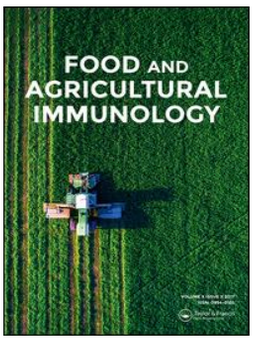

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Development of an immunochromatography assay for salinomycin and methyl salinomycin in honey

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ABSTRACT

This study developed a sensitive monoclonal antibody, 3A3, to salinomycin (SLM) and methyl salinomycin (MLN). The monoclonal antibody 3A3 is highly specific for SLM with an antibody halfinhibitory concentration (IC 50) of 0.86 ng/mL, a detection limit of 0.28 ng/mL, and a linear detection range of 0.28–2.26 ng/mL. The cross-reaction values of SLM and MLN were 100% and 74.48%, respectively. The recovery of honey samples ranged from 94.6% to 100.4%, thus indicating that this analytical method can be used for the analysis of real honey samples. In addition, immunochromatographic bands were developed for qualitative, semi-quantitative and speckle detection analysis. The critical values of SLM and MLN in honey were 40 and 40 ng/mL, respectively. The results showed that these analyses can be used for real samples, with the most stringent MRL of 40 ng/mL, for SLM and MLN.

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Immunochromatography; assay; salinomycin; methyl salinomycin

1. Introduction

Salinomycin (Figure 1(a); SLM) and methyl salinomycin (Figure 1(b); MLN) are polyether antibioticsand are widely used as anticoccidial agents for poultry worldwide. Because of its low drug resistance and in food, SLM has broad market expectation (Jiang, Li, et al., 2018). SLM and MLN have strong antibacterial activity against Gram-positive bacteria and various anaerobic bacteria (Huang et al., 2018). However, they are highly toxic and may cause headaches, nausea, nosebleeds, ocular opacity, and myocardial damage in humans. European and American countries have set maximum allowable added amounts in livestock and poultry production. The 235 Announcement of the Ministry of Agriculture of China also stipulates that the maximum allowable residue in different animal source products shall not exceed 4500 ng/g (Tian et al., 2017).

To protect consumers, assessment and control of antibiotic residues in food is important. Several methods for monitoring SLM and MLN have been developed, including high performance liquid chromatography (HPLC) (Zhao et al., 2018), gas chromatography

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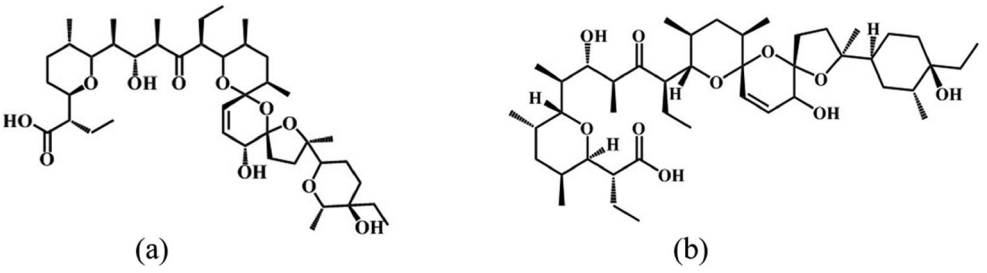

Figure 1. Chemical structure of salinomycin (a) and methyl salinomycin (b).

(Rudnicki, Domagała, Burnat, & Skrzypek, 2016), and liquid chromatography-tandem mass spectrometry. These methods do not have significant advantages in terms of sensitivity and accuracy, and they require specific instruments and trained laboratory assistant. The detection limit of SLM is 18 ng/g through the HPLC/MS method, a level significantly higher than the detection limit of the enzyme-linked immunosorbent assay (ELISA) method, and the treatment process is cumbersome and time consuming (Zhao et al., 2018). Liquid chromatography-tandem mass spectrometry has been used to detect SLM in mouse plasma with a detection limit of 2000 ng/mL (Sparidans, Lagas, Schinkel, Schellens, & Beijnen, 2007). Furthermore, substantial time is needed for sample preparation (Bak et al., 2013). Thus, these methods are not applicable for the analysis of numerous samples.

Immunological methods based on specific binding reactions of antibodies and antigens have been rapidly developed for food safety screening (Kong, Xie et al. 2017b). ELISA and immunochromatographic strips (ICS) have been successfully used to monitor many drug residues because of their rapidity, user friendliness, and sensitivity (Kong et al., 2017a; Kong, Liu, Song, Kuang, & Xu, 2016; Xie, Kong, Liu, Song, & Kuang, 2017). In this work, a SLM based prodrugs was used to prepare a hapten through a one-step reaction, and a high affinity monoclonal antibody (mAb) was produced to simultaneously recognize SLM and MLN. Honey samples were screened for evaluation with indirect competition ELISA (ic-ELISA) and ICS methods.

2. Materials and methods

2.1. Reagents and apparatus

SLM was provided by J&K Scientific Ltd. Keyhole limpet hemocyanin (KLH; MW 500,000), bovine serum albumin (BSA; MW 67,000), and ovalbumin (OVA; MW 45,000) were obtained from Sigma Chemical Company (St. Louis, MO, USA). 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), complete Freund's adjuvant (FCA), incomplete Freund's adjuvant (FIA), gelatin, and 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (goat anti-mouse IgG–HRP) was purchased from the Military Medical Institute (Beijing, China). Reagents for cell fusion and culture (e.g. polyethylene glycol 1500 solution, HAT supplement, HT medium supplement, and 1640 cell culture medium) were obtained from Life

Technologies Co., Ltd. (Shanghai, China). N,N-dimethylformamide and Tween 20 were purchased from Donghong Chemical Company (Guangzhou, China) was obtained from Amresco (Solon, OH). All other reagents used were of chemical grade and were obtained from CB, (pH 9.6) containing Na_2CO_3 (0.795 g) and $NaHCO_3$ (1.47 g) was used as the coating buffer. (3) The coating buffer contained 0.2% gelatin as the blocking buffer. (4) PBS with 0.05% (v/v) Tween 20 and 0.1% (w/v) gelatin was used as the antibody dilution buffer; (5) 2 mol/L H_2SO_4 was used as the stop solution.

2.3. Synthesis of haptens and conjugated antigen

SLM contains a carboxyl group in its molecular formula, and it can be synthesized through an activated ester method, thus yielding a protein-PLA conjugate (Kolosova, Samsonova, & Egorov, 2000). SLM (10 mg) was added to 600 μL of N-dimethylformamide, and 6 mg of EDC and 4 mg of NHS were weighed and dissolved in 200 μL of 0.01 mol MES solution; the mixture was stirred at room temperature for 4–6 h to obtain solution A. In addition, 20 mg of BSA was dissolved in 2 mL of CB to obtain solution B. Solution A was added dropwise to solution B with constant stirring and was incubated overnight at room temperature. Finally, the mixture was purified by dialysis against 0.01 M PBS, and the dialysate was changed three times per day for 3 days at room temperature, during which the PBS was replaced three or four times per day to remove any uncoupled free hapten. The conjugates were Characterized by UV and polyacrylamide gel electrophoresis. Immunogen were stored at −20°C until future use (Lei, Xu, Song, Liu, & Kuang, 2018).

2.4. Preparation of monoclonal antibody

Female BALB/c mice (6–8 weeks old) were immunized by subcutaneous multiple injection of immunogen (Feng et al., 2018). For the first immunization, a 1:1 mixture of SLM-EDC-BSA (0.1 mg) and complete Freund's adjuvant was fully emulsified, and the resulting mixture was subcutaneously injected into the mice. Booster immunization was then performed after 3 weeks by injection of a 1:1 mixture of SLM-EDC-BSA (0.05 mg) and incomplete Freund's adjuvant (Hao, Suryoprabowo, Song, Liu, & Kuang, 2018; Peng et al., 2017). After the third immunization, blood was collected from the tail of each mouse 1 week after each booster and examined by ic-ELISA. The BALB/c mice with the highest inhibition were immunized with 25 μg of SLM-EDC-BSA through intraperitoneal injection. After 3–5 days, the mice were used for cell fusion. After fusion, the number of positive cells were high, and the well-inhibited cells were subjected to multiple cloning and selection. Validated cell lines were further expanded and injected into BALB/c mice to produce ascites. The ascites were purified by caprylic acid and ammonium sulphate precipitation, and then dialyzed against PBS for 72 h at 4°C (Li, Liu, Song, & Kuang 2018; Yao, Liu, Song, Kuang, & Xu, 2017). The subtypes of the purified mAbs were identified with a mouse mAb subtyping kit.

2.5. Procedure of indirect ELISA and ic-ELISA

As previously described, indirect immunoassays were performed in 96-well polystyrene plates to determine the concentrations of antibody and coating antigen (Guo, Xu, Song,

Liu, & Kuang, 2018; Liu, Suryoprabowo, Zheng, Song, & Kuang, 2017). Briefly, 96-well plates were coated with 100 μL coating antigen (SLM-EDC-OVA, 0.1 μg/mL) diluted in 0.05 M CBS (pH 9.6) in each well, and the plates were incubated for 2 h at 37°C or overnight at 4°C. After the wells were washed three times with PBST, 200 μL/well blocking buffer was added to the plates and incubated for 2 h at 37°C or overnight at 4°C. After removal of the blocking solution, the antibody at the optimal dilution (50 μL) and standard solution (50 μL) were added and incubated at 37°C for 0.5 h. Then, after three washes, 100 μL/well of goat anti-mouse HRP (1:4000 in PBST with gelatin) was added and incubated for 0.5 h at 37°C. The plates were washed with PBST and incubated with 100 μL of substrate solution for 15 min at 37°C. Finally, 50 μL/well of stop solution was added to halt colour enhancement, and the optical density was measured at 450 nm with a micro-plate detector.

2.6. Specificity and sensitivity assessment

The specificity of mAb was assessed on the basis of cross-reactivity (CR). CR values were tested after addition of different concentrations of structurally similar analogs, including MLN, maduramycin, moxidectin, lasalocid, and nigerin. The CR value was calculated with the following formula:

$$\mathrm{CR}(\%) = (\mathrm{IC}_{50}\text{of SLM}/\mathrm{IC}_{50}\text{ of related analogs}) \times 100\%.$$

The sensitivity was evaluated on the basis of the IC_{50}, and the IC_{20} was used as the detection limit of the assay (LOD) (Ge, Suryoprabowo, Zheng, & Kuang, 2017).

2.7. Synthesis of gold nanoparticles

The glass instruments required for the experiment were soaked in aqua regia and washed several times with ultra-pure water. Colloidal gold nanoparticles with a diameter of 40 nm were prepared according to the classical sodium citrate reduction method frequently used in our previous work (Jiang, Zeng, Liu, Song, & Kuang, 2018). First, 100 mL of chloroauric acid (0.01 g/L) was heated. Next, 2 mL of trisodium citrate solution (1%) was added quickly and stirred continuously. Within 1 min, the colour of the reaction solution became wine red, and the solution was heated with stirring for 15 min until the reaction was complete. Finally, ultra-pure water was added to a final volume of 100 mL, cooled to room temperature and stored at 4°C for usage.

The colloidal gold particles were characterized by transmission electron microscopy, and an almost uniform 25 nm particle size was observed; the UV-visible spectrum showed a maximum absorption peak at 524 nm (Liu, Xu, Suryoprabowo, Song, & Kuang, 2018).

2.8. Preparation of colloidal gold-labeled mAb

The coupling between the gold nanoparticles and antibodies was based on electrostatic absorption. After adjustment of 10 mL of CG solution to pH 8.8 with 0.1 M K_2CO_3, the mAb was added to 1 mL of CG solution with continuous stirring.

The mixture was stirred continuously for 50 min at room temperature. Then 1 mL of blocking buffer (0.5% casein, w/v) was added, and the solution was stirred for 2 h. Next, the mixture was centrifuged at $8{,}000 \times g$ for 25 min to remove the blocking agent and

excess antibody. The precipitate was collected and washed with gold-labeled resuspension buffer three times (20 mM Tris [pH 8.2], 0.1% PEG, 0.1% Tween, 5% sucrose, 5% trehalose, 0.2% BSA, and 5% Brij) (Jiang, Zeng, et al., 2018). Finally, the mAb was reconstituted to a volume of 1 mL with gold-labeled resuspension buffer including 0.02% NaN_3 and stored at 4°C until use (Kong, Xie et al. 2017; Xie et al., 2018).

2.9. Preparation of immunochromatographic strips

Under the action of electrostatic absorption, a positively charged antibody was coupled to a negatively charged CG nanoparticle. The test strip consisted of an NC membrane attached to a PVC backing pad, a bottom sample pad, and a top absorption pad. Both of these were pressed in the NC film. To prepare a control line (C-line) and a test line (T-line), 40 μL of goat anti-mouse antibody (1 mg/mL) and coated antigen (0.5 mg/mL SLM-EDC-BSA) were sprayed onto the NC membrane, and drying was maintained at 37°C for 2 h. The distance between them was 0.5 cm to ensure complete reaction of the reactants. A 3 mm wide test strip was cut with a cutter (Li, Zhi, Jia, Nie, & Ai, 2018). The gold-labeled antibody was immobilized on a solid support, as previously described (Briggs, Tapper, Sprosen, Mace, & Finch, 2017). Specifically, the gold-labeled antibody was dried to a powder and dissolved by application of a liquid sample. The sample was reacted at room temperature for approximately 3 min. The mixture was added to the test strip and migrated to the coated antigen within 5 min. The coated antigen was able to bind an excess of gold-labeled antibody.

2.10. Test strip assay

The principle of this assay was that in the absence of SLM or MLN in the sample, the colloidal gold-labeled mAb is captured by the coated antigen, the test line turns red, while the excess MAb will be captured by the goat anti-mouse IgG, with the control line also turns red. In contrast, if the sample contains SLM or MLN, the colloidal gold-labeled mAb binds the free standard and the coated antigen, resulting in a weaker colour or disappearance of colour from the standard on the test line, although the control line remains red (Sun, Liu, Song, Kuang, & Xu, 2016).

2.11. Honey sample preparation

Honey samples without SLM and MLN (determined by HPLC) were used for spiked samples. The SLM-negative honey samples, as confirmed by HPLC, were provided by the Jiangsu Entry-Exit Food and Agricultural Immunology 833 Inspection and Quarantine Bureau. For the ELISA, 1 g of honey sample was dissolved in 1 mL CBS and 2 mL acetonitrile, and mixed by shaking for 5 min. The mixture was then centrifuged for 10 min at 4000 rpm. The supernatant was evaporated under gentle nitrogen flow and dissolved in 2 mL PBS for analysis. The dilution ratio was four-fold. Another method was direct dilution, with a four-fold dilution in the SLM-spiked sample and four-fold dilution in the sample added to the MLN. Each analysis was repeated three times, with three replicates per concentration. The honey samples used in the test strip were directly diluted four-fold with PBS (Sun et al., 2016).

3. Results and discussion

3.1. Characterization of hapten and conjugated antigen

SLM is weakly immunogenic, owing to its low molecular weight, and must be attached to a carrier protein (BSA or OVA). The salt of the SLM contains a carboxyl group that reacts with the protein and is covalently linked to BSA and OVA through an active ester method (Mukunzi et al., 2016; Xu et al., 2016). Characterization was performed by UV spectroscopy and gel electrophoresis. As shown in Figure 2(a), BSA had an absorption peak at 280 nm, and SLM had a peak at ~282 nm. Small molecules and proteins peak at similar positions, and the SLM-BSA peaks smilar to the peaks of SLM and BSA proteins but higher than them. These findings were also observed in the spectroscopy of SLM-OVA (Figure 2(b)). Because this result did not necessarily indicate that the hapten was successfully conjugated to the carrier protein, electrophoretic characterization was performed for validation. As shown in Figure 3(a), BSA and small molecules each showed a band, and the band of SLM clearly lagged behind the band of BSA protein. These findings were also observed in the electropherogram of SLM-OVA (Figure 3(b)), except that OVA showed two bands. These results confirmed the successful conjugation of the immunogen and coating antigen.

3.2. Mab characterization

The selected hybridoma cell line was obtained after cell fusion, and the selected mAb 3A3 was purified from the prepared ascites. The titre of mAb 3A3 was determined by ELISA to be $>6.5 \times 10^5$. IC_{50} values are key in assessing ELISA sensitivity. The lower the IC_{50} value, the higher the sensitivity of the measurement. In this study, mAb 3A3 was highly sensitive, with an IC_{50} of 0.86 ng/mL for 3A3 and a measured LOD of 0.28 ng/mL, and a linear detection range of 0.28–2.26 ng/mL. (Figure 4).

3.3. Specificity of mAbs

We studied the specificity of the mAbs by testing IC_{50} and CR values obtained for a panel of antibiotics. The specificity of 3A3 was assessed through competitive assays of seven

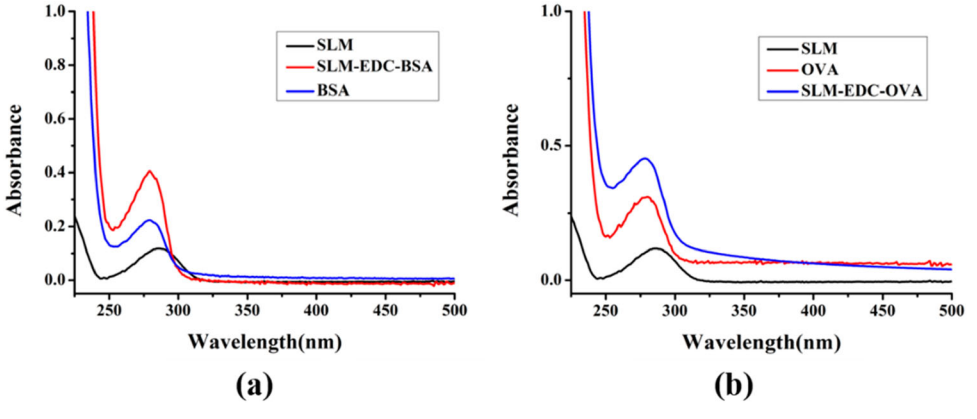

Figure 2. (a) UV–VIS spectra of SLM immunogen (SLM-EDC-BSA); (b) UV–VIS spectra of SLM immunogen (SLM-EDC-OVA).

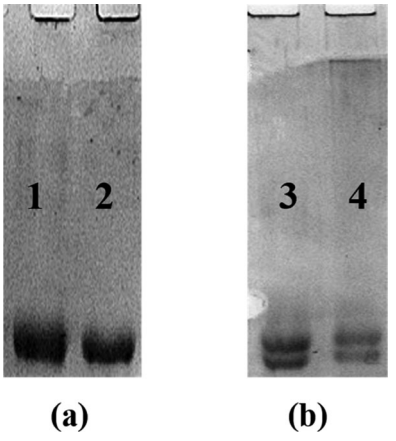

Figure 3. polyacrylamide gel electrophoresis image (a) (1: SLM-EDC-BSA; 2: BSA); (b) (3: OVA; 4: SLM-EDC-OVA). Bovine serum albumin (BSA; MW 67,000), and ovalbumin (OVA; MW 45,000) were obtained from Sigma Chemical Company (St. Louis, MO, USA).

antibiotics and feed additives (Table 1). As shown in Table 1, the CR values of the analogs and related compounds were determined by ic-ELISA. The antibody had the highest affinity for SLM (100%) followed by MLN (74.78%), and other compounds had a CR value of less than 1%, indicating no CR. The CR results indicated that mycinose is an important recognition site, because both analogs with mycinose were recognized by the mAb. We also analyzed erythromycin and some aminoglycosides, including neomycin kanamycin and streptomycin, which showed no CR.

3.4. Spiked and recovery tests by ic-ELISA

For sensitivity and convenience, the honey samples were spiked through the direct dilution method. The SLM spike concentrations were 1.0, 3.0, and 6.0 ng/mL (direct

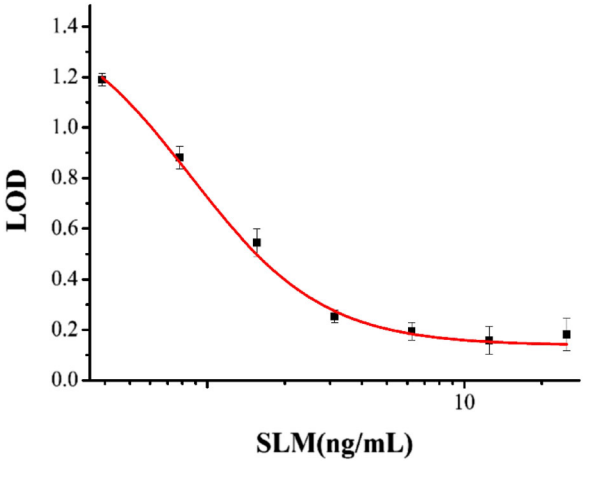

Figure 4. The standard curve for SLM with ic-ELISA.

Table 1. CR results of MD-mAb. ($n = 4$).

Chemicals	IC_{50} (ng/mL)	CR (%)
Narasin	1.15	74.48
Maduramycin	>100	<0.1
Monensin	>100	<0.1
Nigericin	>100	<0.1
Lasalocid	>100	<0.1

dilution four times), and the MLN spike concentrations were 1.0, 3.0, and 6.0 ng/mL (directly added to the diluent three times). As clearly shown in Table 2, the recovery of SLM in the honey sample was 94.6% to 100.4%, and the recovery of MLN was 75.8% to 82.9%. The coefficient of variation was less than 9.34%; therefore, this method can reliably detect SLM and MLN residues in honey.

3.5. Sensitivity of the immunochromatographic assay

Immunological testing is often used to detect veterinary drug residues in animal foods. If a kit is used, running the test does not require much knowledge. When 1 mL of gold nanoparticles was conjugated with 10 ng/mL of 3A3 antibody, the developed band was found to be coloured and the colloidal gold solution was found to be stable at pH 8. The optimum coating concentration was 0.8 mg/mL in 5 min, and the measurement sensitivity of the developed strip was evaluated by using serial dilutions of SLM and MLN standards, as shown in Figure 5. The characteristics of the negative test criteria were as follows: SLM or MLN samples produced clear visible lines at the control and detection lines, whereas positive tests produced only one visible line at the control line. Figure 6 shows that the test line gradually decreased in colour intensity until it completely disappeared at an SLM concentration of 25 ng/mL, and the concentration of MLN was 25 ng/mL.

3.6. Immunochromatographic strip assay

The honey samples were diluted four-fold before analysis and were spiked with SLM and MLN at different concentrations. SLM and MLN solutions were added to each sample at concentrations of 0, 0.5, 1, 2.5, 5, and 10 ng/mL and then tested with test strips. As shown in Figure 5, when 0 ng/mL SLM and MLN were added, a dark colour was observed on the T line with the naked eye. As the concentration of SLM and MLN

Table 2. Recovery of clorprenaline in urine by ic-ELISA and strip assay ($n = 4$).

Samples	Spiked level (ng/mL)	Detection level (ng/mL), mean ± SD	Recovery rate (%)	CV (%)
Honey (SLM)	1.0	0.473 ± 0.003	94.6 ± 0.6	8.32
	3.0	1.004 ± 0.002	100.4 ± 0.2	7.63
	6.0	1.968 ± 0.008	98.4 ± 0.4	6.45
Honey (MLN)	1.0	0.379 ± 0.012	75.8 ± 2.4	3.27
	3.0	0.814 ± 0.009	81.4 ± 0.9	9.34
	6.0	1.657 ± 0.016	82.9 ± 0.8	6.39

Note: The honey samples were diluted four-fold.

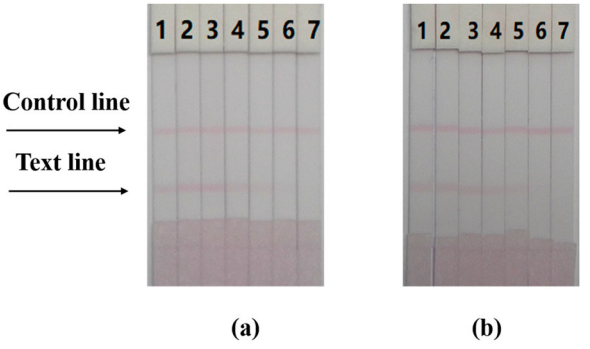

Figure 5. Image of immunochromatographic band analysis of SLM (a) and MLN (b) in 0.01 M PBS. SLM concentration: (0 = blank sample, 1 = 0.5 ng/mL, 2 = 1 ng/mL, 3 = 2.5 ng/ml, 4 = 5 ng/mL, 5 = 10 ng/mL, 6 = 25 ng/mL); MLN Concentration: (0 = blank sample, 1 = 0.5 ng/mL, 2 = 1 ng/mL, 3 = 2.5 ng/mL, 4 = 5 ng/mL, 5 = 10 ng/mL, 6 = 25 ng/mL).

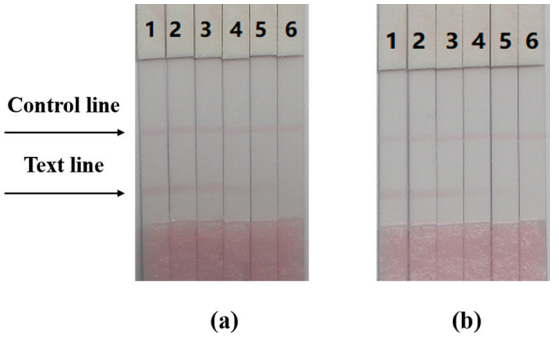

Figure 6. Immunochromatographic strip analysis image of SLM (a) and MLN (b) in honey samples. The concentration of SLM added to the honey sample was: (0 = blank sample, 1 = 0.5 ng/mL, 2 = 1 ng/mL, 3 = 2.5 ng/mL, 4 = 5 ng/mL and 5 = 10 ng/mL); The concentration of MLN added to the honey sample was: (0 = blank sample, 1 = 0.5 ng/mL, 2 = 1 ng/mL, 3 = 2.5 ng/mL, 4 = 5 ng/mL and 5 = 10 ng/mL).

increased, the colour on the T line became weaker. Therefore, the vLOD was 1 ng/mL. When the SLM concentration reached 10 ng/mL, the colour disappeared completely; when the MLN concentration reached 10 ng/mL, the colour disappeared completely. The concentration of 10 ng/mL was thus defined as the cutoff value in evaluating the band sensitivity. Therefore, in the lateral flow ICA band analysis, the semi-quantitative results assessed by the naked eye could be detected only as negative (SLM < 4 ng/mL), weakly positive (SLM 4–40 ng/mL), or positive (SLM > 40 ng/mL). The samples were able to be analyzed by the ICS within 5 min; moreover, the pretreatment and operation procedures were simple.

4. Conclusion

We prepared a monoclonal antibody (3A3) that recognizes both SLM and MLN and is based on the innovative hapten SLM-EDC, and had IC_{50} values of 0.86 ng/mL and 1.15 ng/mL, respectively. The isotype was found to be IgG1 by using a mouse mAb

isotype kit. The cross-reactivity values of SLM and MLN were 100% and 74.78%, respectively. We established an ic-ELISA method to detect SLM and MLN in honey samples. In addition, the recovery for the honey samples ranged from 94.6% to 100.4%. In addition, the ICS method was developed with cutoff values of SLM and MLN of 40 and 40 ng/mL, respectively. The test strip analysis was able to be completed in less than 5 min, thus meeting the requirements of simplicity, efficiency, on-site inspection, and ease of visualization.

Disclosure statement

No potential conflict of interest was reported by the authors.

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