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Simultaneous determination of thiamphenicol, florfenicol and florfenicol amine in swine muscle by liquid chromatography–tandem mass spectrometry with immunoaffinity chromatography clean-up☆

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ABSTRACT

A rapid and sensitive liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) method to quantify thiamphenicol (TAP), florfenicol (FF), and florfenicol amine (FFA) in swine muscle is described. An immunoaffinity chromatography (IAC) column based on polyclonal antibodies and protein A-sepharose CL 4B was used to clean-up extracted samples. IAC optimized conditions were found that allowed the IAC to be reused for selective binding of TAP, FF, and FFA. The dynamic column capacity was more than 512 ng/mL of gel after being used for 15 cycles. From fortified swine muscle samples at levels of 0.4–50 ng/g, the average recoveries were 85.2–98.9% with intra- and inter-day variations less than 9.8% and 12.4%, respectively. The limit of quantitation ranged from 0.4 to 4.0 μg/kg.

1. Introduction

Thiamphenicol (TAP) and florfenicol (FF) are both synthetic and broad-spectrum antibiotics belonging to the fenicol drug family and are widely used in veterinary medicine practice. Because of concerns related to drug residues in animal tissues and the potential for emergence of drug resistant bacteria, they are strictly controlled in many countries including China, USA, Japan, and the European Union (EU) [1–4]. For example, maximum residue limits (MRLs) have been set for TAP (50 μg/kg), and the sum of FF and its major metabolite florfenicol amine (FFA) (200 μg/kg) in swine muscle by China and the EU. A rapid and sensitive method for the simultaneous determination and confirmation of TAP, FF, and FFA residues in animal tissues is urgently needed.

Various analytical methods have been reported for the determination of TAP, FF, and FFA in animal tissues, such as gas chromatography (GC) [5,6], liquid chromatography (LC) [7–10], GC–mass spectrometry (MS) [11,12], LC–MS [13], and LC–MS/MS [14–16]. However, these approaches usually use solid-phase extraction techniques or liquid–liquid extraction to isolate and concentrate the target analytes from sample matrices. The methods require a large amount of organic solvents and many steps. Several immunoassays for these fenicols have been reported [17–19]. But the performance of antibodies has limited the use of immunoassays to single-residue analysis. Immunoaffinity chromatography (IAC) clean-up based on the specific interactions of the antigen–antibody is a good alternative method to prepare the samples. It is a simple and selective way to purify extracts and can save organic solvents during pretreatment. Many methods have been reported for determining chloramphenicol (CAP) in animal tissues using IAC clean-up [20,21]. However, no method has been published for the simultaneous determination of TAP, FF, and FFA in swine tissue using IAC clean-up.

The aim of the present study was to develop an IAC using a polyclonal antibody covalently immobilized on protein A-sepharose CL 4B for simultaneous determination of the 3 fenicol residues in swine muscle tissue with further quantification by liquid chromatography–electrospray

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ionization-tandem mass spectrometry (LC–ESI-MS/MS) [16].

2. Materials and methods

2.1. Reagents and materials

CAP (99.0%) and TAP (99.0%) standards, dimethyl pimelimidate, bovine serum albumin (BSA), and ovalbumin (OVA) were obtained from Sigma (St. Louis, MO, USA). FF (99.0%) and FFA (97.6%) were from Schering–Plough (Lafayette, NJ, USA). Protein A-sepharose CL 4B was purchased from Pharmacia Corporation (Uppsala, Sweden). Methanol (MeOH), acetonitrile, formaldehyde (F), and formic acid of HPLC grade were from Fisher Scientific Inc. (Pittsburgh, PA, USA). Other reagents of analytical grade were supplied by Beijing Chemical Co. (Beijing, China). Water was purified using a Milli-Q Synthesis system from Millipore (Bedford, MA, USA).

2.2. Solutions

Stock solutions at a concentration of 100 μg/mL were prepared by dissolving each analyte in acetonitrile. Working standard solutions at different concentrations for TAP, FF, and FFA were prepared by diluting the stock solutions with acetonitrile. The stock solutions were stable for 6 months at −20 °C.

The 0.01 M phosphate-buffered solution (PBS, pH 7.4) was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 0.2 g of KH_2PO_4, and 2.9 g of $Na_2HPO_4{\cdot}12H_2O$ in 1 L of purified water. PBS_1 was made up with 0.01 M PBS and 20.5 g of NaCl. PBS_2 was prepared by 0.02 M PBS, 0.062 M trichloroacetic acid, and 10% MeOH (v/v).

2.3. Instrumentation

Polystyrene microtiter plates were purchased from Beijing Weideweikang Bio-technology Co., Ltd. (Beijing, China). The ELISA plate reader was obtained from Tecan Inc. (Tecan Sunrise, Durham, NC, USA). The ultraviolet–visible (UV–vis) spectrometer was obtained from Shanghai Analytical Instrument (type 751GW, Shanghai, China). The LC equipment was a Waters Alliance 2690 quarternary solvent delivery system (Waters, Milford, MA, USA). The chromatographic separation was performed using a Waters Symmetry Shield RP_{18} (150 mm × 2.1 mm i.d., 5 μm) column. The injection volume was 10 μL and the analysis was carried out with gradient elution using acetonitrile and water as the mobile phase at a flow rate of 0.20 mL/min [16]. The ESI-MS/MS detection of the 3 fenicol residues was achieved using a Quattro LC triple stage quadrupole instrument from Micromass (Manchester, UK). The analysis of TAP and FF was performed in negative ionization (NI) mode, whereas FFA was analyzed in positive ionization (PI) mode. The temperatures of desolvation and source were set at 300 and 80 °C, respectively. Nitrogen was used as the nebulization and desolvation gas, at flow rates of 30 and 446 L/h, respectively. For quantitative purposes, multiple reaction monitoring (MRM) mode was applied to analyze samples. Argon was used as the collision gas. Cone voltage and collision energy were optimized for each analyte separately.

2.4. Preparation of rabbit serum

Immunogen (FFA-F-BSA) and coating antigen (FFA-F-OVA) were prepared by formaldehyde coupling method [22]. Briefly, FFA (50 mg, 0.202 mmol) and BSA (150 mg, 0.0022 mmol) or OVA (100 mg, 0.0023 mmol) were dissolved in the mixture solution of 10 mL of PBS and 2 mL of N,N-dimethylformamide, the pH value of which was adjusted to 6.5. Then 3 mL of freshly prepared 1% F solution was added dropwise. After the mixture was gently stirred at room temperature for 6 h, the reaction product was dialyzed against PBS for 72 h at 4 °C. Two female New Zealand white rabbits (about 2.0 kg) were immunized with FFA-F-BSA. Serum was isolated by centrifugation and stored at −20 °C. To obtain the control serum, each rabbit was pre-bled a week before immunization. Routinely, 2 mg of the conjugate dissolved in 1 mL of NaCl (0.9%) was emulsified with Freund's complete adjuvant (1:1, v/v) and injected intradermally at multiple sites on the back. For booster immunizations, 2 mg of the immunogen was dissolved in 1 mL of NaCl (0.9%) and emulsified with 1 mL of Freund's incomplete adjuvant. The booster immunizations were given every three weeks for a total of 5 booster immunizations. The rabbits were bled from the marginal ear vein one week after each booster (following the third immunization). Serum was isolated by centrifugation and stored at −20 °C. The antibody was purified crudely by saturated ammonium sulfate [23]. Cross-reactivity (CR) was calculated using the following equation,

$$\mathrm{CR\%} = \frac{\mathrm{IC_{50}\ of\ FFA}}{\mathrm{IC_{50}\ of\ analytes}} \times 100 \quad (1)$$

where IC_{50} is the concentration at which 50% of the antibody is bound to the analyte and has the units of pmol/mL [24]. CR was calculated for FFA, FF, TAP, and CAP.

2.5. Immunosorbent preparation

Immunosorbent preparation followed the previously published method [25], with some modification. Briefly, 1.5 g of protein A-sepharose CL 4B was dissolved in 100 mL of water and poured into a sintered-glass funnel (40–60 μm). The gel was washed with PB (0.1 M, pH 8.0). The gel was suspended in the same PB, and mixed with a total of 35 mg of antibody. The mixture was stirred end over end for 30 min at room temperature. It was then washed with 50 mL of PB to remove the unreacted antibody. The eluted solution was collected to detect the amount of antibody by UV–vis spectrometry and the coupling efficiency was determined. The gel was washed with triethanolamine buffer (0.2 M, pH 8.2) and then treated with dimethyl pimelimidate (100 mL, 0.1 M freshly made up in triethanolamine buffer) and mixed end over end for 50 min at room temperature. The gel was recovered by the sintered-glass funnel and treated with aqueous buffered ethanolamine (100 mL, 0.1 M in triethanolamine buffer) for 5 min to block unreacted cross-linking agent. The gel was washed well with PBS and recovered. Finally, 1 mL of bed volume gel was transferred to a glass column (10 mm × 0.8 mm i.d.), and stored in PBS containing 0.01% (v/w) sodium azide at 4 °C. The same procedure was used to obtain a control sepharose column without the antibody.

2.6. Column capacity determination

5000 ng of each antibiotic was mixed with 20 mL of PBS containing 10% MeOH. This solution was introduced into the column and drawn through the IAC at 0.8 mL/min. The antibiotic-saturated column was washed with 20 mL of PBS_1 and 30 mL of water. Finally, 6 mL of MeOH/formic acid (9:1, v/v) was used to elute the analytes. Immediately after the elution step, 20 mL of PBS were added in order to regenerate the column. The column was stored in PBS containing 0.01% sodium azide (v/w) at 4 °C when not in use. All the steps were performed at room temperature. The eluate was evaporated to dryness by a stream of N_2 at 40 °C. The residue was dissolved in 0.5 mL of water/acetonitrile (7:3, v/v). After filtering the redissolved samples through a 0.2 μm PTFE filter (Jinteng Ltd., Tianjin, China), they were injected into the LC–MS/MS system.

2.7. Sample preparation and IAC clean-up

Swine muscle was minced and homogenized in a homogenizer (Nissei AM-6, ACC, Japan, room temperature) for 2 min, and 5.0 g of the sample was weighed into a 50 mL polypropylene centrifuge tube. About 10 mL of PBS_2 was added, the sample was homogenized on a vortex mixer for 1 min, and then centrifuged at 3000 × *g* for 10 min, the supernatant was decanted into a clean tube and the extraction step was repeated once. The supernatants were combined and adjusted pH to 7.4. After filtering this solution through a 0.4 μm PTFE filter, it was subjected to IAC clean-up. The following procedures were the same as described in Section 2.6.

2.8. Calibration curve and fortification

Matrix-matched external standard calibrations were employed in our study. 5.0 g of control muscle tissue was extracted and processed with clean-up procedures as described above. The eluate was spiked with mixed fortified standard solutions to obtain working standard solutions. The spiked eluate was evaporated to dryness by a stream of N_2 at 40 °C. The residue was dissolved in 0.5 mL of water/acetonitrile (7:3, v/v). After filtering the redissolved samples through a 0.2 μm PTFE filter, they were injected into the LC–MS/MS system.

Blank tissue homogenates were spiked by adding 50 μL of standard solution of the desired concentration. The spiked samples were pretreated with the method in sample preparation and IAC clean-up section and analyzed by LC–MS/MS as described in Section 3.7.

3. Results and discussion

3.1. Antibody characterization

The antibody plays an important role on the potential use of the immunosorbent, whether the IAC is used for a single analyte or for class-selective purposes. The antibody against FFA showed high titer (1/128,000). The antibody had CR of 225%, 31%, and 0.6% with FF, TAP and CAP, respectively. Thus, the antibody was suitable for establishing a single IAC column that could simultaneously trap all three compounds.

3.2. Preparation of IAC column

The IAC gel was prepared by means of directional coupling method [25]. The IgG fraction from the antiserum was conjugated to protein A-sepharose CL 4B by using a bifunctional cross-linking agent, dimethyl pimelimidate. The advantage of this method over other methods using gels is that protein A binds specifically to the Fc region of IgG, which could keep the antigen binding site maximally exposed. The high stability of the resulting affinity columns makes protein A-sepharose CL 4B gels extremely useful.

3.3. Elution conditions

A preliminary study which we called the elution study was used to determine the volumes as well as the kinds of elution solution needed for the subsequent IAC procedure. The salt ions present in the elution buffer would cause ionization suppression of the analytes during the electrospray process of LC–MS/MS. Therefore, elution solutions contained salt ions were not used. However, a pure aqueous solution such as 0.1 M glycin–HCl (pH 3.0) was not suitable since it needed 20 mL of elution volume that was not evaporated easily, even protein A could be dissociated from the gels according to the supplier directions. Elution with only pure MeOH [21] resulted in mean recoveries of only 52% for the drugs. We evaluated the use of acidic or basic MeOH to potentially enhance drug recovery [26]. The acidic or basic MeOH solutions were made of formic acid or ammonia water and MeOH. Mean 58% analytes could be removed from the antibody using basic MeOH. MeOH/formic acid (9:1, v/v) resulted in better recovery of mean 93%. And 6 mL of this elution solution was the most satisfactory elution solution in this study (Table 1). It was concluded that MeOH/formic acid (9:1, v/v) may cause a change in pH and polarity of the medium, which had a large influence on the binding of antibody–analyte complex and resulted in release of the analytes from the gel.

Table 1
Influence of elution condition on the three drugs recovery.

Eluting solution	Recovery (%)
4 mL of MeOH	30–35
4 mL of MeOH/ammonia (99.8:0.2)	41–56
4 mL of MeOH/ammonia (99.4:0.6)	46–58
4 mL of MeOH//formic acid (95: 5)	52–82
4 mL of MeOH//formic acid (90:10)	87–93
6 mL of MeOH//formic acid (90:10)	98–100

3.4. Loading conditions

In our experiments, the sample extract was directly loaded onto the IAC. During the analysis of animal muscle tissue, the first extraction step uses an organic solvent for adequate dissolution of the analyte of interest, and the three analytes are soluble in MeOH. In order to study whether the loading medium had an effect on analyte recovery, solutions containing small amounts of MeOH were used. An amount of 20 mL of TAP, FF and FFA, at a level of 200 ng/mL were loaded in 0.02 M PBS containing different levels of MeOH, and washed with 20 mL of PBS_1 followed by 30 mL of water, and finally eluted with 6 mL of MeOH/formic acid (9:1, v/v). The drugs were determined by LC–MS/MS. Recovery results demonstrated that adding up to 15% MeOH in this PBS showed no significant influence on recovery. A small percentage of organic solvent could reduce non-specific interactions and protect the antibody [27], thus 0.02 M PBS containing 10% MeOH was selected as the loading medium.

3.5. Flow rate conditions

The generated IAC was a gravity-flow column with a flow rate of about 0.2–1 mL/min. A decrease in the loading and elution flow rate made an increase in recovery of the analytes in this study. The flow rate at 0.8 mL/min was chosen, at which satisfactory recoveries for the drugs were obtained.

3.6. Washing conditions

Analytes of interest in matrix samples can be selectively adsorbed by specific antibodies, and the interfering matrix may also be retained because of non-specific binding to the gel. Therefore, immediately after the sample percolation, 20 mL of PBS_1 followed by 30 mL of pure water was added in order to elute any analyte unspecifically bound to the gel. The mean recovery of each drug was more than 97%, indicating that 20 mL of PBS_1 combined with 30 mL of pure water used as wash medium could remove the interfering components well. Meanwhile, the residual MeOH remaining on the loading step may assist to elute interfering matrix. The ions from PBS would cause severe ionization suppression of the analytes during the electrospray process, and they could worsen the analytic sensitivity of the MS and contaminate ion source. Thus, sufficient pure water was subsequently applied to remove the PBS ions.

Table 2
The dynamic column capacity and specific column capacity.

Drugs	Dynamic column capacity (ng/mL gel)	Specific column capacity (ng/mg IgG)
FF	2568	447
FFA	1895	330
TAP	1250	217

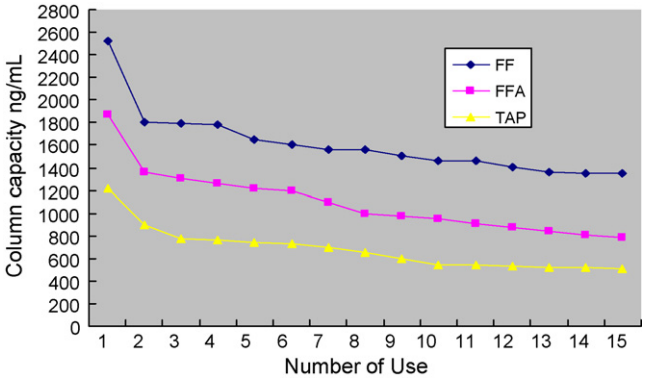

Fig. 1. Variation curves of the immunoaffinity column capacity at 30 days.

3.7. Column capacity determination

From the amount of IgG determined and added before the coupling reaction, the coupling efficiency of the antibody to the gel was observed to be 98.7%. The column capacity was determined by loading 5000 ng each of the analytes standard solution at a flow rate of 0.8 mL/min using optimized loading, washing, and elution conditions. The IAC was washed with 20 mL of PBS_1 followed by 30 mL of water, and eluted with 6 mL of MeOH/formic acid (90:10, v/v). The column capacity for the drugs are summarized in Table 2. The dynamic column capacity for TAP was lower (1250 ng/mL) due to the lower affinity of the antibody for TAP. However, this column was suitable for a class-selective extraction scheme. The reusability of the IAC was evaluated following 15 cycles of use in 45 days. The column capacity curve was shown in Fig. 1. The column capacity gradually decreases as the number of cycles increases because organic solvents caused antibody denaturation and shortened column life. However, the dynamic capacity of the IAC column was still greater than 512 ng/mL after being used for 15 cycles. If the IAC was frequently used it was simply stored in PBS at 4 °C. For a long-term storage it was stored in PBS containing 0.01% NaN_3 at 4 °C.

In order to demonstrate that the analytes retention observed was due to antibody–antigen interactions and not due to non-specific adsorption on the solid support, the control column was used to measure the capacity in the same way. No analyte was found in the eluate, which helped prove the specificity of retention of the analytes.

3.8. Method validation

3.8.1. Sample extraction

Phosphate buffer containing 1% trichloroacetic acid was used as the extraction solution without any further treatment. Trichloroacetic acid was used to precipitate protein from the sample, and the other purpose was to increase the recovery of FFA from samples because FFA is an alkaline drug referred to as a weak base. Meanwhile, 10% MeOH was applied for adequate dissolution of the analytes of interest. The pH of extraction was adjusted to 7.4 with 10 M NaOH prior to loading onto the IAC column.

3.8.2. Linearity

Matrix-matched external standard calibrations were employed in our study to minimize potential matrix interferences [28,29]. Because matrix effects could be minimized or eliminated by adopting selective extraction methods [26,30], minimal matrix effects were observed on comparing peak areas of standard spiked and extraction of control muscle tissue based on high selectivity of our IAC. Control sample (5.0 g) was pretreated with clean-up procedures as described in Section 2.7. The eluate was spiked with mixed fortified standard solutions to obtain working standard solutions (Table 3). The standard calibration curve for each analyte was linear with relative coefficients (R^2) higher than 0.9996.

3.8.3. Selectivity and sensitivity

The selectivity of the method was estimated by analysis and comparison of 20 blank and spiked samples. No interference was observed at the retention time of the 3 analytes and the internal

Table 3
Regress linear formulation and coefficient of TAP, FF and FFA; The LOD and LOQ of the method.

Drug	Regress linear formulations	Relative coefficients (R^2)	LOD (μg/kg)	LOQ (μg/kg)
TAP	$y = 30.833x - 27.081$	0.9997	1.2	4.0
FF	$y = 104.46x - 120.14$	0.9999	0.6	2.0
FFA	$y = 1073.8x - 149.99$	0.9996	0.12	0.4

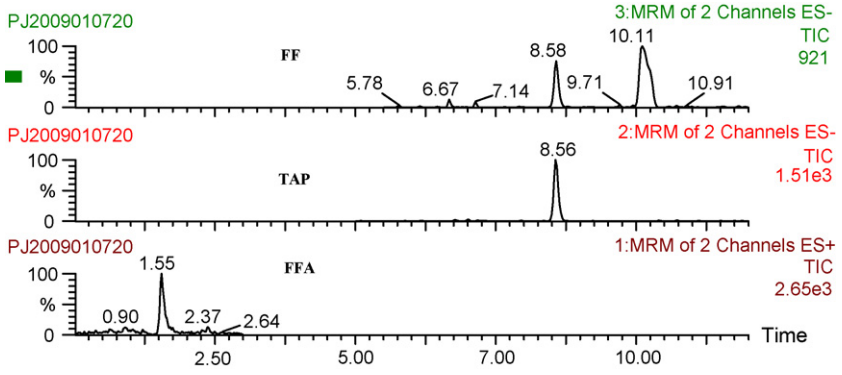

Fig. 2. MRM chromatograms of matrix-matched standard solution in fortified swine muscle.

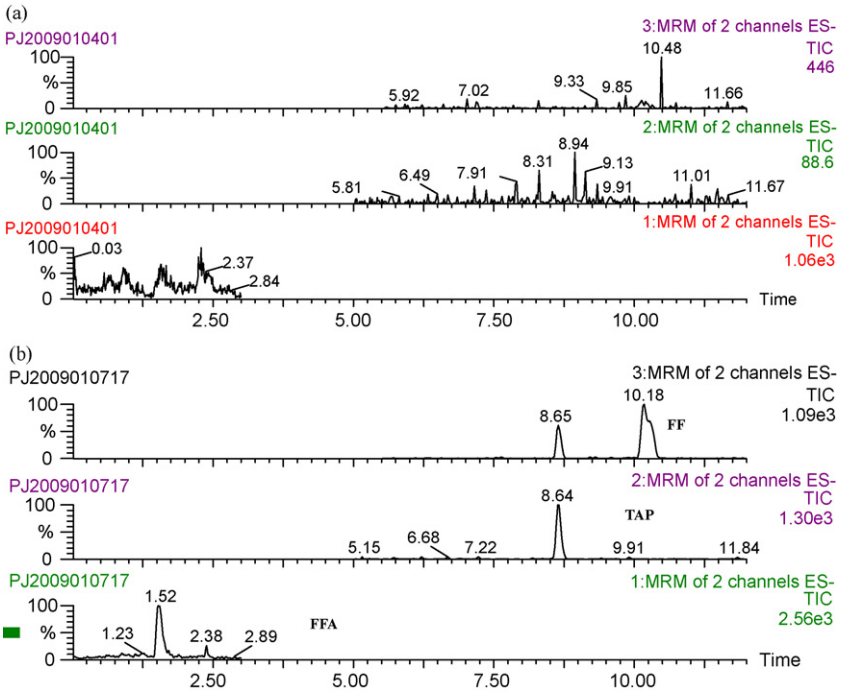

Fig. 3. (a) MRM chromatograms of the blank control of swine muscle and (b) MRM chromatograms of the fortified swine muscle spiked at 4.0 μg/kg for TAP, 2.0 μg/kg for FF, and 0.4 μg/kg for FFA.

standard. Representative MRM chromatograms of blank and spiked samples are shown in Fig. 2.

The sensitivity of the method was estimated by limits of detection (LOD) and limits of quantitation (LOQ). The LOD and LOQ were defined as signal-to-noise ratio of 3:1 and 10:1, respectively. The LODs were 0.6 μg/kg for FF, 1.2 μg/kg for TAP, and 0.12 μg/kg for FFA, respectively. The LOQs were 2 μg/kg for FF, 4 μg/kg for TAP, and 0.4 μg/kg FFA, respectively (Fig. 3).

3.8.4. Accuracy and precision

The accuracy and precision of the method were evaluated by determining recoveries of the 3 analytes in spiked muscle tissue samples. As shown in Table 4, from fortified swine muscle samples at levels of 0.4–50 ng/g, the mean recoveries of the 3 analytes ranged from 85.2% to 98.9%. The intra-day and inter-day variations were less than 9.8% and 12.4%, respectively.

3.9. Methods comparison

In the present method, sample was extracted directly by PBS, which saved much organic solvent that used in our previously work [16]. Secondly, IAC purification was more simple and saved organic solvent. Thirdly, the sensitivity of the two methods was different.

In the study [16], the mean recoveries of the 4 analytes ranged from 95.1% to 107.3%. The intra-day and inter-day variations, expressed as relative standard deviation, were less than 10.9% and 10.6%, respectively. The LODs for each of the 4 analytes, were 0.1 μg/kg for CAP, 0.2 μg/kg for FF and 1 μg/kg for TAP and FFA. The LOQs were 0.3 μg/kg for CAP, 0.5 μg/kg for FF and 3 μg/kg for TAP and FFA.

Comparison of the two methods, in this study the LOD and LOQ for FFA were lower than the first one [16], however, the LODs and LOQs for TAP and FF were higher. The main reason was that the mobile phase was different. The sensitivity of the first method for each of CAP, TAP, and FF was higher when using acetonitrile and water as the mobile phase. But in this work, the mobile phase was acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid, which can facilitate the ionization of alkaline FFA. Thus, the sensitivity of the method for FFA was higher. Secondly, the samples were different. Different types of sample may have different matrix effects that could affect the sensitivity of the method.

Table 4
Recoveries and coefficient variation (CV) of TAP, FF, and FFA in fortified swine muscle tissue samples by IAC–LC–MS/MS.

Drug	Spiked (ng/g)	Intra-day ($n = 5$)		Inter-day ($n = 3$)	
		Recovery (%)	CV (%)	Recovery (%)	CV (%)
TAP	4	86.5 ± 6.9	8.0	87.9 ± 8.7	9.9
	10	92.4 ± 7.1	7.7	93.9 ± 11.6	12.4
	50	96.8 ± 4.1	4.2	97.3 ± 8.4	8.6
FF	2	86.0 ± 7.0	8.1	85.6 ± 6.2	7.2
	10	95.1 ± 5.8	6.1	94.5 ± 7.3	7.7
	50	97.4 ± 7.9	8.1	98.2 ± 10.3	10.5
FFA	0.4	85.2 ± 4.8	5.6	88.8 ± 5.9	6.6
	2	94.3 ± 6.5	6.9	93.9 ± 7.9	8.4
	20	98.9 ± 9.7	9.8	97.0 ± 10.6	10.9

4. Conclusions

In this study, an IAC–LC–MS/MS method was successfully developed for simultaneous determination of TAP, FF and FFA residues in swine muscle. The analytes were extracted from swine muscle by a one step IAC clean-up prior to LC–MS/MS analysis. Satisfactory detection limits, recoveries, and intra- and inter-day variations of the 3 drugs from fortified samples were all obtained, which indicated the method is suitable for the routine analysis of TAP, FF and FFA in food samples of animal-origin.

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