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Simultaneous determination and confirmation of chloramphenicol, thiamphenicol, florfenicol and florfenicol amine in chicken muscle by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A reliable and sensitive liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) confirmation method has been developed for the simultaneous determination of chloramphenicol (CAP), thiamphenicol (TAP), florfenicol (FF), and florfenicol amine (FFA) in chicken muscle. Samples were extracted with basic ethyl acetate, defatted with hexane, and cleaned up on Oasis MCX cartridges. LC separation was achieved on a XTerra C_{18} column with gradient elution using a mobile phase composed of acetonitrile and water at a flow rate of 0.20 mL/min. The analysis was carried out on a triple–quadrupole tandem mass spectrometer in the multiple reaction monitoring (MRM) mode via electrospray interface operated in the positive and negative ionization modes, with deuterated chloramphenicol-d5 (d_5-CAP) as the internal standard. The method validation was performed according to the criteria of Commission Decision 2002/657/EC. Four identification points were obtained for each analyte with one precursor ion and two product ions. Limits of detection (LODs) were 0.1 μg/kg for CAP, 0.2 μg/kg for FF and 1 μg/kg for TAP and FFA in chicken muscle. Linear calibration curves were obtained over concentration ranges of 0.3–20 μg/kg for CAP, 0.5–20 μg/kg for FF and 3–100 μg/kg for TAP and FFA in tissues. Mean recoveries of the 4 analytes ranged from 95.1% to 107.3%, with the corresponding intra- and inter-day variation (relative standard deviation, R.S.D.) less than 10.9% and 10.6%, respectively. The decision limit (CCα) and detection capability (CCβ) of the method were also reported.

1. Introduction

Chloramphenicol (CAP), thiamphenicol (TAP), and florfenicol (FF) are all synthetic and broad-spectrum antibiotics belonging to the fenicol drug family which have been widely used in veterinary medicine for treatments of various infections. Because of toxic side effects on the haemopoietic system [1–3] and the emergence of drug resistant bacteria [4–6], their clinical applications are strictly controlled in many countries including China, USA and member states of European Union (EU) [7–10]. For example, CAP has been banned for use in food-producing animals in EU and minimum required performance limit (MRPL) or maximum residue limits (MRLs) have also been set for CAP (0.3 μg/kg), TAP (50 μg/kg), the sum (100 μg/kg) of FF and its major metabolite florfenicol amine (FFA) in foodstuffs of animal origin. Therefore, it is of great importance to develop sensitive methods for the simultaneous determination and confirmation of the 4 fenicol residues in animal tissues.

Many different analytical methods have been developed for the determination of the 4 fenicols in animal tissues, such as gas chromatography (GC) [11,12], liquid chromatography (LC) [13–16], GC–mass spectrometry (MS) [17,18], LC–MS [19], and LC–MS/MS [20,21]. However, few methods were found in the literature for the simultaneous determination of the 4 fenicol compounds. Pfenning et al. [11] applied a GC method with electron capture detection (ECD) for the simultaneous determination of CAP, TAP, FF and FFA in shrimp. van de Riet et al. [19] reported a liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) method for the simultaneous determination of the 4 fenicol compounds in farmed aquatic species. Dumont et al. developed a qualitative screening method for the 4 fenicol compounds using a surface plasmon resonance (SPR) biosensor [22]. The above three methods could not obtain four identification points for the confirmation of the 4 fenicol compounds according to European Commission Decision 2002/657/EC [23]. In addition, the performance of antibodies limits the applications of immunoassays such as SPR biosensor to the multi-residue analysis. For example, the

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method of Dumont et al. [22] had a much lower detection sensitivity for FFA (CCβ = 250 μg/kg) than FF (CCβ = 0.2 μg/kg), CAP (CCβ = 0.1 μg/kg) and TAP (CCβ = 0.5 μg/kg) in shrimps. Therefore, it is essential to establish a reliable and sensitive LC–MS/MS method which could meet the confirmatory criteria of the 2002/657/EC Decision. However, no LC–MS/MS method has been reported in previous studies for the simultaneous determination and confirmation of 4 fenicols in animal tissues.

The aim of the present study was to develop and validate a confirmative LC–MS/MS method for the simultaneous determination of CAP, TAP, FF and FFA in chicken muscle according to the criteria of the 2002/657/EC Decision. In this method, samples were prepared with liquid–liquid extraction and the subsequent solid-phase extraction (SPE). The LC–MS/MS analysis was carried out in both positive and negative electrospray ionization modes.

2. Experimental

2.1. Reagents and materials

CAP (99.0%) and TAP (99.0%) standards were obtained from Sigma (St. Louis, MO, USA). FF (99.0%) and FFA (97.6%) were from Schering–Plough (Lafayette, NJ, USA). Deuterated chloramphenicol-d5 (d_5-CAP, 98.0%) as the internal standard was from Dr. Ehrenstorfer GmbH (Augsburg, German). Methanol, acetonitrile, ethyl acetate and hexane of HPLC grade were from DIMA Technology Inc. (Richmond, 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). Oasis MCX cartridges (60 mg, 3 cm^3) and extraction manifold were from Waters (Milford, MA, USA).

2.2. Standard solutions

Stock solutions at a concentration of 100 μg/mL were prepared by dissolving each analyte in acetonitrile. Working standard solutions at concentrations of 15, 50 and 250 ng/mL for CAP, 150, 250 and 500 ng/mL for TAP and FFA, 25, 50 and 250 ng/mL for FF were prepared by diluting the stock solutions with acetonitrile. A d_5-CAP internal standard solution of 250 ng/mL was prepared by dissolving the ampoule with 100 μg/mL in acetonitrile. The stock solutions were stable for 6 months at −20 °C. The standard solutions were stable for 1 month at 4 °C.

2.3. Samples

The blank samples of chicken muscle were obtained from slaughterhouse as well as from different supermarkets and different producers. The blank samples were previously screened using an enzyme-linked immunosorbent assay (ELISA) kit (Beijing Wanger Biotechnology Co. Ltd., Beijing, China) and stored at −20 °C until analysis.

Table 1
Chromatographic gradient conditions for the separation of CAP, TAP, FF and FFA.

Time (min)	Acetonitrile (%)	Water (%)	Flow rate (mL/min)
0	10	90	0.2
10	100	0	0.2
12	100	0	0.2
13	10	90	0.2
20	10	90	0.2

2.4. Instrumentation and conditions

2.4.1. Liquid chromatography (LC)

Chromatography was performed on a Waters Alliance 2690 LC system (Waters Corp., Milford, MA, USA) with a vacuum degasser and autosampler. The separation was achieved on a XTerra C_{18} column (100 mm × 2.1 mm i.d., 5 μm; Waters Corp., Milford, MA, USA). The column temperature was maintained at 20 °C. The injection volume was 10 μL and the analysis was carried out with gradient elution using (A) acetonitrile and (B) water as the mobile phase at a flow rate of 0.20 mL/min. The gradient conditions are shown in Table 1.

2.4.2. Mass spectrometer

The Quattro LC triple–quadrupole mass spectrometer (Micromass, Manchester, UK) was connected to the LC system via an electrospray ionization (ESI) interface. The analysis of CAP, TAP and FF was performed in negative ionization (NI) mode, whereas FFA was analyzed in positive ionization (PI) mode. The ESI source was operated with the capillary voltage set at 2.8 kV. The extractor and RF lens voltages were 3.0 and 0.3 V, respectively. The temperature of the source and desolvation was set at 80 and 300 °C, respectively. The nitrogen flows were adjusted to 30 L/h for the cone gas and 446 L/h for the desolvation gas. For collision induced dissociation (CID), argon was used as the collision gas with a collision cell pressure of 2×10^{-3} mbar. For quantitative purposes, samples were analyzed by multiple reaction monitoring (MRM) mode. The MRM parameters for the optimal yield of product ions were defined in individual time windows for each analyte and the internal standard as they eluted from the LC column (Table 2). All data were acquired using MassLynx (Version 3.5) software (Micromass, Manchester, UK).

2.5. Sample preparation

5.0 g of homogenized chicken muscle was weighed into a 50-mL polypropylene centrifuge tube and spiked with 100 μL of d_5-CAP

Table 2
LC–MS/MS conditions for the analysis of CAP, TAP, FF and FFA.

Analyte	t_R (min)	Parent ion (m/z)	Daughter ions (m/z)	Cone voltage (V)	Collision energy (eV)	Mode
CAP	8.46	321	152[a]	25	15	ESI−
			257	25	12	
TAP	6.55	354	185[a]	36	18	ESI−
			290	36	11	
FF	8.18	356	185	28	15	ESI−
			336[a]	28	11	
FFA	1.53	248	130	25	20	ESI+
			230[a]	25	15	
d_5-CAP	8.43	326	157[a]	35	15	ESI−

[a] Ion for quantification.

internal standard solution (250 ng/mL) to obtain a concentration of 5 μg/kg. The sample was vortexed for 30 s and allowed to stand for 20 min. After the addition of 20 mL of ethyl acetate–ammonium hydroxide (98:2, v/v), the mixture was vortexed for 1 min and centrifuged for 5 min at 2300 × *g*. The supernatant was transferred to a 100-mL pear-shaped flask and the extraction step was repeated twice. Then 2 mL of 5% acetic acid solution was added into the combined extracts which were evaporated to a volume of 1–2 mL under vacuum on a rotary evaporator at 40 °C. The residual solution was transferred to a 10-mL polypropylene centrifuge tube. The flask was washed with 1 mL 5% acetic acid solution. 5 mL of hexane was added to the tube and the mixture was vortexed for 1 min. After centrifugation for 5 min at 2300 × *g*, the hexane layer was discarded. This de-fatting step was repeated with another 5 mL of hexane.

The aqueous phase was applied to Oasis MCX cartridge which was previously conditioned with 2 mL of methanol and 2 mL of water. The cartridge was washed with 2 mL of 5% acetic acid solution and eluted with 5 mL of methanol–ammonium hydroxide (90:10, v/v). The eluate was evaporated to dryness under nitrogen stream at 40 °C. The residue was reconstituted by 500 μL of acetonitrile–water (30:70, v/v) and poured through a 0.20-μm filter into LC–MS/MS autosampler vial.

2.6. Calibration

Calibration curves were constructed using analyte/internal standard peak area ratio versus concentration of analyte. Matrix-matched calibration curves were prepared at six spiking levels over ranges of 0.3–20 μg/kg for CAP, 0.5–20 μg/kg for FF and 3–100 μg/kg for TAP and FFA. The spiked samples were pre-treated in three triplicates at each spiking level with the method in Section 2.4 and analyzed by LC–MS/MS as described in Section 2.3.

2.7. Decision limit (CCα) and detection capability (CCβ)

Two critical parameters CCα and CCβ were required by the 2002/657/EC Decision. For CAP, the CCα and CCβ were determined by the matrix calibration curve procedure according to the method of Nicolich et al. [24]. Samples spiked at levels of 0.30, 0.45 and 0.60 μg/kg were prepared in six replicates and samples at levels of 1.00, 3.00 and 5.00 μg/kg were not prepared in replicate (only one sample prepared at each level). For TAP, FF and FFA, the CCα and CCβ were calculated with the method of Samanidou and coworkers [25]. For the measurement of CCα, samples were prepared at the spiking level of limits of quantitation (LOQs) as well as maximum residue limits (MRLs). The CCα was calculated as the mean value of the found concentration plus 1.64 times the corresponding standard deviation. The CCβ was obtained after spiking the samples at the CCα level by adding 1.64 times the corresponding standard deviation. The number of replicates for analysis was 20.

3. Results and discussion

3.1. Optimization of LC–MS/MS analysis

The chromatographic separation was optimized by testing different mobile phase compositions and gradient elution conditions. The mobile phase containing 0.1% acetic acid gave a higher detection sensitivity for FFA but a lower sensitivity for CAP. Since CAP is a banned substance in edible tissues and has a much higher requirement for detection sensitivity compared with FFA, the mobile phase composed of acetonitrile and water without acetic acid was finally chosen to separate the 4 analytes. However, the peak shape of FFA was influenced because the acetic acid was helpful in the FFA ionization.

As CAP, TAP, FF and d_5-CAP contain halogen atoms and hydroxy groups which have high electronegativities, high sensitivity could be obtained in NI mode; whereas FFA with a amino group is more sensitive in PI mode, which is in accordance with the description of van de Riet et al. [19]. The deprotonated molecular ions $[M-H]^-$ were selected as precursor ions for CAP, TAP, FF and d_5-CAP in NI mode and the precursor ion for FFA was the protonated molecular ion $[M+H]^+$ in PI mode. For each analyte, two different mass transitions were monitored using the conditions given in Table 2. The most abundant product ion was used for quantification and the second one for confirmation in compliance with the 2002/657/EC Decision. The determination of the internal standard d_5-CAP was based on one transition.

3.2. Optimization of sample preparation

Acetonitrile, acetone and ethyl acetate were used to optimize the extraction efficiency. Ethyl acetate would permit an efficient extraction as well as high recoveries for CAP, TAP and FF. Basification of ethyl acetate with ammonium hydroxide was selected because FFA is a weak base compound and has a better solubility at high pH. The choice of basic ethyl acetate as the extraction solvent was consistent with the method of Pfenning et al. [11]. It was found that the recovery for FFA was low when the combined extracts were evaporated to dryness followed by reconstitution. The loss of recovery for FFA could be avoided by adding acetic acid solution to the flask before evaporation.

Several SPE approaches have been tried for cleanup optimization. Preliminary experiments with C_{18} SPE cartridges, Oasis HLB cartridges and Florisil cartridges gave satisfactory recoveries for CAP, TAP and FF but not for FFA. As FFA is a weak base compound and mainly exists as cationic form in acid solution (pH < 4), cationic exchange cartridges such as propylsulfonic acid (PRS) cartridges [11] had been used for the isolation of FFA. The best cleanup effect was achieved with Oasis MCX cartridges as described in our previous study [12] for the simultaneous determination of FF and FFA. The properties of Oasis MCX cartridges allow one-step purification of samples containing CAP, TAP, FF and FFA. The sample applied to the MCX cartridge was acidified (pH < 3) with 5% acetic acid solution and the 4 analytes were retained on the cartridges based on cation-exchanges and reversed-phase interactions, respectively. The testing results showed that when 2 mL of 5% acetic acid solution was used as washing solutions and 5 mL of methanol–ammonium hydroxide (90:10, v/v) as eluting solutions, there were high recoveries for the 4 analytes and also minimum matrix interference peaks around the 4 analytes peaks in chromatograms.

3.3. Method validation

3.3.1. Identification and confirmation

According to the 2002/657/EC Decision, the confirmation of banned substances of Group A such as CAP requires a minimum of four identification points and the confirmation of substances

Table 3
Ion ratios of two transition reactions of the 4 analytes in standard solutions and spiked samples.

Analyte	Ion ratios of standard solutions	Maximum permitted tolerances according to Decision 2002/657/EC	Ion ratios of spiked samples
CAP	0.49	0.37–0.62 (0.49 ± 25%)	0.42–0.58
TAP	0.85	0.68–1.02 (0.85 ± 20%)	0.71–1.00
FF	0.89	0.71–1.06 (0.89 ± 20%)	0.74–0.97
FFA	0.28	0.21–0.35 (0.28 ± 25%)	0.23–0.32

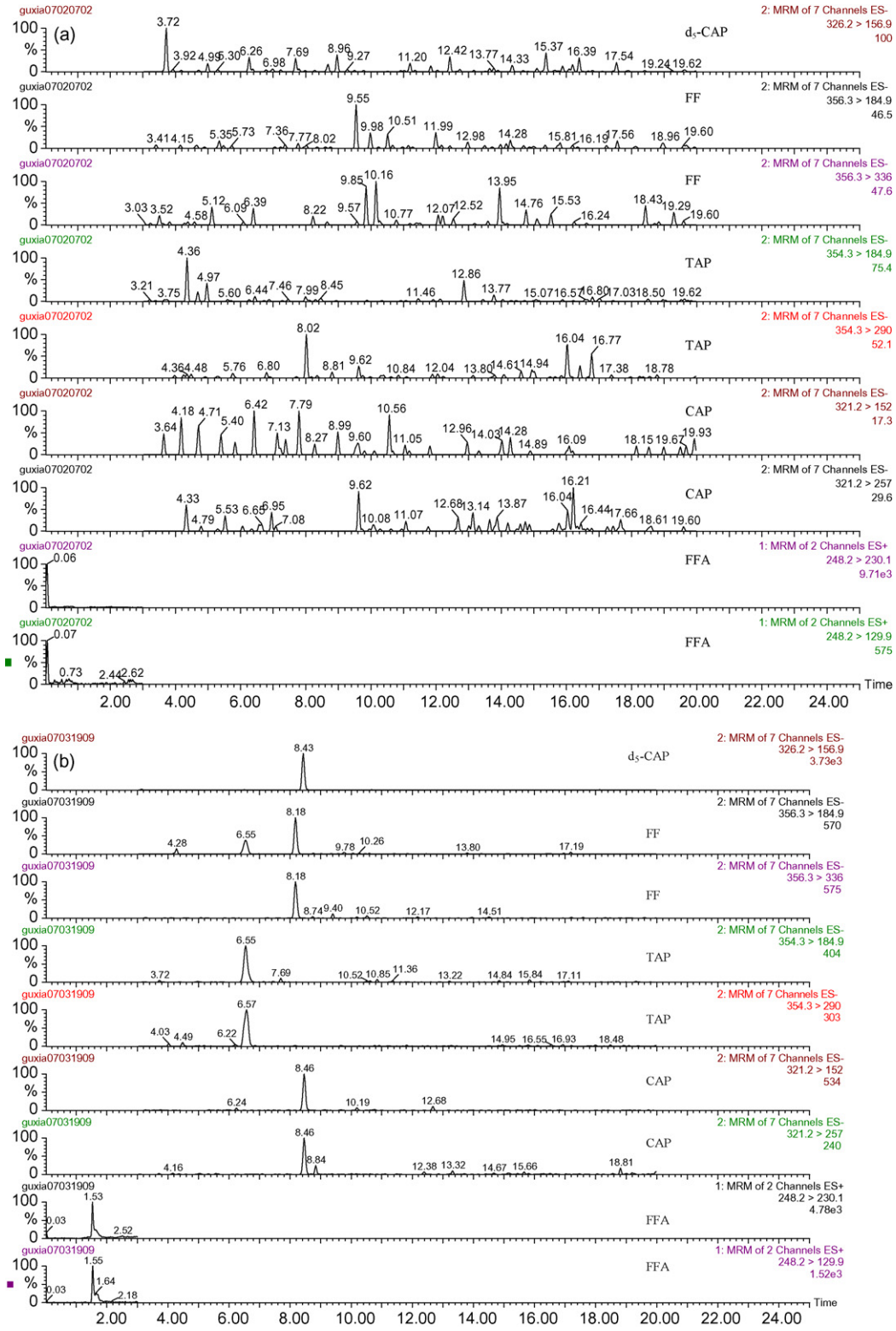

Fig. 1. (a) MRM chromatograms of blank chicken muscle and (b) MRM chromatograms of chicken muscle spiked at 5 μg/kg for d_5-CAP, 0.3 μg/kg for CAP, 0.5 μg/kg for FF and 3 μg/kg for TAP and FFA.

of Group B such as TAP, FF and FFA requires a minimum of three identification points. The four identification points were obtained using LC–MS/MS with one precursor and two product ions in the presented study. Thus, the performance criteria for confirmation were fulfilled.

Table 3 shows ion ratios of two transition reactions of the 4 analytes in standard solutions and spiked samples together with maximum permitted tolerances given in the 2002/657/EC Decision. The ion ratios of each analyte in spiked samples fell within the maximum permitted tolerances for positive identifications. The

Table 4
Performances of the method in term of recoveries and precision.

Analyte	Concentration added (μg/kg)	Recovery (mean ± S.D.%)	Intra-day variation (R.S.D.%, $n = 6$)	Inter-day variation (R.S.D.%, $n = 3$)
CAP	0.3[a]	102.5 ± 9.16	7.1	8.9
	0.45	101.7 ± 9.75	10.2	9.3
	0.6	103.5 ± 8.48	9.0	8.1
TAP	3	98.7 ± 7.49	7.9	10.2
	25	97.8 ± 7.19	6.5	9.0
	50[b]	102.2 ± 8.56	9.3	10.6
	75	103.4 ± 8.78	8.4	8.9
FF	0.5	103.4 ± 7.37	7.4	7.1
	50	99.1 ± 6.73	6.9	5.8
	100[b]	97.6 ± 8.02	7.8	8.0
	150	98.2 ± 7.45	6.4	9.1
FFA	3	107.3 ± 7.63	4.4	9.1
	50	101.2 ± 6.25	6.9	5.8
	100[b]	98.1 ± 8.92	8.1	7.2
	150	95.1 ± 9.57	10.9	9.3

[a] Minimum required performance limit (MRPL).
[b] Maximum residue limit (MRL).

signal-to-noise ratio for each diagnostic ion was >3:1. The relative retention time of each analyte in spiked samples corresponded to that of the standard solution well within a tolerance of ±2.5% according to the 2002/657/EC Decision.

3.3.2. Selectivity and sensitivity

The selectivity of the method was checked by preparation and analysis of 20 blank samples and spiked samples. No interference was observed at the retention time of the 4 analytes and the internal standard. Representative MRM chromatograms of blank samples and spiked samples are shown in Fig. 1.

The sensitivity of the method was assessed by limits of detection (LODs) and limits of quantitation (LOQs). The LODs, defined as the spiking level in chicken muscle that produce a signal-to-noise ratio of 3:1 of the weaker transition 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, the lowest point on the calibration curve for each of the 4 analytes, were 0.3 μg/kg for CAP, 0.5 μg/kg for FF and 3 μg/kg for TAP and FFA based on a signal-to-noise ratio of 10:1.

3.3.3. Linearity

Response linearity was evaluated by matrix-matched calibration curves. For each analyte, 3 calibration curves were made on 3 validation days. No significant differences were observed for the slope and the intercept among the 3 calibration curves prepared in the 3 validation days ($p < 0.05$). Good linearity was obtained throughout all the tested concentrations for each analyte with the corresponding correlation coefficients (r^2) higher than 0.993.

3.3.4. Accuracy and precision

Accuracy and precision were evaluated by determining recoveries of the 4 analytes in spiked samples using 6 replicates on 3 validation days. According to the 2002/657/EC Decision, for CAP the spiking levels were 1× MRPL, 1.5× MRPL and 2× MRPL and for TAP, FF and FFA the spiking levels were 0.5× MRLs, 1× MRLs and 1.5× MRLs, respectively. As shown in Table 4, mean recoveries of the 4 analytes ranged from 95.1% to 107.3%. The intra-day and inter-day variation, expressed as relative standard deviation (R.S.D.), were less than 10.9% and 10.6%, respectively.

3.3.5. CCα and CCβ

For CAP, the CCα and CCβ were calculated with calibration curves which give more weight to the lower spiking levels and thus allows a more representative estimation of the standard deviation of the intercept ($S_{intercept}$) associated with uncertainly at lower levels. CCα was calculated as the concentration corresponding to the intercept value $+2.33 \times S_{intercept}$, whereas CCβ was calculated as the concentration corresponding to the intercept value $+3.97 \times S_{intercept}$. The CCα and CCβ obtained from three calibration curves were presented in Table 5. The mean of these values was considered as CCα and CCβ of the method: 0.07 and 0.11 μg/kg, respectively. Table 6 shows the CCα and CCβ based on the spiking levels of LOQs and MRLs for TAP, FF and FFA.

Table 5
CCα and CCβ values for CAP.

Calibration curve	Slope	y-Intercept	$S_{intercept}$	CCα (μg/kg)	CCβ (μg/kg)
Curve 1	0.5320	0.0542	0.0151	0.07	0.11
Curve 2	0.5054	0.0470	0.0133	0.06	0.10
Curve 3	0.6342	0.0351	0.0203	0.07	0.13
Mean				0.07	0.11

Table 6
CCα and CCβ values for TAP, FF and FFA.

Analyte	Concentration added (μg/kg)	CCα (μg/kg)	CCβ (μg/kg)
TAP	3[a]	3.41	3.83
	50[b]	57.38	65.82
FF	0.5[a]	0.57	0.64
	100[b]	113.12	126.47
FFA	3[a]	3.40	3.81
	100[b]	114.75	132.06

[a] Limits of quantitation (LOQ).
[b] Maximum residue limit (MRL).

4. Conclusions

In this study, a LC–MS/MS method for the simultaneous determination and confirmation of CAP, TAP, FF and FFA residues in chicken muscle was developed. The 4 analytes were extracted from chicken muscle by liquid–liquid extraction and SPE cleanup prior to the LC–MS/MS analysis. The method could fulfill the requirement of the confirmatory criteria according to European Commission Decision 2002/657/EC by four identification points obtained for each analyte with high sensitivity and selectivity. At different spiking levels for

each analyte, good accuracy and precision were obtained, which indicated that the presented method is suitable for the routine analysis of the 4 analytes in chicken muscle.

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