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Development of an ic-ELISA and immunochromatographic strip for detection of sparfloxacin in honey

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ABSTRACT

In this study, a sensitive monoclonal antibody (mAb) 3D5 against sparfloxacin (SPFX) was generated. Based on the mAb 3D5, an indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) method was developed for the detection of SPFX in honey. The half maximal inhibitory concentration and limit of detection for the ic-ELISA method were 0.12 and 0.02 ng/mL, respectively. The experiments showed a negligible cross-reaction with other drugs. The average recovery rates for spiked SPFX honey extracts ranged from 90% to 101%, indicating an accepted accuracy. Furthermore, a lateral-flow immunochromatographic assay strip method with a cut-off value of 2 ng/mL was developed. Therefore, both of the developed methods are suitable for future use as rapid screening methods to detect and control the content of SPFX residues in honey samples.

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KEYWORDS

Sparfloxacin; monoclonal antibody; ic-ELISA; ICA strip; honey

Introduction

The use of sparfloxacin (SPFX), a third-generation fluoroquinolone antibiotic, is widespread in animals for preventing disease and promoting growth due to its broad-spectrum antibacterial properties (Peng et al., 2016). A deficiency in SPFX leads to phototoxicity, renal-hepatic system damage, and genotoxicity (Beberok et al., 2015; Giacometti, Cirioni, Barchiesi, & Scalise, 2000). Furthermore, long-term exposure to SPFX can result in the emergence of drug-resistant pathogens which increases the risk of death from common infections (Martín-Galiano & de la Campa, 2003; Minnick, Wilson, Smitherman, & Samuels, 2003; Tlili et al., 2016; Weigel, Anderson, & Tenover, 2002). Due to these concerns, many countries have set maximum residue limits (MRLs) for several quinolones to protect consumers (Tochi, Khaemba, et al., 2016; Zhi et al., 2010). China and the USA do not permit use of SPFX in honey or honey products. Therefore, it is essential that a sensitive, rapid, and accurate SPFX detection method is required to assess the safety of foods.

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Many analytical methods have been used to detect fluoroquinolones, including high-performance liquid chromatography (HPLC), liquid chromatography–tandem mass spectrometry, fluorescence analyses, spectrophotometric analyses, and capillary electrophoresis (Alcaráz, Siano, Culzoni, Muñoz de la Peña, & Goicoechea, 2014; Gul, Sultana, Saeed Arayne, Shamim, & Akhtar, 2012; He & Blaney, 2015; Li et al., 2009; Pinacho, Sánchez-Baeza, Pividori, & Marco, 2014), which are all complex, time-consuming, and expensive. Compared with these methods, an immunoassay is a sensitive, low-cost, and rapid technique that has already been reported to detect fluoroquinolones (Chen & Jiang, 2013; Jiang et al., 2011; Liu et al., 2014; Peng, Liu, Kuang, Cui, & Xu, 2017; Tian, Zhang, Sun, Qian, & Ji, 2013; Tochi, Khaemba, et al., 2016; Tochi, Peng, et al., 2016; Wang, Zhang, Ni, Zhang, & Shen, 2014; Zhang et al., 2011). Although the enzyme-linked immunosorbent assay (ELISA) with a half maximal inhibitory concentration (IC_{50}) of 31 ng/mL was established (Deng et al., 2013), it does not meet the requirements for field applications. Furthermore, no studies have yet investigated immunochromatographic assays (ICAs) for the detection of SPFX.

Therefore, the main aim of this study was to develop an indirect competitive (ic)-ELISA method and lateral-flow ICA strip for detection of SPFX in honey samples.

Materials and methods

Reagents and materials

Sparfloxacin, enrofloxacin, fleroxacin, flumequine, ciprofloxacin, ractopamine, gentamicin, terbutaline, clenbuterol, and sabutamol were provided by the National Institute of Control of Pharmaceutical and Biological Products (Beijing, China). Bovine serum albumin (BSA) and ovalbumin (OVA) were purchased from Shanghai Shangbao Biological Science and Technology Co., Ltd (Shanghai, China). *N,N'*-Dicyclohexylcarbodiimide (DCC) and Freund's complete and incomplete adjuvants (cFA and iFA, respectively) were obtained from Sigma-Aldrich (St. Louis, MO, USA). *N*-Hydroxysuccinimide (NHS) was purchased from J&K Chemical Co., Ltd (Shanghai, China). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (goat anti-mouse IgG–HRP) was purchased from the Military Medical Institute (Beijing, China). *N,N*-Dimethylformamide (DMF) and Tween 20 were purchased from Donghong Chemical Company (Guangzhou, China). 3,3',5,5'-Tetramethylbenzidine (TMB) was obtained from Amresco (Solon, OH). All other reagents used were of chemical grade and obtained from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China).

Instrumentation

The ELISA was performed in polystyrene 96-well microtiter plates (Bio Basic Inc., Markham, Canada). The Microplate Reader 450/550 was from Bio-Rad (Richmond, CA, USA). UV data were collected with a U-4100 spectrophotometer from Hitachi Co. Milli-Q water was obtained from Milli-Q® Integral Water Purification System (Millipore, Bedford, USA).

Buffers and solutions

Ultrapure deionized water was used for the preparation of all the buffers and reagents used in the immunoassays. (1) 0.01 mol/L Phosphate-buffered saline (PBS, pH 7.4) contained NaCl (4 g), KH_2PO_4 (0.135 g), $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (1.8 g), and KCl (0.1 g) as the dilution to the SPFX standard substance. (2) 0.05 mol/L carbonate buffer (CBS, pH 9.6) contained Na_2CO_3 (0.795 g) and NaHCO_3 (1.47 g) as the coating buffer. (3) Washing buffer contained 5% porcine serum as the blocking buffer. (4) PBS with 0.05% (v/v) Tween 20 and 0.1% (w/v) gelatin was the antibody dilution buffer; (5) 2 mol/L H_2SO_4 was the stop solution.

Preparation of immunogen and coating antigen

The immunogen was synthesized by conjugating SPFX to BSA with the active ester method with some modifications (Figure 1). SPFX (3.6 mg) was dissolved in a mixture

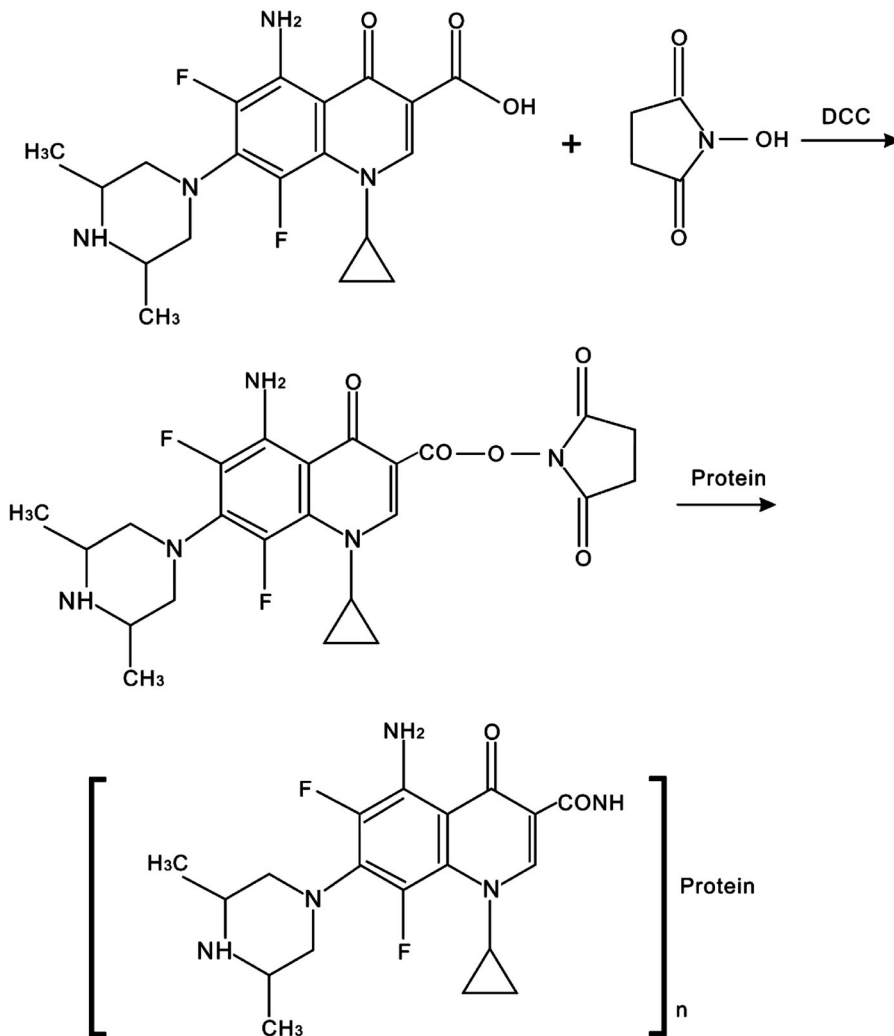


Figure 1. Synthesis of immunogen and coating antigen (Protein: BSA or OVA).

of NHS (2.5 mg) and DCC (5 mg) in 2 mL DMF. Then the mixture was incubated at room temperature for 12 h without light. Finally, the reaction mixture was drop-wise added into 5 mL PBS containing 8 mg BSA under stirring condition and stirred at 4°C for 8 h.

The hapten–BSA conjugate solutions were dialyzed stirring against PBS at 4°C for 72 h, during which the PBS was renewed three times per day to remove any uncoupled free hapten. The conjugates were freeze-dried and stored at –20°C until future use. The coating antigen SPFX–OVA was generated in a similar way. The conjugates were characterized by their UV absorbance spectra and with the antiserum of mice immunized with the SPFX–BSA conjugate.

Immunization, cell fusion, and monoclonal antibody (mAb) generation

Three female BALB/c mice (6–10 weeks old) were subcutaneously immunized with the immunogen BSA–SPFX at multiple sites on their backs. To monitor the quality of the antiserum produced by the immunized mice, the serum titers were measured with the ELISA procedures as previously described (Wang et al., 2014). The mice with high serum antibody titer and low IC₅₀ were selected for cell fusion. Briefly, the mouse spleen cells were aseptically harvested and fused with myeloma cells in a 10:1 ratio, using polyethylene glycol 1500 as the fusing agent. In order to obtain stable cell lines, the fused cells were screened and subcloned using the ic-ELISA method and limiting dilution method, respectively (Kong et al., 2017).

Mature female BALB/c mice were injected intraperitoneally (i.p.) with 0.5 mL of paraffin 10 days before an i.p. injection of the positive hybridoma cells. The ascites fluid was collected 10 days after injection. The mAb was purified with the modified caprylic acid ammonium sulfate precipitation method. The mAb from the mouse that showed the highest titer and sensitivity was used in this study.

Development of the ic-ELISA

Checkerboard tests were performed to determine the optimal dilutions of the coating antigen and the primary antibody (Buchberger, 2007). Ninety-six-well microtiter plates were coated using the SPFX–OVA solution at different concentrations (from 50 to 10,000 ng/mL) at 37°C for 1 h. After the wells were washed four times with washing buffer, the plates were blocked with 5% porcine serum (200 µL/well) at 37°C for 1 h. After washing, 50 µL of antiserum (dilutions ranged from 1×10^{-5} to 6.5×10^{-5}) and 50 µL of SPFX standard solution were added using a standard checkerboard titration method and incubated at 37°C for 30 min. After the wells were washed four times, goat anti-mouse IgG–HRP solution (1:2000) was added to the plates (50 µL/well) and the plates were incubated at 37°C for 30 min. For color development, substrate solution was added to each well (50 µL/well) and the plates were incubated for 10 min at room temperature. The reaction was terminated by the addition of the stop solution at 50 µL/well. The absorbance was measured with a microplate reader at 450 nm.

Sensitivity and specificity of the assay

The IC₅₀ value and cross-reaction (CR) were determined to evaluate the sensitivity and specificity of the assay, respectively. Competitive immunoassays were performed using

four related compounds (enrofloxacin, fleroxacin, flumequine, and ciprofloxacin) and several other compounds. The four fluoroquinolones were chosen because they are structurally similar to SPFX. The tested fluoroquinolones were subjected to the ic-ELISA, as described above for SPFX. The CR values were then calculated with the following equation:

$$\text{CR} = \frac{\text{IC}_{50} \text{ sparfloxacin (ng/ml)}}{\text{IC}_{50} \text{ tested compound (ng/ml)}} \times 100\%.$$

Preparation of the lateral-flow ICA strip

A polyvinyl chloride (PVC) backing card, an nitrocellulose (NC) membrane, the sample pad and absorption pad were assembled to prepare the lateral-flow ICA strip as described previously (Yang et al., 2015). Sample and absorbent pads were made from nonwoven, 100% pure cellulose fiber. The sample pad was cut to 15 × 280 mm and saturated with a buffer (pH 8.0) containing 20 mmol/L sodium borate, 2.0% (w/v) sucrose, 2.0% (w/v) BSA, and 0.1% (w/v) NaN₃ to reduce the matrix interference, and then dried and stored. The absorbent pad was cut to 40 × 280 mm. 1 mg/mL SPFX-BSA and 1 mg/mL goat anti-mouse IgG were individually applied on the nitrocellulose membrane as the test and control lines. The assembled strip card was dried at 37°C for 30 min and then cut into individual test strips.

The gold nanoparticles (GNPs) were prepared as described previously (Jiang et al., 2013). (1) The pH of the GNP solution was adjusted to pH 9.0 by 0.2 M K₂CO₃ and then 0.15 mg mAb was added to 10 mL GNP solution. The mixture was kept for 15 min at RT. (2) 0.1 mL of 20 mmol/L sodium borate (pH 9.0) containing 10% BSA (w/v) was slowly added and the mixture was incubated for another 15 min at RT for blocking and stabilizing the GNP-labeled mAb. (3) The GNP-mAb solution was collected by repeated centrifugation (25,000 g) at 4°C for 30 min after adding 20 mmol/L sodium borate (pH 9.0) containing 3% BSA and 3% sucrose. The precipitate was the labeled mAb and stored in the PBS at 4°C for later use.

Principle of the lateral-flow ICA strip

The competitive/inhibitory reaction between the SPFX in samples and the coating antigen (SPFX-OVA) proceeded on the T line for the GNP-labeled anti-SPFX mAb. If SPFX is present in the sample, it competes with the immobilized capture reagent for the limited amount of detection reagent. The more SPFX in the sample, the weaker the test line color. If abundant SPFX is present in the sample, it will completely block the reaction with the capture reagent, thus no test line is visible. Provided that the test strip and the test procedure are correct, the control line is always visible. If no colored capture line or only a red color at the test line appears, the strip was invalid, and the test should be repeated using a new strip.

For the lateral-flow ICA strip, the visual limit of detection (vLOD) was defined as the lowest concentration of SPFX that led to a definitely weaker color intensity of the T line compared to a negative sample (Kong et al., 2017). The cut-off value was the SPFX concentration that led to the disappearance of the T line (Li et al., 2013)

Honey sample preparation and HPLC analysis

Honey samples were obtained directly from a market. They were assayed with HPLC in the Supervision and Verification Center of the Ministry of Agriculture (Zhengzhou, China). The same samples were analyzed with the ic-ELISA and HPLC to compare the quantitative concordance of the two methods. The HPLC analysis was performed on an Agilent 1200 system, equipped with an Agilent UV-VIS detector set at 290 nm, and the Chromeleon™ software was used to calculate the peak areas. An Agilent Zorbax Eclipse XDB-C₁₈ column (150 mm × 4.6 mm, 5.0 μm particle size) was used at a flow rate of 1 mL/min at 25 °C. The column conditions included an eluent of acetonitrile–0.05 mol/L phosphate buffer and an injection volume of 20 μL. Under these conditions, the retention time of SPFX was 13 min. The concentrations of SPFX in the honey samples were 0.8, 2, and 5 ng/mL, representing low, medium, and high levels of residue.

Results and discussion

Design and synthesis of the hapten

The design of hapten is one of the crucial steps in producing a high-quality antibody (Cao, Meng, Lu, & Xi, 2011; Sheng et al., 2009). The spacer-arm folding and steric-shielding effects of the hapten play important roles in the specificity and selectivity of an antibody (Wang et al., 2014). SPFX (molecular mass, 392.41 Da) as a small molecule requires the appropriate modification. The active carboxylic acid group at position 3 in the SPFX molecule allows its direct conjugation to the most commonly used carrier proteins (BSA and OVA). UV absorbance was used to characterize the BSA, SPFX, and SPFX–BSA. The hapten showed one specific ultraviolet absorption peak at 292 nm, whereas the BSA showed maximum absorption at 278 nm. The hapten–BSA conjugate showed typical absorbance peaks at both 224 and 292 nm (Figure 2). These data demonstrate that the conjugation of SPFX–BSA was successfully synthesized.

The mAb characterization

The selected hybridoma cell line was obtained after cell fusion, and the selected mAb, 3D5, was purified from the prepared ascites. The titer of the mAb 3D5 was determined to be $>6.5 \times 10^5$ by an ELISA. The IC₅₀ value is a key criterion for evaluating the sensitivity of an ELISA. The lower the IC₅₀ value, the higher the sensitivity of the assay (Zhi et al., 2010). In this study, the mAb 3D5 is highly sensitive, the IC₅₀ of 3D5 was 0.3 ng/mL, and the LOD of the assay was 0.04 ng/mL (Figure 3).

Specificity of mAb

The specificity of mAb was investigated by testing the IC₅₀ and CR values obtained for a set of related compounds (Cao et al., 2011; Duan & Yuan, 2001; Holtzapple, Buckley, & Stanker, 1997). The specificity of 3D5 was evaluated by performing competitive assays against four fluoroquinolones and five other veterinary drugs (Table 1). The CR values for the antibody against four structurally related compounds were 1.68–4.01%, and the

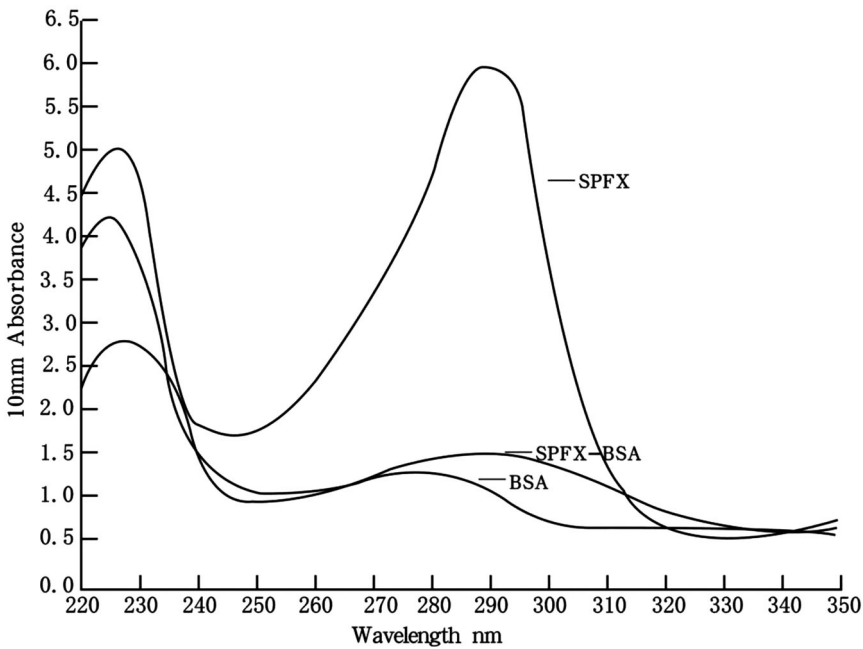


Figure 2. UV scanning of BSA, SPFX-BSA, SPFX (BSA: Bovine serum albumin; SPFX-BSA: The conjugate of SPFX with BSA).

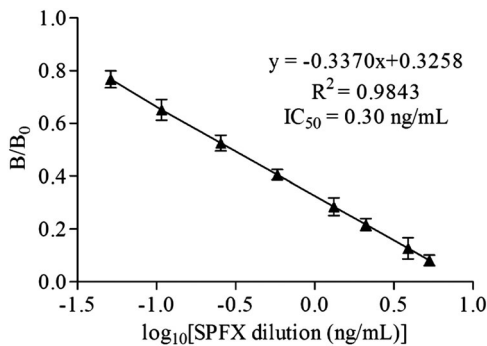


Figure 3. Inhibitive curve of SPFX mAb by ic-ELISA.

antibody shows no CR to other compounds (CR < 0.009%). The results clearly demonstrate that the mAb 3D5 is highly specific for SPFX.

Optimization of ic-ELISA

Different concentration standards were prepared, and ic-ELISA was developed to evaluate the sensitivity of mAb 3D5 with a coating antigen concentration of 1000 ng/mL and mAb concentration of 2×10^{-5} . Under the optimal assay conditions, a standard curve for the ic-ELISA (Figure 4) was constructed with nonlinear regression using a four-parameter

Table 1. The CR of SPFX mAb 3D5 with compete compounds.

Compound	IC ₅₀ (ng/mL)	CR (%)
Sparfloxacin	0.30	100
Flumequine	7.47	4.01
Fleroxacin	88.21	3.40
Enrofloxacin	97.36	3.08
Ciprofloxacin	178.23	1.68
Ractopamine	$>3.2 \times 10^3$	<0.009
Gentamicin	$>3.2 \times 10^3$	<0.009
Terbutaline	$>3.2 \times 10^3$	<0.009
Clenbuterol	$>3.2 \times 10^3$	<0.009
Sabutamol	$>3.2 \times 10^3$	<0.009

logistic equation. According to the standard curve, the IC₅₀ was 0.12 ng/mL and the LOD was 0.02 ng/mL.

Immunochromatographic strip assay

Each sample was spiked with the SPFX solution at concentrations of 0, 0.25, 0.5, 1, 1.25, 1.5, 2, and 2.5 ng/mL and then detected with a test strip. As shown in Figure 5, a deep color was observed on the T line by the naked eye when 0 ng/mL SPFX was added. As the concentration of SPFX increased, the color on the T line became weaker. Accordingly, the vLOD was 1 ng/mL. The color disappeared completely when the concentration of SPFX reached 2 ng/mL. This concentration of 2 ng/mL was defined as the cut-off value when evaluating the sensitivity of the strip. Therefore, in the lateral-flow ICA strip analysis, the semiquantitative results evaluated with the naked eye were only detectable as negative (<1 ng/mL for SPFX), weakly positive (1–2 ng/mL for SPFX), or positive (>2 ng/mL for SPFX).

Sample analysis

SPFX (at concentrations of 0.8, 2, and 5 ng/mL) was added to the honey samples and measured with the ic-ELISA, the lateral-flow ICA strip, and HPLC. The average recoveries of SPFX by the ic-ELISA were 90.0–101.0% (Table 2). As expected, the semiquantitative results of samples (at concentrations of 0.8, 2, and 5 ng/mL) detected by the lateral-flow ICA strip were negative, weakly positive, and positive, respectively (Table 2).

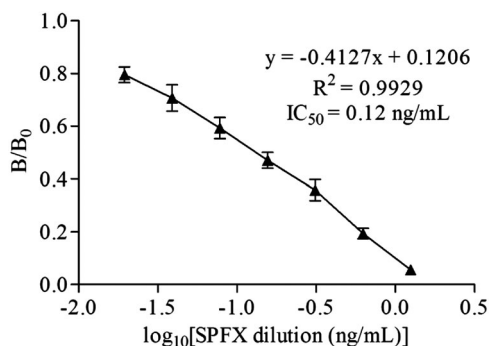
**Figure 4.** Standard curve of SPFX by ic-ELISA.



Figure 5. The spiked sample analysis with lateral-flow ICA strip by visual ($n = 6$). The spiked sample with SPFX: (A) 0 ng/mL; (B) 0.25 ng/mL; (C) 0.5 ng/mL; (D) 1 ng/mL; (E) 1.25 ng/mL; (F) 1.5 ng/mL; (G) 2 ng/mL; (H) 2.5 ng/mL. Negative result: the test line can be observed. Positive result: no test line is observed.

Table 2. The spiked sample analysis with ic-ELISA and lateral-flow ICA strip ($n = 6$).

Spiked SPFX (ng/mL)	ic-ELISA		Lateral-flow ICA strip
	Results (ng/mL)	Recovery (%)	Results (visual)
0.8	0.78 ± 0.49	97.5 ± 4.90	–
2	1.87 ± 0.87	93.5 ± 4.35	+
5	5.05 ± 0.62	101.0 ± 3.97	++

Note: –: negative; +: weakly positive; ++: positive.

Table 3. Concentrations of SPFX in honey samples determined by ic-ELISA and HPLC.

Sample (ng/mL)	ic-ELISA (ng/mL)	HPLC (ng/mL)
Sample1, 0.8	0.78 ± 0.49	0.799 ± 0.001
Sample2, 2	1.87 ± 0.87	1.985 ± 0.040
Sample3, 5	5.05 ± 0.62	4.963 ± 0.011

Good consistency was observed between the ic-ELISA and HPLC as shown in Table 3 ($R^2 = 0.999$). This further confirms the reliability of the two methods.

Conclusion

In this study, a sensitive and specific mAb 3D5 was obtained. Both the ic-ELISA method and the lateral-flow ICA strip method were established for the detection of SPFX in honey products. For the ic-ELISA method, under the optimum conditions, the IC_{50} for sample analysis was 0.12 ng/mL and the LOD was 0.02 ng/mL. The recovery rates ranged from 90.0% to 101.0% in honey samples. Good consistency was obtained between the ic-ELISA and HPLC. In the ICA strip, the $vLOD$ and cut-off values were 1 and 2 ng/mL, respectively. In conclusion, both methods were effective for the analysis of honey samples, and these tools will play a significant role in future analytical applications.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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