-well microtiter plate to determine the absorbance values. B/B_0 was calculated.

Recovery study in samples: Citrus samples spiked with 100, 10 and 1 μg/mL, respectively trifloxystrobin standard material were analyzed by the IMB-ELISA method to calculate the recovery rates of standard addition. The procedure was repeated for three times on the same day and three different days to calculate the recovery and inter-assay and intra-assay Coefficients of Variation (CVs). Recovery:

$$R = (VP-VB)/VA\times100\%$$

VA: Theoretical value of trifloxystrobin
VB: Value of sample without trifloxystrobin
VP: Value of sample with trifloxystrobin

RESULTS

Estimation of conjugated IMB: The composition and structure of IMB samples were qualitatively estimated by means of the Infrared Spectroscopy (Franke *et al*., 2005). From the Fig. 3, the Summary from the two different curves showed that the hydroxyl groups of nano ferroferric oxide and protein amino were condensated to an amide groups. The structure of ferromagnetic oxide magnetic beads could be showed from MB curve (A curve in Fig. 3). The stretching vibration of Fe-O could cause the characteristic peak at 588 cm^{-1}. These characteristic absorption peaks were attributed to the lattice absorption of magnetite and the absorbance band at 631 cm^{-1} was attributed to the phenyl structure (Zhong *et al*., 2010). According to the IMB curve (B curve in Fig. 3), there was a strong characteristic peak at 1070 cm^{-1} due to the stretching vibration without -H, which was the fingerprint region of C-N structure. The presence of two peaks at 1402 and 1456 cm^{-1}, was attributed to stretching vibration of single bond C-N of amide. Moreover, the weaker characteristic peak at 1557 cm^{-1} and the strong peak at 1648 cm^{-1} suggested that there were respectively a secondary amide groups, including the flexural vibration of N-H and stretching vibration of C = O. The antisymmetric and symmetric vibrations were at 2928 cm^{-1} due to the aliphatic alkyl chains. In general, the IMB curve (B curve in Fig. 3) keep the characteristic peak of nano ferroferric oxide in MB curve (A curve in Fig. 3) after coupling and the new peaks at 1070 cm^{-1} to 1648 cm^{-1} in IMB curve suggested amide corresponding to the principle of condensation reaction. Based on above information, the antibody IgG was bound to the MB.

The best reactive concentration: In order to prevent the low sensitivity and high background in the enzyme-linked immunosorbent assay, the antibody and enzyme labeled antibody needed to be determined for the best reactive concentration by using a phalanx experiment. IMB and IgG-HRP concentrations in IMB-ELLISA method were examined by the OD_{450} value (close to 1) of reactive solution according to the characteristics of

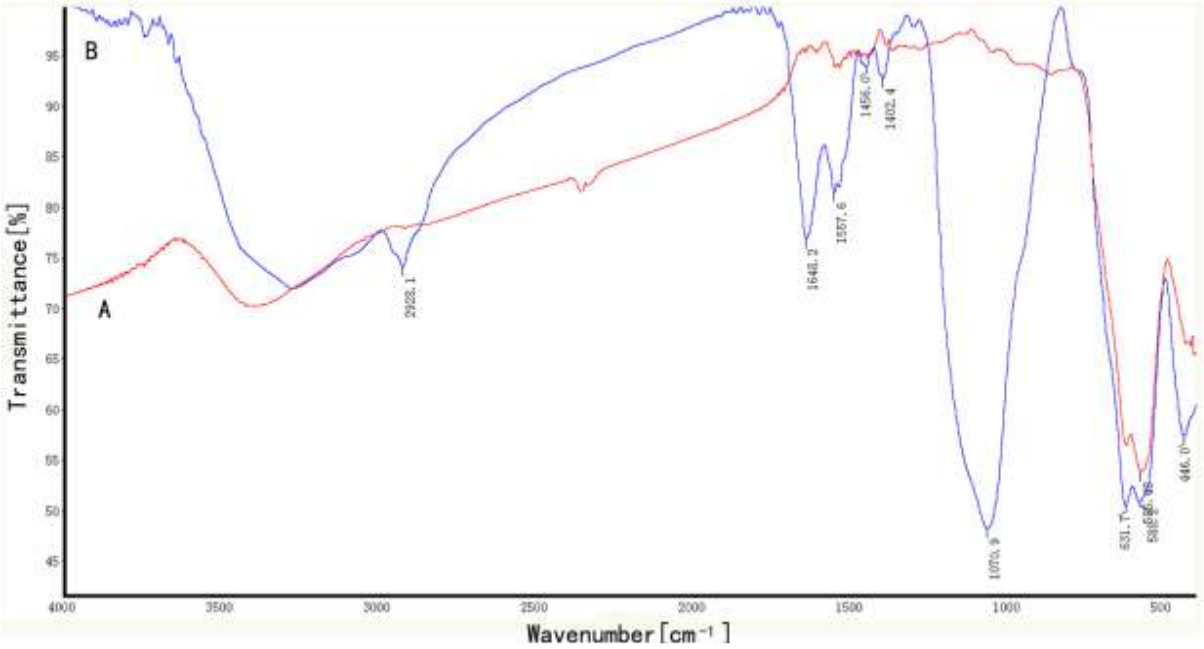

Fig. 3: FT-IR spectra of the IgG-IMB. The spectral range measured by the FT-IR was from 4000 cm^{-1} to 400 cm^{-1}. A, B curves, respectively represent MB and IMB. The range of 1070 cm^{-1} to 1648 cm^{-1} in B curve was the characteristics peaks of the formation of amide and 588 cm^{-1} in both A and B curve was attributed to the magnetic microsphere, nano-iron oxide

Table 1: The absorbance values of the reactive concentration in IMB-ELISA method

	IMB							
IgG-HRP	1/1000	1/2000	1/4000	1/8000	1/16000	1/32000	1/64000	1/128000
1000	1.834	1.796	1.519	0.961	0.713	663	0.577	0.401
1500	1.617	1.475	1.058	0.751	0.568	0.475	0.346	0.231
2000	1.312	0.921	0.786	0.581	0.475	0.413	0.297	0.212
3000	0.707	0.568	0.435	0.349	0.297	0.245	0.238	0.176
4000	0.458	0.454	0.411	0.309	0.295	0.225	0.219	0.174

*IMB was diluted to 1/1000-1/12800 and IgG-HRP was 1/1000-1/4000; The wavelength of the microplate reader was 450 nm; The 1.058 value was chosen as the best concentration

Table 2: The absorbance values of the reactive concentration in ELISA method

	Serum antibody							
IgG-HRP	1/1000	1/2000	1/4000	1/8000	1/16000	1/32000	1/64000	1/128000
1000	1.632	1.556	1.459	1.212	0.887	0.714	0.686	0.438
1500	1.204	1.126	1.024	0.727	0.673	0.521	0.417	0.331
2000	0.781	0.681	0.654	0.483	0.398	0.316	0.256	0.223
3000	0.531	0.455	0.438	0.312	0.301	0.278	0.212	0.164
4000	0.393	0.331	0.314	0.298	0.256	0.198	0.154	0.112

*Serum Antibody was diluted to 1/1000-1/12800 and IgG-HRP was 1/1000-1/4000; The wavelength of the microplate reader was 450 nm; The 1.024 value was chosen as the best concentration

automatic microplate reader (Li *et al*., 2011; Xu *et al*., 2012). As a result in the Table 1 and 2, the optimum dilution of IMB was 1/4000, which is the same as conventional ELISA and the dilution of IgG-HRP solution was 1/1500. Two methods had the same dilution level, which was attributed to the similar concentration of MB-antibody and the antibody in reactive process. However, the absorbance value in IMB-ELISA way was higher than conventional ELISA method, which suggested that the IMB-ELISA method could have higher capture efficiency in the same condition. It can also be confirmed from the IC50 value in followed study.

Sensitivity and specificity of IMB-ELISA: With these optimal conditions, the sensitivity of antibody could be summarized from the inhibition curve in Fig. 4. For assay characterisation, the concentration of trifloxystrobin was necessary to induce a 50% inhibition of the antibody-conjugate reaction (IC50) taken as a reference (Mercader *et al*., 2014). The IC50 value obtained by IMB-ELISA method was 2.28 μg/mL and ELISA method was 12.57 μg/mL. It can be summarized from the Fig. 4 that B/B_0 was lower in IMB-ELISA method than conventional ELISA at the condition of low concentration of trifloxystrobin standard materials. It was identical with those reported by Wang *et al*. (2014), this IMB-ELISA method was more accurate and sensitive and less time-consuming than the conventional ELISA. The IMB-ELISA method could have high capture efficiency in solution condition, which contributed low binding rate of IMB and ELISA plate. According to the two curve bending trend, IMB-ELISA method was more suitable for detection of low concentration of trifloxystrobin standard materials. Both methods showed excellent linear tendency in the measured range. Respectively, the linear relationship of B/B_0 value versus

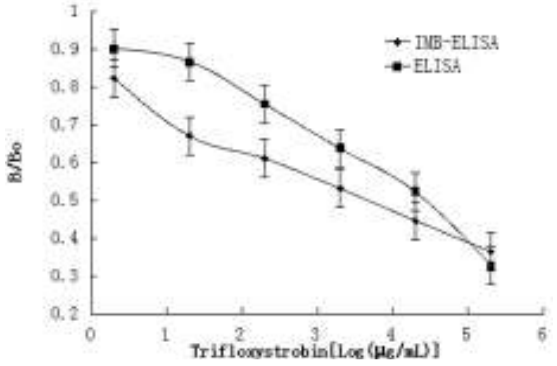

Fig. 4: Inhibition curves of IMB-ELISA and ELISA method. 6 dilution concentrations of trifloxystrobin were taken from 0.002, 0.02, 0.2, 2, 20, 200 μg/mL, respectively and B/B_0 was lower in IMB-ELISA method than conventional ELISA

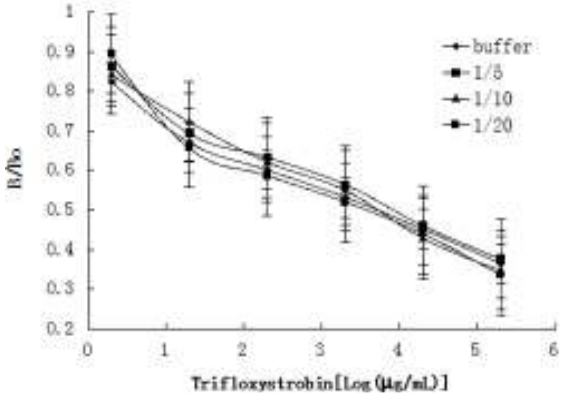

Fig. 5: Inhibition curves of trifloxystrobin in buffer and Sugar orange juice of different dilutions. Sugar orange juice was made by centrifuge and diluted to 1/5, 1/10, 1/20

trifloxystrobin was y = -0.0873x+0.5312 (IMB-ELISA $R^2 = 0.9837$) and y = -0.1097x+0.6206 (ELISA $R^2 =$

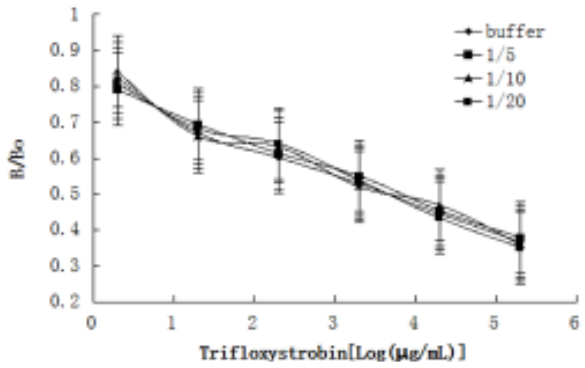

Fig. 6: Inhibition curves of trifloxystrobin in buffer and Ponkan citrus juice of different dilutions. Ponkan citrus juice was made by centrifuge and diluted to 1/5, 1/10, 1/20

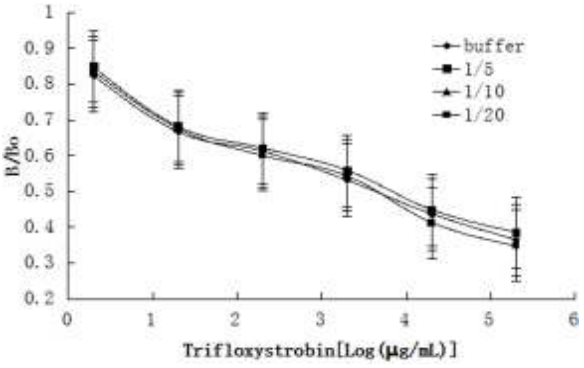

Fig. 7: Inhibition curves of trifloxystrobin in buffer and Honghe Papeda citrus juice of different dilutions. Honghe Papeda citrus juice was made by centrifuge and diluted to 1/5, 1/10, 1/20

Table 3: Recoveries for detection of trifloxystrobin in citrus sample

		Trifloxystrobin standard materials					
		100 μg/mL		10 μg/mL		1 μg/mL	
Citrus samples		R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
NO.1	inter-assay	92.52	7.8	91.69	6.8	90.12	8.9
	intra-assay	93.64	8.9	92.54	8.5	90.14	10.3
NO.2	inter-assay	91.73	4.5	93.25	7.3	95.74	6.8
	intra-assay	94.83	7.9	94.63	8.5	103.65	11.2
NO.3	inter-assay	93.29	6.4	93.27	7.8	95.44	8.2
	intra-assay	94.28	8.2	98.47	10.6	97.61	9.7

*Citrus samples were spiked with trifloxystrobin standard materials in 100 μg/mL; 10 μg/mL and 1 μg/mL; The inter-assay and intra-assay coefficients of variation were calculated by repeating in one day (n = 3) and different days (n = 3)

0.9747), where y was the B/B_0 values and x was the logarithmic concentration of trifloxystrobin. Finally, the CR = IC50 (trifloxystrobin)/IC50 (Pyraclostrobin)× 100%<0.1%. The pyraclostrobin could not couple the antibody without the methoxy imino acetic acid methyl ester, which displayed that the immunomagnetic beads had good specificity.

Matrix effects of IMB-ELISA: Three kinds of citrus were juiced and the sedimentation was removed by centrifugation. The supernatant would be diluted to different concentrations to detect the result of capture. In the Fig. 5 (Sugar orange), Fig. 6 (Ponkan citrus) and Fig. 7 (Honghe Papeda citrus), B/B_0 values were respective 0.335-0.3640.435-0.469, 0.521-0.551, 0.605-0.641, 0.661-0.695 and 0.792-0.824 when the concentrations of trifloxystrobin were 200, 20, 2, 0.2, 0.02, 0.002 μg/mL, respectively. There was no linear relationship of B/B_0 value with the increase of the juice concentration. The results showed that matrix effect was not important for the trifloxystrobin detection in the IMB-ELISA, which contributed to reduce the difficulty and time of the sample pretreatment.

Recovery rates of the assay in citrus samples: In the experiment, citrus was chosen as an actual system to study the stability and recovery rates of the assay. Citrus samples were spiked with different dilutions of trifloxystrobin standard materials which were detected by the IMB-ELISA assay. The recoveries determined according to the standard curve were summarized in Table 3, which showed that the recoveries were 90.12-103.65% and the CVs were in the range of 4.5-11.2%, indicating the good stability of the IMB-ELISA method. According to above results, the IMB-ELISA method could meet the requirements of trace analysis of trifloxystrobin in the citrus samples.

CONCLUSION

The IMB-ELISA detection was established for trifloxystrobin detection in citrus on the basis of immune magnetic beads. It could rapidly enrich the trifloxystrobin in sample, which provided a simple and highly effective pretreatment technology. An average IC50 value of 12.57 μg/m was obtained for IMB-ELISA method, which increased five times in contrast to IC50 value of 2.28 μg/m by conventional ELISA. This IMB-ELISA method was more suitable for low and medium concentration of trifloxystrobin in sample with high sensitivity and stability. There was only negligible low cross-reactivity in IMB-ELISA method. Therefore, it could be to detect trifloxystrobin content for different citrus species. The recovery rates ranges

from 90.12-103.65% suggested the IMB-ELISA method was satisfactory in citrus system with high accuracy. Based on above experiment, the IMB-ELISA method was an efficient and sensitive method for Trifloxystrobin detection, which had prospective potential in practical applications.

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