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A strip-based immunoassay for rapid determination of fenpropathrin

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We report the generation of monoclonal antibodies against fenpropathrin, originating from a BALB/c mouse immunized with a conjugate of hapten 1 [(RS)-cyano-3-phenoxybenzyl-2,2,3,3-tetramethylcyclopropane-carboxylic acid)] and keyhole limpet hemocyanin and the establishment of a monoclonal antibody-based immunochromatographic test strip. A coating antigen (test band) and a goat anti-mouse antibody (control band) were separately immobilized on nitrocellulose membrane strips as capture reagents. The anti-fenpropathrin monoclonal antibody was labeled with gold nanoparticles as a detection probe. This test strip had a wide linear range (15.6–250 μg L^{-1}) with a low detection limit of 62 ± 6 μg L^{-1} for fenpropathrin, evaluated using a strip reader, and could be read within 10 min. This novel test strip was used to test fenpropathrin in fortified samples and found to have a high recovery rate (>80%). In summary, our monoclonal antibody-based immunochromatographic strip offers a rapid screening tool for fenpropathrin detection in agricultural commodities.

1. Introduction

Fenpropathrin (FP, see Fig. 1a) was launched in 1971, classified as a type II synthetic pyrethroid and used to control various pests and mites in cotton, apple trees, vegetables and tea trees worldwide. In recent decades, the increasing use of FP has resulted in the presence of residues in agricultural products. Several studies have revealed that synthetic pyrethroids can cause serious health effects in humans such as headache, dizziness and nausea.[1] Consequently, legislation has been enacted to control pyrethroid residues and FP has been forbidden by the Chinese government in some agricultural plantings.[2]

Many analytical methods have been established for the determination of FP residues. Among them, chromatographic techniques with good accuracy and reproducibility have been described for FP detection including gas chromatography coupled mass spectrometry (GC-MS) or electron capture detection[3–5] and liquid chromatography,[6,7] but these formats are unsuitable for routine analysis due to the high cost and the time-consuming and complex sample cleanup required. In addition, immunoassays have been extensively employed to detect FP residues.[8–12] Wengatz *et al.* first prepared a polyclonal antibody specific to FP with a 50% inhibition concentration (IC_{50}) of 20 μg L^{-1}. Shi *et al.* developed an enzyme-linked immunosorbent assay (ELISA) to detect FP in aquatic samples. ELISA offers advantages of cost-effective and high throughput capability, but this approach requires 2–3 h and thus is not suitable for rapid detection. Consequently, a strip-based

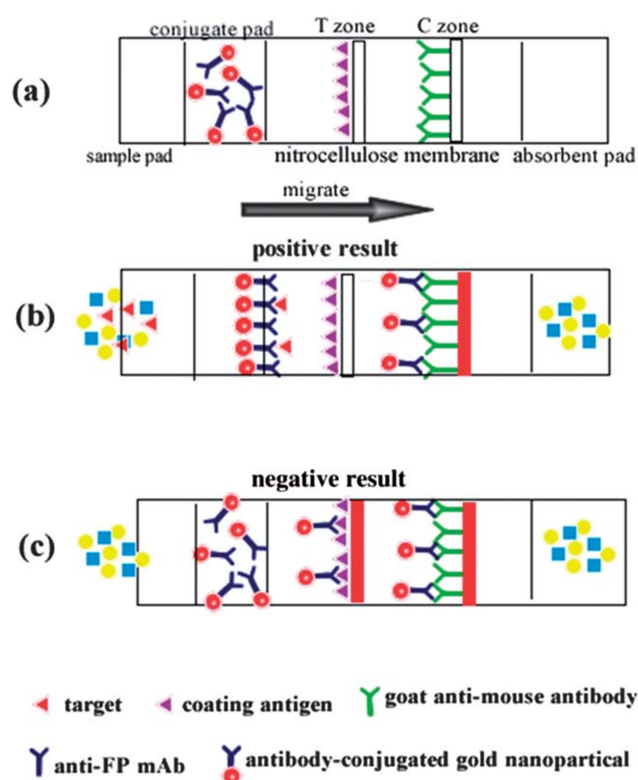

Fig. 1 The configuration diagram of the ICT strip (a); the schematics of the ICT strip for FP detection (b and c).

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immunoassay has been proposed for rapid detection of FP residues in fruits and vegetables.

Colloidal gold has previously been used in immunochromatographic strips for the detection of pesticides[13–15] due to its vivid color and excellent chemical stability. Kranthi *et al.* developed a simple lateral-flow immunochromatographic kit with a minimum detection concentration of cypermethrin (800 μg L^{-1}), deltamethrin (1000 μg L^{-1}) and fenvalerate (1400 μg L^{-1}). The strip-based immunoassay for FP measurement is an emerging technique. To the best of our knowledge, there is no published report on an immunochromatographic test strip specific for FP detection.

In this study, we aimed to prepare an anti-FP monoclonal antibody (mAb) and to use this to create a mAb-based immunochromatographic test strip for the rapid detection of FP. As a model, the test strip was then employed to detect FP residues in spiked apples and cucumbers. And then the result could be read within 10 min.

2. Materials and methods

2.1. Reagents and materials

All pyrethroids (fenpropathrin, high effect cypermethrin, cypermethrin, deltamethrin, cyfluthrin, cyhalothrin, tau-fluvalinate, cyphenothrin and esfenvalerate, 99% purity) were purchased from the Tianjin Institute for Environmental Protection (Tianjin, China). QuickAntibody adjuvant was obtained from Kang Biquan biological Co., Ltd. (Beijing, China). Keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), goat anti-mouse IgG antibody (GAM antibody) and chloroauric acid ($HAuCl_4 \cdot 4H_2O$) were obtained from Sigma-Aldrich (St. Louis, MO). All other reagents were purchased from Aladdin Co., Ltd. (Shanghai, China).

Other materials used to prepare test strips were purchased from JieYi Biotech. Co., Ltd. (Shanghai, China). These comprised a nitrocellulose membrane (NC membrane) used for immobilizing the coating antigen and GAM antibody, a glass fiber membrane used as the sample pad, Ahlstrom 8964 used as the conjugate pad, H5076 filter paper for the absorbent pad and a polyethylene (PVC) adhesive card.

2.2. The principle of the immunochromatographic test strip (ICT strip)

The lateral flow strip was assembled by mounting the NC membrane, conjugate pad, sample pad and absorbent pad on the adhesive backing card with appropriate overlaps (see Fig. 1a). The NC membrane was attached directly to the center of the adhesive card. The conjugate pad was pasted onto the end of the NC membrane, and the sample pad was overlapped with the conjugate pad and placed at one end, while the absorbent pad was pasted at the other end next to the control band and overlapping the NC membrane. The principle of our strip-based immunoassay is similar to that of a one-step competitive ELISA.

When samples were tested, the sample solution rapidly spreads along the test strip if it is applied to the sample pad. At the same time the antibody-conjugated gold nanoparticles (conjugated particles) on the conjugate pad begin to migrate along with the flowing sample. After 10 min, test results can be observed. A positive result is indicated by a red zone on the control line because FP in the sample blocks the conjugated particles from combining with the coating antigen (Fig. 1b), whereas a negative result is presented as two red zones on the NC membrane because the conjugated particles are trapped by the immobilized coating antigen and the GAM antibody (Fig. 1c). Furthermore, the color intensity in the test band is inversely proportional to the amount of FP in the sample, namely, the more FP is present in the sample, the weaker the test line appears.

2.3. Production of the monoclonal antibody (mAb)

Hapten 1 (Fig. 2b) was prepared and linked to KLH by the carbodiimide method using a previously reported method.[16] Hapten 1-BSA (coating antigen) was prepared using the *N*,*N*′-carbonyldiimidazole method.[17] BALB/c mice were immunized using the mixture of hapten 1-KLH and quickantibody adjuvant every two weeks. The quickantibody adjuvant is used without the need for time-consuming emulsification, resulting in a shorter immunization period. A mAb specific to FP was generated by typical cell fusion techniques. The mAb was purified by the caprylic acid and ammonium sulfate precipitation method[18] and then dialyzed for 3 days at 4 °C using phosphate buffer (10 mM PB, pH 7.2). The mAb concentration was tested by UV-visible (UV-vis) spectroscopy (Bokin Instruments, Tsushima, Japan) and adjusted to 0.2 g L^{-1} using borate buffer solution (2 mM BB, pH 8.2) before use.[19]

2.4. Preparation of gold nanoparticles (GNPs)

GNPs were synthesized by the sodium citrate reduction method, referring to previous reports.[20,21] All glassware was cleaned by soaking for 2 days in aqua regia ($HCl/HNO_3 = 3:1$, v/v), rinsed thoroughly with Millipore-Q water, and dried before further use. To prepare the GNPs, 100 mL of $HAuCl_4 \cdot 4H_2O$ solution (0.01%, w/v) was heated to boiling under continuous stirring, and then 1.5 mL of freshly prepared 1% (w/v) sodium citrate solution was quickly added while stirring. The color of the solution changed progressively to dark red within 1 min. The solution was boiled for 15 min, constantly topped-up with Millipore-Q water to 100 mL, then allowed to cool to room temperature before being stored at 4 °C. The GNP solution was characterized by UV-vis spectrometry at a wavelength of 300–800 nm and by transmission electron microscopy (JEOL, Tokyo, Japan).

2.5. Labeling of the mAb with GNPs

The antibody was labeled with GNPs according to the reported method.[22] After 20 mL of GNP solution was adjusted to pH 8.2 with 0.1 M K_2CO_3, the anti-FP mAb (0.2 g L^{-1}, 70 μL) was added dropwise under stirring. The resulting solution was shaken constantly for 2 h at ambient temperature. Blocking solution (10% BSA, 800 μL) was added to reduce non-specific binding and the mixture was incubated for a further 2 h under continuous stirring. At the end of this period the solution was centrifuged 3 times at 8000 rpm for 12 min to remove the excess antibody and the blocking agent. The soft sediment was resuspended in 10 mL

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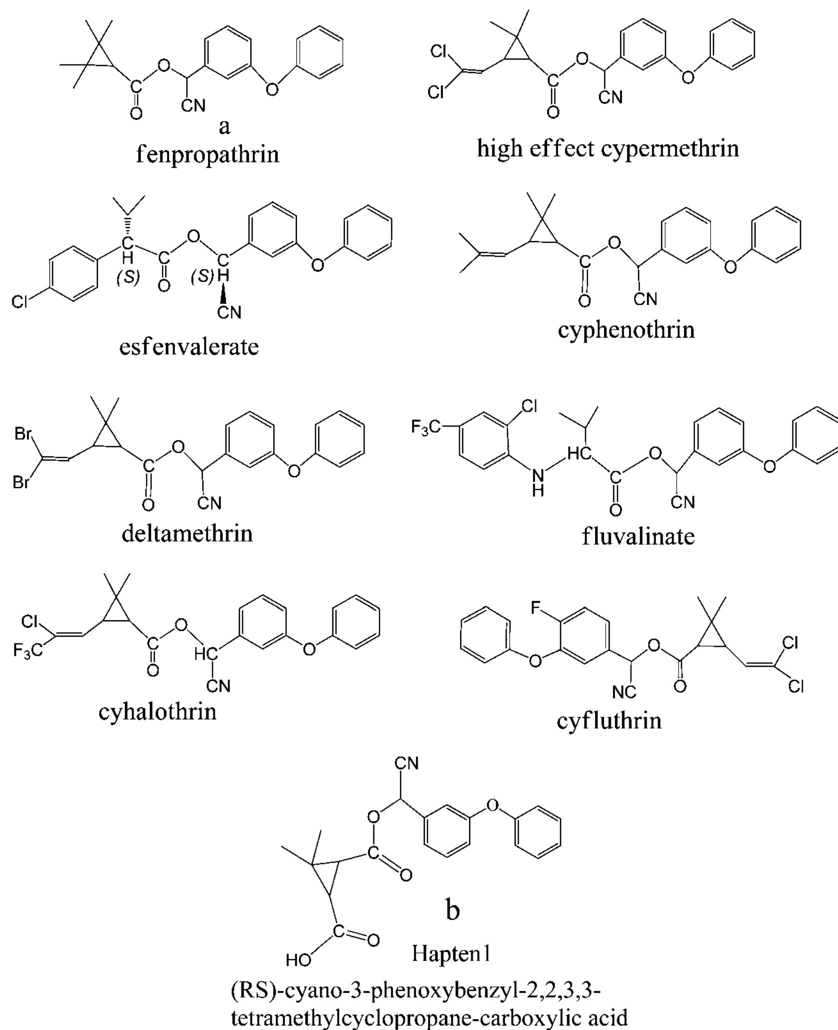

Fig. 2 Chemical structures of pyrethroids and hapten 1.

of gold-labeled resuspension buffer (10 mM PB, 0.5% PEG 6000, 5% sucrose, 1% BSA and 0.01% sodium azide, pH 7.2, w/v)[23] and stored at 4 °C until further use.

2.6. Assembly of the immunochromatographic test strip

Firstly, the conjugated particles were loaded on the fiber membrane to be used as the pad, and then this conjugate pad was dried at 37 °C for 2 h. Next, the coating antigen was adjusted to 0.5 g L^{-1} using carbonate buffer (5 mM CB, pH 9.6) and the GAM antibody was diluted to 0.2 g L^{-1} with phosphate buffer (10 mM PB, pH 7.2). Then, the coating antigen and GAM antibody were sprayed onto the NC membrane to create test bands (T bands) and control bands (C bands) using a Dispensing Platform (BioDot Inc., Irvine, CA). Finally, the entire assembly was then cut into 3 mm wide strips using a CM4000 Guillotine Cutting Module (BioDot Inc.) and dried at 37 °C for 4 h. The final prepared strips were stored in a self-sealing plastic bag.

2.7. Sensitivity of the ICT strip for FP analysis

A stock solution of 100 mg L^{-1} FP was prepared in *n*-hexane. The FP in *n*-hexane was dried under nitrogen before use and then dissolved in methanol. Subsequently, a range of FP standards was prepared by doubling dilution with 10% methanol-PB (10 mM, pH 7.2) to give eight concentrations (0, 7.8, 15.6, 31.2, 62.5, 125, 250, and 500 μg L^{-1}). The strip was dipped into a standard solution of FP for 3 min and then placed horizontally to allow the sample to flow easily. Seven minutes later, the color intensity of the test zones was quantified using the BioDot TSR3000 Membrane Strip Reader (BioDot Inc.). Each point was calculated by five determinations in this experiment. The calibration curve was obtained by plotting the color intensity values of the test band against FP concentration.

To evaluate the assay on real samples we used apples and cucumbers which were verified by GC-MS (Varian GC 3800 with 1079 injector, Varian Inc.) and found not to contain FP. Ten grams of samples were fortified with FP in *n*-hexane (100, 150 and 250 μg L^{-1}) and then allowed to infuse for 20 min. The spiked samples were extracted by shaking gently in 50 mL of *n*-hexane for 30 min and then filtered. The extraction process was repeated, and the organic phase was combined with that from the first extraction and dried under vacuum. The residue was completely dissolved in methanol and adjusted to an appropriate concentration in 10% methanol-PB prior to analysis using the test strips.

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A GC-MS assay [24] was performed to evaluate the performance of the test strips. The Florisil solid phase extraction was applied for sample cleanup, and the capillary column used to separate FP effectively was a DB-5ms (30 m × 0.25 mm i.d. × 0.25 μm) capillary column. The GC oven temperature program was as follows: 80 °C raised to 200 °C at the rate of 20 °C min^{-1}, then raised to 240 °C at the rate of 15 °C min^{-1}, and then raised to 286 °C at the rate of 5 °C min^{-1} and held for 5 min, and finally raised to 300 °C at the rate of 20 °C min^{-1} and held for 5 min. The analyte was tested by ion trap detection and the injection volume was 1 μL.

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3. Results and discussion

3.1. Production of the anti-FP mAb

The hybridoma named 7G5 secreting anti-FP mAb was obtained and found to have an IC_{50} value of 15 μg L^{-1}, which demonstrated that the sensitivity of the mAb increased approximately 20-fold over that of the antiserum. This mAb was specific to FP with little cross-reactivity (CR < 5%) with high effect cypermethrin or cyphenothrin. Essentially, no CR (<0.1%) was observed with cypermethrin, cyfluthrin, deltamethrin, cyhalothrin, tau-fluvalinate and esfenvalerate. Using IsoQuick strips from Envirologix (in Portland, ME) to test the supernatant of 7G5 cells, the types of heavy chain and light chain were identified as IgG_{2a} and kappa, respectively. Based on the format reported previously,[25] the affinity constant (K_a) of the mAb was calculated to be 3.37 × 10^9 L mol^{-1}.

3.2. Preparation of conjugated particles

The maximum absorbance of the GNP solution was at a wavelength of 523 nm. Transmission electron microscopy showed that the gold nanoparticles had a uniform size distribution, with an average diameter of 25 nm, which is the most popular size used for labeling antibodies.[26]

Some key parameters were evaluated to assess stabilization of colloidal gold and conjugation efficiency. First, different amounts of the antibody (3, 5, 7, 9, and 11 μg mL^{-1} of gold) were tested because this could influence the aggregation of GNPs. In addition, the pH of the GNP solution was changed by adding different amounts of 0.1 M K_2CO_3 (1, 1.5, 2, 2.5, 3, 3.5, and 4 μL mL^{-1} of gold). The GNPs coated with a citrate layer were negatively charged, while the antibodies were positively charged when the pH value of the GNP system was less than the isoelectric point of the antibodies (pH 9.0). Based on these findings, anti-FP antibodies were targeted for linking with GNPs through electrostatic interaction. Thus, the best conjugation efficiency was achieved when 7 μg of antibody was linked with 1 mL of gold nanoparticles after adding 2.5 μL of 0.1 M K_2CO_3.

3.3. Optimization of the ICT strip

ICT conditions were optimized to achieve the best performance. In the negative sample, different concentrations of the GAM antibody (0.1, 0.2, 0.3, 0.4 and 0.5 g L^{-1}) were evaluated based on the color intensity of the control zone. Then, various

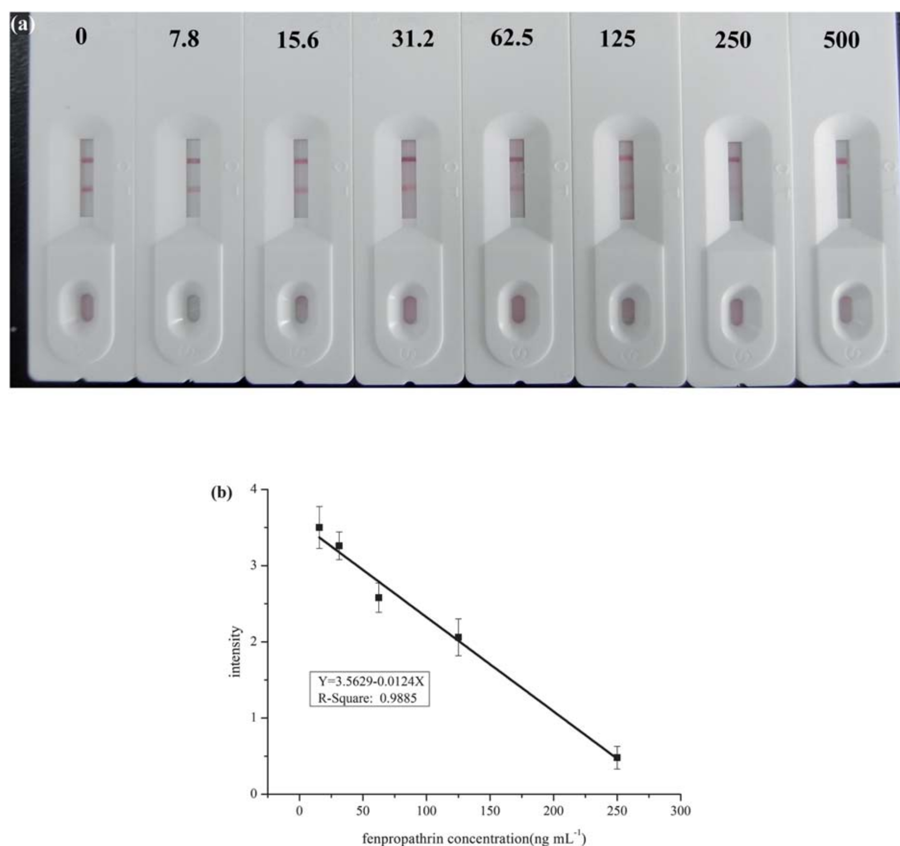

Fig. 3 Images of FP analysis using the ICT strip (a); standard curve for the determination of FP with the ICT strip (b).

Table 1 Determination of FP in spiked samples

	ELISA		Coefficients of variation (CV, %)		GC-MS
Samples	Fortified level (μg L^{-1})	Mean recovery (%)	Intra-day ($n = 5$)	Inter-day ($n = 3$)	Mean recovery (%) (CV) ($n = 15$)
Apples	100	87	3.12	5.36	82 (5.5)
	150	90	2.96	5.57	87 (6.2)
	250	96	3.35	5.48	95 (7.8)
Cucumbers	100	85	3.25	5.13	80 (6.2)
	150	89	3.41	5.46	84 (7.1)
	250	95	3.02	5.53	93 (8.3)

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concentrations of the coating antigen (0.1, 0.2, 0.3, 0.4 and 0.5 g L^{-1}) and concentrations of conjugated particles (8, 4, and 2 nmol L^{-1}) were investigated to obtain the desired color density of the test band and the lowest detection limit. The best sensitivity was found under the following conditions: coating antigen 0.5 g L^{-1}, GAM antibody 0.2 g L^{-1}, conjugated particles 4 nmol L^{-1}.

Under these optimized conditions, the ICT sensitivity was determined over the FP concentration range of 0 to 500 μg L^{-1} in 10% methanol-PB. As the FP concentration increased, the color density of the test band became significantly weaker. The visual limit of detection was defined as the minimum FP concentration at which the test line disappeared completely. As shown in Fig. 3a, 250 μg L^{-1} of FP resulted in a colorless test band. Consequently, 250 μg L^{-1} of FP was considered to be the detection limit (LOD) of the ICT strip by the naked eye.

An inhibition curve was established based on data recorded by the test strip reader (Fig. 3b). The concentration of FP ranged from 15.6 to 250 μg L^{-1} with a correlation coefficient of 0.9885. The detection limit was defined as 20% inhibition concentration, as reported in a previous published paper[27] and was calculated to be 62 ± 6 μg L^{-1}.

The apple and cucumber samples were spiked with FP in *n*-hexane to estimate the recovery rate from agricultural produces (Table 1). Each test was performed on five replicates over three consecutive days. The recovery rates varied from 81.6% to 103.8% and the coefficient of variation was less than 6%. The strip detection was found to have good correlations to the results of GC-MS (Table 1).

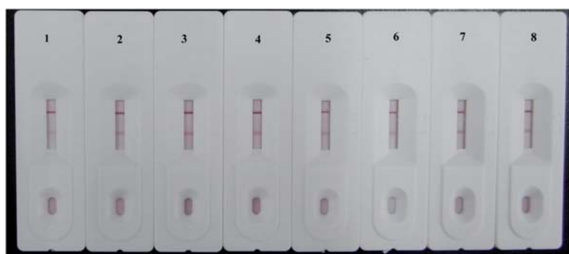

Fig. 4 Cross-reactivity of the ICT strip. (1) High effect cypermethrin, (2) cypermethrin, (3) deltamethrin, (4) cyhalothrin, (5) cyfluthrin, (6) cyphenothrin, (7) tau-fluvalinate, and (8) esfenvalerate.

3.4. Specificity of the strip test

The cross-reactivity of the ICT assay was examined using high effect cypermethrin, cypermethrin, deltamethrin, cyfluthrin, cyhalothrin, tau-fluvalinate, cyphenothrin, and esfenvalerate. All these pyrethroids were used to create spiked samples at 2 mg L^{-1}. As shown in Fig. 4, the density of the test zone with high effect cypermethrin and cyphenothrin was weaker than other pyrethroids, indicating low cross-reactivity. Six other pyrethroids exhibited satisfactory color intensity of the test zone, indicating a clear specificity of the strips to FP.

4. Conclusions

We established a sensitive strip-based immunoassay for the rapid detection of FP in food using a mAb conjugated to GNPs. The ICT strip had a low detection limit of 62 ± 6 μg L^{-1} with the aid of a portable strip reader. And the strip could meet the screening requirements for FP residues in agricultural products. Testing was only possible if test strips could be dipped into the sample solution. Results were available within 10 min. Thus, this developed strip is a fast, simple, and sensitive method of detecting FP in agricultural produces.

Acknowledgements

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