Development of Indirect Competitive Enzyme-Linked Immunosorbenent and Immunochromatographic Strip Assays for Tiamulin Detection in Chicken

Xianlu Lei,†§§ Shanshan Song,†§§ Hong Tao,† Liqiang Liu,†§§ Qiankun Zheng,† Chuanlai Xu,*†§§ and Hua Kuang†§§

†State Key Laboratory of Food Science and Technology, University of Science and Technology of China, No. 99 Kexue Road, Hefei 230029, People’s Republic of China
§International Joint Research Laboratory for Biointerface and Biodetection, and School of Food Science and Technology, and §Collaborative Innovation Center of Food Safety and Quality Control in Jiangsu Province, Jiangnan University, Wuxi, Jiangsu 214122, People’s Republic of China
∥National Institute of Metrology, No. 18 Beisanhuan Donglu, Chaoyang District, Beijing 100029, People’s Republic of China
‡Delishi Group, Weifang, Shandong 262216, People’s Republic of China

ABSTRACT: Tiamulin (TML) is a diterpenoid antibiotic used in animals. In this study, a gold nanoparticle immunochromatographic strip assay and an indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) were developed to detect the residue of TML in chicken. TML aminobutyric was synthesized and conjugated to keyhole limpet hemocyanin by mixed anhydride method as immunogen, whereas TML was connected to ovalbumin with 1,1′-carbonyldimidazole as coating antigen. Under optimized conditions, the ultrasensitive monoclonal antibody-based ic-ELISA exhibited a half-maximal inhibitory concentration (IC50) value of 0.36 ng/mL with a working range of 0.14–0.9 ng/mL for TML. A rapid and sensitive immunochromatographic strip assay was developed with a TML cutoff value of 2.5 ng/mL. On the basis of these results, both developed methods are useful for TML detection in chicken.

INTRODUCTION

Tiamulin (TML), a diterpenoid compound, is one of the world’s top 10 used veterinary antibiotics with strong activity against mycoplasma, Streptococcus spp., and Staphylococcus aureus.†‡§ TML is widely used as a veterinary drug and feed additive to treat respiratory diseases and encourage growth in cattle.¶ However, TML, similarly to other antibiotics, has been reported to have adverse effects on human health, which have been linked to drug residues found in food-animal tissues. These residues may have the potential to contribute to the emergence of drug-resistant bacteria. Officially, residual TML has been strictly limited in the European Union and Japan as well as China where the maximum residual limits for TML and its metabolite 8-α-hydroxymutilin are between 100 and 3000 ng/g in food matrix. Therefore, a rapid and sensitive detection method for quantitation of TML and its metabolites is needed.

There have been many methods developed for TML detection, including liquid chromatography—mass spectrometry,† high-performance liquid chromatography (HPLC),§ ultra-HPLC,¶ and gas chromatography. Although these instrument-based analytical methods have low detection limits and good accuracy, they require skilled and trained labor and highly specialized equipment. Furthermore, these techniques were hindered by low sample throughput and lengthy analysis times. Thus, a rapid and sensitive TML detection method is required.

In recent years, a number of immunological assays have been developed and widely adopted for the detection of a wide variety of antibiotics, such as chloramphenicol, florfenicol, and tetracycline.††–††† The key to establishing an immunological method is to prepare a high-quality primary antibody. Wang et al.‡‡ established an indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) for TML detection using a polyclonal antibody with an IC50 value of 200 ng/mL. In our study, we based our ic-ELISA development on an ultrasensitive monoclonal antibody (mAb) for TML and also developed an immunochromatographic strip (ICS) assay for the determination of TML in chickens.

MATERIALS AND METHODS

Chemicals. TML, TML fumarate, and aminobutyric standards were purchased from J&K Scientific Ltd. (Beijing, China). Keyhole limpet hemocyanin (KLH; MW 4 500 000 Da), bovine serum albumin (BSA; MW 67 000 Da), ovalbumin (OVA; MW 45 000 Da), complete Freund’s adjuvant, incomplete Freund’s adjuvant, 3,3′,5,5′-tetramethylbenzidine (TMB), tributylamine, isobutyl chloroformate, Tween-20, horseradish peroxidase, and gelatin were obtained from Sigma Chemical Company (St. Louis, MO). Enzyme immunoassay-grade horseradish peroxidase-labeled goat anti-mouse immunoglobulin was purchased from Kang-cheng Bioengineering...
Co. (Shanghai, China). Cell culture reagents, including poly(ethylene glycol) solution, HT supplement, HAT supplement, and 1640 cell culture medium, were obtained from Life Technologies Corporation (Shanghai, China).

Jieyi Biotech. Co., Ltd. (Shanghai, China) provided all of the materials used for creating test strips; these include glass fiber membrane (Ahlstrom 8964) used for the sample pad, nitrocellulose (NC) membrane used to immobilize the coating antigen, an HS076 filter paper for the absorbent pad, and the backing material (poly(vinyl chloride), PVC). Other reagents were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

**Solution.** We prepared phosphate-buffered saline (PBS, 0.01 M, pH 7.4) in our laboratory, and the washing buffer was obtained by adding 0.05% Tween-20 (v/v) to 0.01 M PBS. The coating buffer (CB, 0.05 M, pH 9.6) was prepared by dissolving 2.93 g of NaHCO₃ and 1.59 g of Na₂CO₃ in 1 L of purified water. The blocking buffer contained 0.2% gelatin in the CB. The substrate solution consisted of solutions A (Na₂HPO₄, citric acid, and H₂O₂) and B (0.06% v/v TMB in glycol) at a ratio of 5:1 (v/v). The stop solution was prepared by diluting 12 M sulfuric acid into 2 M.

**Hapten Synthesis.** The hapten TML aminobutyric (TML-AS) was synthesized using the modified method of Yao et al. (Figure 1). Briefly, TML fumarate (610 mg, 1 mmol) was dissolved in 10 mL of dichloromethane and triethylamine (100 mg, 1 mmol) was added, the solution was stirred under ice cooling for 30 min. Then, bis(p-nitrophenyl) carbonate (310 mg, 1 mmol) was added and the solution recovered to room temperature (28 °C). After stirring for 1 h, the degree of chemical reaction was monitored with thin-layer chromatography until completion. Subsequently, methyl 4-aminobutyrate hydrochloride (170 mg, 1 mmol) was used to basify with trimethylamine in ice baths overnight at room temperature. Water and saturated brine were used to rins the reaction system and anhydrous sodium sulfate for drying. After the drying agent was removed by filtration, vacuum column chromatography (silica gel of 200–300 mesh, mobile phase consisted of petroleum ether/ethyl acetate = 15:1 to 10:1 elution) and drying were used for purification resulting in 220 mg of intermediate products. Finally, the ester products (220 mg, 0.44 mmol), dissolved in 1,4-dioxane (2 mL), were hydrolyzed with trifluoroacetic acid (2 mL) in water (15 mL) at 45 °C for 6 h. After rinsing and purifying as aforementioned, 85 mg of the TML-AS hapten was obtained.

**Preparation of Immunogen and Coating Antigen.** For synthesis of the immunogen, we based our method on historical methods found in the literature (reference) with some modifications. TML-AS (15 mg) was dissolved in 1.5 mL of dried N,N-dimethylformamide and kept at 4 °C for 15 min. Then, 12 μL of tributylamine and 6.5 μL of isobutyl chloroformate were added to the cooled solution at 10 min intervals while stirring. The reaction mixture was kept at 4 °C and stirred for 1 h (solution A). Meanwhile, BSA (50 mg) was dissolved with 5.0 mL of CB and cooled to 4 °C (solution B). Solution A was added to solution B dropwise and maintained at pH 8.0, the mixture was stirred for 6 h at 4 °C and dialyzed against PBS for 3 days at room temperature. Finally, the presence of TML-AS-MA-BSA was confirmed with UV and polyacrylamide gel electrophoreses.

**Synthesis of Coating Antigen TML-carbodiimide (CDI)-OVA.** The TML hapten was conjugated to OVA via the linker carbodiimide (CDI) on the basis of a modified procedure. Specifically, 10 mg of TML and 20 mg of 1,1′-carbon-yldiimidazole were added to 3 mL of dried N,N-dimethylformamide and the reaction incubated overnight at 60 °C with stirring. Next, the mixture was added to 50 mg of OVA in 5 mL of 0.1 mol/L CBS (pH 9.0) slowly with stirring. After 12 h incubation at room temperature, the conjugates were purified and confirmed as described earlier.

**TML mAb Preparation.** Female BALB/c mice (aged 6–8 weeks, weight 18–20 g) were the most suitable for immune experiments. The immunogen TML-AS-MA-BSA was emulsified with the same amount of Freund’s complete adjuvant for primary immunization, ensuring 100 μg of immunogen per mouse was used. Incomplete Freund’s adjuvant was used for booster immunizations with only 50 μg of immunogen. Each immunization, at 3 week intervals, was executed with the same volume of immunogen (0.1 mL) by subcutaneous multisite injections. After three immunizations, blood samples were collected and analyzed with ic-ELISA 1 week after booster administration. Positive BALB/c mice with the highest inhibition and lowest IC₅₀ values were given the

![Figure 1. Hapten synthesis of TML-AS.](image-url)
final immunization with 25 μg of immunogen by intraperitoneal injection. The mice were fused with Sp 2/0 myeloma cells after 3 days. The qualified cell was obtained through subcloning 3 times, selection, and followed by injection into mice for the production of ascites. The TML mAb was prepared by bitterness-saturated ammonium sulfate method from ascites and dialyzed against PBS.24,25

ic-ELISA. A 96-well polystyrene titration plates were used to perform the ic-ELISA, as described earlier. The optimized concentration of antibody and antigen was obtained initially; then with the aim of improving the stability and sensitivity of the assay, several additional experimental factors were evaluated, including pH (5.5, 6.5, 7.5, and 8.5), NaCl concentration (0, 0.4, 0.8, 1.6, and 3.2%), and methanol concentration (0, 10, 20, and 30%, v/v). The optical density values of zero concentration and IC50 were two core indicators to determine optimal values. Finally, mAb standard curve was established on the basis of optimization experiment parameters as well as the nominal concentration for the coating and antibody. Antibody performance was evaluated with maximum absorbance (Absmax), IC50, Absmax/IC50, and detection limit (the range of standard concentration corresponding to IC20−IC80).

Recovery Test in Chicken Samples Using ic-ELISA. TML-negative chicken sample (confirmed with HPLC) was used for this assay. Different amounts of TML (0.5, 1, 2, 5, and 10 ng) were spiked into 1 g of chicken sample in 15 mL centrifuge tubes, respectively. The spiked chicken sample was mixed well and immersed in 4 mL of acetonitrile. After rotating for 5 min, the mixture was centrifuged for 5 min at 4500 × g. The upper organic solution (1 mL) was transferred to a glass tube and evaporated under nitrogen. Then, 2 mL of freshly prepared 1% (w/v) HAuCl4·4H2O solution was added quickly under stirring and kept for boiling for another 10 min until claret. The GNPs were cooled to room temperature and stored at 4 °C for further use. Transmission electron microscopy and UV–vis spectrometry were used for the characterization of GNPs.

Preparation of Gold-Labeled Anti-TML mAb. The protocol used for the connection of the anti-TML mAb and GNPs was previously described. First, the pH of GNPs (10 mL) was adjusted to 8.0 by adding 0.1 M K2CO3 and then the purified anti-TML mAb (2 g/L, 80 μL) was added dropwise into the solution. After mixing for 2 h at room temperature, 1 mL of 50 mL of 10% (m/v) BSA was added to eliminate nonspecific binding and the mixture was incubated for another 2 h under constant stirring. The solution was centrifuged 3 times for 25 min at 6000 × g to remove excess reagents, and the residue was resuspended in resuspension buffer (0.02 M PBS, 2% BSA, 1% sucrose, 0.04% NaN3, and 0.01% Tween-20, pH 7.2, w/v) each time. The gold-labeled anti-TML mAb was stored at 4 °C for further use.

ICS Preparation. The ICS structure was divided into four parts: the sample pad, the absorbent pad, NC membrane, and PVC backing card.29,30 The goat anti-mouse IgG (0.5 mg/mL) and the coating antigen TML-CDI-OVA (1 mg/mL) were loaded on the NC membrane with 0.5 cm separating the control line (C-line) and test line (T-line). The NC membrane was dried at 37 °C for 1 h and pasted on the center of the PVC backing card and then the sample pad was attached on the side of the card closest to the test line with the absorbent pad on the other side. Finally, the card was cut into 2.8 mm wide strips after vacuum drying overnight.

Principle and Characteristics of the ICS Assay. The sample solution and gold-labeled anti-TML mAb were added into microwell plates and reacted for 5 min.51 After the sample pad of the strip was inserted, the solution quickly travels up the strip due to capillary action. The gold-labeled mAb was combined with coating antigen on the T-line, which results in a visible red color in the absence of TML in the sample solution. Conversely, when TML is abundant in the sample solution, the T-line remains colorless due to the competitive action between TML and the coating antigen. The C-line functions as a control line to prevent false positive results.

Development of an ICS Assay for TML Detection. Preparation of Gold Nanoparticles (GNPs). Colloidal GNPs were prepared by the sodium citrate reduction method.26,27 Specifically, 50 mL of 0.01% (m/v) HAuCl4·4H2O solution was heated to boiling with constant stirring in a clean flask. Then, 2 mL of freshly prepared 1% (w/v) trisodium citrate solution was added quickly under stirring and kept for boiling for another 10 min until claret. The GNPs were cooled to room temperature and further use.

Transmission electron microscopy and UV–vis spectrometry were used for the characterization of GNP.

Figure 2. Ultraviolet–visible absorption spectra of antigen. (a) Coating antigen TML-CDI-OVA and (b) immunogen TML-AS-MA-KLH.
using PBS. Subsequently, 50 μL of each standard dilution was mixed with the labeled mAb in the microwell plate for 5 min, and the sample pad of the ICS was inserted into the mixture. After 5 min, the color intensity of the T and C bands was quantified with a BioDot TSR3000 Membrane Strip Reader (BioDot Inc.).

**RESULTS AND DISCUSSION**

**Antigen Characterization.** In this study, artificial antigen was synthesized by conjugating a small molecule to the carrier protein based on previously published methods (Figure 1). KLH was selected to combine TML-AS by the mixed anhydride method as immunogen TML-AS-KLH, whereas OVA was selected to combine by the CDI method as coating antigen TML-CDI-OVA.

UV spectroscopy was used for the characterization of immunogen and coating antigen. TML and TML-AS kept increasing in the 230–280 nm photometric range, with OVA demonstrating the characteristic peaks at 280 nm and KLH at 280 and 350 nm. The conjugated antigen displayed strong concentration-dependent absorption at 280 nm revealing that TML-AS-KLH and TML-CDI-OVA were synthetized successfully (Figure 2).

**ic-ELISA Optimization and Specificity.** To optimize experimental parameters, the concentration of the selected 3B1 antibody was set at 0.025 μg/mL, whereas the TML-CDI-OVA coating antigen was set at 0.1 μg/mL. The methanol content, ionic strength, and pH of the PBS buffer were several factors that influenced the ic-ELISA performance. To be specific, they could affect protein conformation and significantly affect the combination of antigen and antibody. Ionic strength had a significant impact on absorbance values and IC50 (Figure 3a). There was a perfect S curve at 0.8% NaCl content with the lowest IC50 value. Results of pH optimization experiments are depicted in Figure 3b, pH 5.5 and pH 7.5 had a similar lower IC50, but Absmax was the highest at pH 7.4. Finally, the results showed that with increasing methanol concentration, Absmax decreased and IC50 increased (Figure 3c), demonstrating that a 0% methanol content is the most optimal condition for the assay.

From the foregoing, 0.8% NaCl, pH 7.4, and 0% methanol in PBS buffer were selected as the optimal conditions for ic-ELISA. The standard curve was created on the basis of the
optimal conditions (Figure 3d), the IC<sub>50</sub> value was 0.36 ng/mL and the detection limit was 0.14–0.9 ng/mL (IC<sub>20</sub> to IC<sub>80</sub>). ic-ELISA Recovery Test in Chicken Samples. To investigate the interference contributed from sample matrix, the spiked chicken samples were diluted 5 times resulting in gradient TML concentrations between 0.1 and 2 ng/g and analyzed with ic-ELISA. The TML recovery was 87–96% and the coefficient of variation was 2.1–6.6% (Table 1). It was also concluded that the recovery test performed better in the detection limit (2–5 ng/mL) of ic-ELISA.

Table 1. Recovery of TML by the ic-ELISA in Chicken Samples

| samples | spiked level (ng/g) | mean (ng/mL) ± SD | recovery rate (%) | CV (%) (n = 3) |
|---------|---------------------|-------------------|------------------|---------------|
| chicken | 0.5                 | 0.445 ± 0.030     | 89               | 6.6           |
|         | 1                   | 0.870 ± 0.030     | 87               | 3.4           |
|         | 2                   | 1.872 ± 0.041     | 93               | 2.1           |
|         | 5                   | 4.820 ± 0.125     | 96               | 3.0           |
|         | 10                  | 9.425 ± 0.242     | 94               | 2.5           |

Optimization and Characteristics of the Test Strips. The wet method was used in our ICS assay, and the TML standard was diluted with PBS. Different antibodies (2H8 and SE12) and coating antigens (TML-CDI-OVA-30 and TML-CDI-OVA-60) were optimized along with the concentrations of TML standards (0 and 0.5 ng/mL). The results revealed that 2H8 and TML-CDI-OVA-30 were the most suitable, along with the coating antibody concentration of 0.5 mg/mL. The TML standard was diluted with PBS from stock solution (1 mg/mL) resulting in a titer range between 0 and 2.5 ng/mL. In the optimum conditions, the C-line performed well and the cutoff value of the T-line was 2.5 ng/mL (Figure 4).

TML Detection in Chicken Samples Using the ICS Strips. The coating concentration was optimized using 1 mL of GNP with 4 μL of K<sub>2</sub>CO<sub>3</sub> and 8 μg/mL of 2H8. The results can be seen in Figure 5a, where the results indicate that a concentration of 0.5 mg/mL of the coating antibody was optimal for subsequent assays. The gradient spiked chicken samples were prepared following the ic-ELISA preparation protocol, and the extracting solution was analyzed with ICS assay within 5 min. The strip performed well and the cutoff value was 5 ng/mL (Figure 5b).

■ CONCLUSIONS
In this study, we described an ultrasensitive mAb-based assay against TML with a demonstrated IC<sub>50</sub> value of 0.36 ng/mL. Furthermore, an ic-ELISA was established by optimizing ionic strength, pH, and methanol content, with a working range of 0.14–0.9 ng/mL. The assay also had good performance in spike-recovery experiments using chicken samples. Finally, a simple and portable ICS assay was also successfully developed for TML monitoring with a cutoff value of 2.5 ng/mL in PBS and 5 ng/mL in chicken samples.

■ AUTHOR INFORMATION
Corresponding Author
*E-mail: xcl@jiangnan.edu.cn.

ORCID
Chuanlai Xu: 0000-0002-5639-7102

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS
This work is financially supported by the National Key R&D Program (2016YFF0202300), the Natural Science Foundation of Jiangsu Province and MOF (BE2016307, BK20150145, and BX20151038), the Self-determined Research Program of Jiangnan University (JUSRP51715A), and Taishan Industry Leading Talent Special Funds.

■ REFERENCES
(1) Eyal, Z.; Matzov, D.; Krupkin, M.; Paulkner, S.; Riedl, R.; Rozenberg, H.; Zimmerman, E.; Bashan, A.; Yonath, A. A novel pleuromutilin antibacterial compound, its binding mode and selectivity mechanism. Sci. Rep. 2016, 6, No. 39004.
(2) Yi, Y.; Fu, Y.; Dong, P.; Qin, W.; Liu, Y.; Liang, J.; Shang, R. Synthesis and Biological Activity Evaluation of Novel Heterocyclic Pleuromutilin Derivatives. Molecules 2017, 22, No. 996.
(3) Van der Heijden, H. M. J. F.; de Gussem, K.; Landman, W. J. M. Assessment of the Antihistomonal Effect of Paromomycin and
Tiamulin. In Detection, Typing and Control of Histomonas meleagridis; Tijdschrift voor Diergeneeskunde, 2011; Vol. 136, pp 410–417.

(4) van Duijkeren, E.; Greko, C.; Pringle, M.; Baptiste, K. E.; Catry, B.; Jukes, H.; Moreno, M. A.; Pompa, M. C.; Pyoral, S.; Rantal, M.; Ruzauskas, M.; Sanders, P.; Teale, C.; Threlfall, E. J.; Torren-Edo, J.; Torneke, K. Pleuromutilins: use in food-producing animals in the European Union, development of resistance and impact on human and animal health. J. Antimicrob. Chemother. 2014, 69, 2022–2031.

(5) Thakare, R.; Dasgupta, A.; Chopra, S. LEFAMULIN Pleuromutilin antibacterial. Treatment of pneumonia and ABSSI. Drugs Future 2016, 41, 157–167.

(6) Nitikanchana, S.; Tokach, M. D.; DeRouchey, J. M.; Goodband, R. D.; Nelssen, J. L.; Dritz, S. S. Effect of Dietary Addition of Denagard (Tiamulin) and CTC (Chlortetracycline) on Pig Performance Immediately after Placement in the Finishing Barn. J. Agric. Food Chem. 2012, 10, 343–355.

(7) Ben, W.; Qiang, Z.; Adams, C.; Zhang, H.; Chen, L. Simultaneous determination of sulfonamides, tetracyclines and tiamulin in swine wastewater by solid-phase extraction and liquid chromatography-mass spectrometry. J. Chromatogr. A 2008, 1202, 173–180.

(8) De Baere, S.; Devreeze, M.; Maes, A.; De Backer, P.; Croubels, S. Quantification of 8-α-hydroxy-mutilin as marker residue for tiamulin in rabbit tissues by high-performance liquid chromatography-mass spectrometry. Anal. Bioanal. Chem. 2015, 407, 4437–4445.

(9) Sun, F.; Yang, S.; Zhang, H.; Zhou, J.; Li, Y.; Zhang, J.; Jin, Y.; Wang, Z.; Li, Y.; Shen, J.; Zhang, S.; Cao, X. Comprehensive characterization of Tiamulin Metabolites in Various Species of Farm Animals Using Ultra-High-Performance Liquid Chromatography Coupled to Quadrupole/Time-of-Flight. J. Agric. Food Chem. 2017, 65, 1992–2003.

(10) Yang, S.; Shi, W.; Hu, D.; Zhang, S.; Zhang, H.; Wang, Z.; Cheng, L.; Sun, F.; Shen, J.; Cao, X. In vitro and in vivo metabolite profiling of valnemulin using ultra performance liquid chromatography–quadrupole/time-of-flight hybrid mass spectrometry. J. Agric. Food Chem. 2014, 62, 9201–9210.

(11) Lei, X.; Xu, L.; Song, S.; Liu, L.; Kuang, H. Development of an ultrasensitive ic-ELISA and immunochromatographic strip assay for the simultaneous detection of florfenicol and thiamphenicol in eggs. Food Agric. Immunol. 2017, 1–13.

(12) Xu, N.; Xu, L.; Ma, W.; Kuang, H.; Xu, C. Development and characterization of an ultrasensitive monoclonal antibody for chlortetracycline. Food Agric. Immunol. 2015, 26, 440–450.

(13) Zhu, Y.; Xu, L.; Ma, W.; Xu, Z.; Kuang, H.; Wang, L.; Xu, C. A one-step homogeneous plasmonic circular dichroism detection of aqueous mercury ions using nucleic acid functionalized gold nanorods. Chem. Commun. 2012, 48, 11889–11891.

(14) Wang, A.; Wang, X.; Zhou, J.; Qi, Y.; Zheng, W. Production and Identification of Tiamulin complete immunogen. Acta Agric. Boreali-Occident. Sin. 2014, 23, 191–195.

(15) Yao, L.; Liu, L.; Song, S.; Kuang, H.; Xu, C. Development of indirect competitive enzyme-linked immunosorbent and immunochromatographic strip assays for carbadox detection in fruits and vegetables. Food Agric. Immunol. 2017, 28, 639–651.

(16) Guo, L.; Song, S.; Liu, L.; Peng, J.; Kuang, H.; Xu, C. Comparison of an immunochromatographic strip with ELISA for simultaneous detection of thiamphenicol, florfenicol and chloramphenicol in food samples. Biomed. Chromatogr. 2015, 29, 1432–1439.

(17) Gu, H.; Liu, L.; Song, S.; Kuang, H.; Xu, C. Development of an immunochromatographic strip assay for ractopamine detection using an ultrasensitive monoclonal antibody. Food Agric. Immunol. 2016, 27, 471–483.

(18) Chen, Y.; Kong, D.; Liu, L.; Song, S.; Kuang, H.; Xu, C. Development of an enzyme-linked immunosorbent assay (ELISA) for natamycin residues in foods based on a specific monoclonal antibody. Anal. Methods 2015, 7, 3559–3565.

(19) Han, W.; Pan, Y.; Wang, Y.; Chen, D.; Liu, Z.; Zhou, Q.; Feng, L.; Peng, D.; Yuan, Z. Development of a monoclonal antibody-based indirect competitive enzyme-linked immunosorbent assay for nitroimidazoles in edible animal tissues and feeds. J. Pharm. Biomed. Anal. 2016, 120, 84–91.