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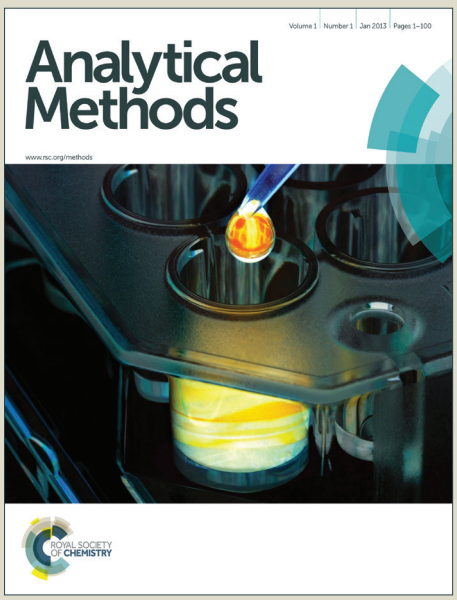

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ARTICLE

High Precision and Selectivity for Quantitation of Enrofloxacin and Ciprofloxacin in Five Chicken Tissues Using Solid Phase Extraction and ESI LC-MS/MS for Application on Monitoring Residues

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Fluoroquinolones (FQs) are synthetic antimicrobials commonly used in intensive poultry farming to treat chronic respiratory disease, colibacillosis, and fowl cholera. As these drugs are also important in treating infections in humans, their use in poultry has been restricted in some countries to avoid the development of antimicrobials -resistant bacteria. In this work, a novel simple and efficient LC-MS/MS method using solid phase extraction (SPE), ESI ionization and multiple reaction mode (MRM) was developed and validated to determine the two most common FQs residues: enrofloxacin (ENR) and ciprofloxacin (CIP) in chicken target tissues (muscle, liver, kidney, fat and skin) and in plasma. The method is shown to be sensitive, with limits of quantification (LOQ) between 1 ng g^{-1} and e 5 ng g^{-1}, recoveries 93-115% and correlation coefficients greater than 0.99. This novel methodology allows faster and simpler sample workflow compared to previously reported methodologies, making it ideal for high-throughput screening and confirmation of both ENR and CIP meeting the international regulation levels.

Introduction

Fluoroquinolones (FQs) are an important group of synthetic antimicrobials that operate by inhibiting bacterial DNA synthesis. FQ display a broad-spectrum antibacterial activity, good absorption via oral administration and extensive tissue distribution. They are therefore widely used to treat many infectious diseases, both in veterinary and human medicine. These antimicrobial drugs have also demonstrated broad-spectrum activity against many pathogenic Gram-negative and Gram-positive bacteria.[1-2]

The broad use of antibiotics in animal husbandry has, however, increased antibiotic-resistant infections, and this drawback has been particularly intense for FQs. As a relevant example in poultry, selection pressure in the presence of FQs rapidly led to resistance in Campylobacter due to the appearance of mutations in DNA gyrase.[3] Due to these issues, the World Health Organization (WHO) and the Food and Drug Administration (FDA) have placed severe restrictions on the veterinary use of FQs. Ciprofloxacin (CIP) and enrofloxacin (ENR) are the most widely used FQ. CIP is used in human medicine, and ENR was developed exclusively for veterinary use. ENR is applied in large-scale poultry settings for the treatment of chronic respiratory disease, colibacillosis, and fowl cholera. In animals, ENR is converted to CIP, which is considered to be an active metabolite, and reaches its peak concentration 4 h after oral administration.[4] When ENR is administered in doses higher than recommended or if the prescribed with drawl period is not respected, ENR residues and its metabolite (CIP) have been detected in considerable amounts in foodstuffs.[5]

To safeguard human health from the risks of multi-resistant and aggressive bacteria, the European Community (EC) has established maximum residue levels (MRLs) for veterinary medicinal products in foodstuffs of animal origin. The EC states, under Commission Regulation (EU) No 37/2010 that the MRL, established as the sum of ENR and CIP is 100 ng g^{-1} in muscle, skin and fat; 200 ng g^{-1} in liver; and 300 ng g^{-1} in kidney.[4] These low levels call for the use of sensitive and high-throughput analytical methods for the mandatory surveillance of possible FQ residues. The validation of these methods should be performed against established analytical gold-standards.[6]

Many methods are available to determine ENR and CIP levels in food-producing animals. Typically, these methods employ high-performance liquid chromatography (HPLC) separation followed by ultraviolet (UV), fluorescence (FLD)[7-9] or mass spectrometry (MS) detection.[10-14] HPLC is often necessary due to the co-elution properties of other compounds belonging to the same chemical family and to pKa differences between the acidic and

amphoteric species of ENR and CIP.[15]Due to the trace analysis requirements, confirmatory techniques based on tandem mass spectrometry (MS/MS) are employed when seeking unequivocal detection of a residue.

LC-MS/MS has proven to be the cornerstone analytical tool for most of the bioactive small-molecule assays[16-17] due to its inherently high compatibility with various drug-like chemicals having basic physiochemical properties.[18-19]It combines efficient separation with sensitive and selective detection of a wide range of molecular species without the need for derivatization. LC-MS/MS is therefore becoming the reference technique for FQ residue monitoring. [20]

This study aimed to develop and validate a highly efficient, most simple and most sensitive LC-MS/MS method for the screening and confirmation of FQ in various chicken tissues. The protocol was developed using ENR and CIP as models for FQ quantification in a triple-quadrupole mass spectrometer after fast SPE extraction. The method was fully validated for each matrix with regards to selectivity, accuracy, and matrix effects. The decision limit (CCα) and detection capability (CCβ) were also determined.

Experimental

Blank and sample chicken tissues

The experimental protocol was approved by the ethics committee for animal experimentation of the University of Campinas, Brazil (Protocol No 3135-1). This study was performed on broiler hybrid lines chickens (commercial *Gallus gallus domesticus*) housed in cages at The Medicine Veterinary College, São Paulo State University-UNESP in Araçatuba, São Paulo, Brazil. All animals were vaccinated against Marek's disease, Gumboro and Bouba flu. Water and food were supplied *ad libitum* starting at day 1 of life. The food provided was formulated according to NRC recommendations (1994) and was free of any antimicrobial drug. On day 25 of the experiment, animals were randomly divided into control and experimental groups. The experimental group was administrated 10 mg mL^{-1} of ENR, mixed in feed as 10% ENR oral solution for poultry (Baytril® Bayer). Six replicates were performed per day of sampling for the control group. The diet containing ENR was fed to the animals for 10 consecutive days. Chickens were euthanized by cervical dislocation during the treatment at days 1, 2, 3 and 7, as well as after the discontinuation of the medication (day 10). From the euthanized animals, blood, kidney, liver, skin/fat and muscle tissues were collected for LC-MS/MS analysis. All samples were stored at –20 °C until analysis.

Blank tissues (muscle, kidney and liver, and skin/fat) and plasma were obtained from the Agrias group (Agrias commerce, Campinas, SP, Brazil). These samples were analyzed several times to confirm that no ENR and CIP were present and were then used to prepare matrix-matched calibration standards and fortified samples. In addition to the blank samples, samples from chickens that consumed feed with ENR were analyzed for a residue depletion study and for validation purposes of the analytical method established in this study.

Chemicals

Standards of ENR and CIP were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), and CIP-d8 hydrochloride hydrate (internal standard, IS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol (MeOH), acetonitrile (ACN), dichloromethane and hexane were purchased from Mallinckrodt (Hazelwood, MO, USA). Formic acid 98%, ammonium hydroxide (NH_4OH) 25%, trifluoroacetic acid (TFA) and ethylenediaminetetraacetic acid (EDTA 5 mM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solid phase extraction (SPE) cartridges Strata-X polymeric Reversed Phase (60 mg/3 mL), were purchased from Phenomenex (Torrance, CA, USA). Water was purified by distillation and passage through a Milli-Q system (Millipore, Bedford, MA, USA).

Standard solutions

Stock standard solutions (5000 μg mL^{-1}) were prepared by dissolving the analytes in acetonitrile/water (v/v) with 0.1% formic acid. The working standard solution was prepared by dilution of stock standard solutions with ACN and contained all analytes at variable concentrations according to their limits of quantification (LOQ) and maximum residue limit (MRL). Stock standard solutions were kept in brown glass to prevent photo degradation and were stored at −20 °C. The stock standard solutions were thus stable for three months.

The concentration of the working standard solution of IS (CIP-d8) was 20 μg L^{-1}. Working solutions of ENR and CIP were prepared with formic acid from stock solutions in ACN at a final concentration of 100 μg mL^{-1}. All working solutions were stored in dark glass at –20 ºC before analysis. Working solutions were used for up to 3 weeks.

Spiked and calibration standards at various concentrations were prepared by combining aliquots of working solutions and IS with the LC mobile phase. These solutions were stored in amber glass at −20 °C for up to 2 days. Tuning solutions (500 ng mL^{-1}) were freshly prepared in ACN containing 0.1% formic acid.

Instrumentation

For sample preparation, an Eppendorf Centrifuge 5418 (Rotor FA-45-18-11, Hamburg, Germany) IKA® Vibrax VXR Basic mixer (IKA Works Inc., Wilmington, NC), and Manifold (Agilent Technologies 1260 series, Waldbronn, Germany) were utilized. For quantitation, a HPLC system (Agilent Technologies 1260 series, Waldbronn, Germany) coupled to a Q-TRAP 5500 tandem mass spectrometer (AB Sciex, Concord, ON, CA) equipped with an electrospray source was used. Data acquisition and processing were performed using the Analyst 1.6.1 and MultiQuant 1.3.1 software packages.

LC-MS/MS conditions

Chromatographic separation of the analytes was performed on a Kinetex PFP 100 Å reversed phase column (00B-4462-E0, 100 mm x 4.6 mm i.d., 2.6 µm particle size, Phenomenex, Torrance, CA, USA) with a compatible pre-column (PFP, PN AJ0-8773, Phenomenex, Torrance, CA) at a flow rate of 850 µL min^{-1}. Analysis time was 8.5 min, and injection volume was 10 µL. The auto sampler was kept at 10 ºC, the column at 30 ºC, and a solution of MeOH: H_2O:NH_4OH (5:4:1) v/v was used during 45 s for needle wash between samples. The isocratic mobile phase was composed of acetonitrile with 0.1% formic acid /water with 0.1% formic acid (60:40, v/v).

The ESI source was operated in the positive ion mode (ESI+). Nitrogen was used as curtain (10 psi), nebulizer (40 psi), auxiliary (15 psi) and collision (high or 12 a.u.) gas. The ion transfer voltage was set to 4500 V and the probe temperature to 650 °C. Sample analysis was performed in the multiple-reaction monitoring (MRM) mode with a dwell time of 50 msec per channel. Source-–dependent parameter optimizations for both analytes were performed by infusion of the standard solutions. The most abundant fragment was selected as the quantifier ion, while the second most abundant fragment was selected as the qualifier ion (**Table 1**).

Table 1. Optimized MS/MS conditions for enrofloxacin (ENR), ciprofloxacin (CIP) and CIP deuterated internal standard (CIP-d8).

Analyte	[M+H]	Type	(*m/z*)	DP(V)	CE(V)	EP (V)	CXP (V)
ENR	359	Quantification	245	146	37	10	16
		Confirmation	203		53	10	6
CIP	331	Quantification	314	91	33	10	6
		Confirmation	231		49	10	12
CIP-d8	339	IS	296	251	30	10	8

Abbreviations: DP=declustering potential; CE=collision energy; EP-entrance potential; CXP=collision cell exit potential.

Sample preparation

Muscle tissue or plasma (0.5 g) and kidney or liver (0.3 g) tissue were accurately weighted and placed in a 2 mL plastic micro tube. Next, 20 µL of the IS solution at 20 µg g^{-1} was added and incubated for 15 min at 5 °C. Then, 1000 µL of MeOH with 0.1% formic acid was added to the sample. Blank samples were spiked by adding the appropriate stock solutions. For quality control (QCs) and unknown samples, only the IS solution was added.

For the preparation of the skin/fat samples, 0.5 g was weighted and 1000 µL of MeOH with 0.1% formic acid was added followed by shaking for 10 min at 1000 g. The mixture was then centrifuged at 18000 g for 5 min. The organic phase was transferred to a 15 mL falcon tube. The residue was washed twice with 800 µL of MeOH with 0.1% formic acid.

Next, 12 mL of water was added to the recovered organic extract of all types of samples (muscle, plasma, kidney, liver and skin/fat). The SPE cartridges used for sample clean up were activated with 2 mL of ACN and 2 mL of water. After passage through the SPE cartridges, the samples were cleaned with 2 mL of water and 3 mL of hexane. For ENR and CIP elution, 5 mL of mobile phase, which consisted of ACN with 0.1% formic acid /water with 0.1% formic acid (60:40, v/v) with 5 mM EDTA. Then, 1000 µL of the SPE resulting extract was placed in brown glass and used for injection into the LC-MS/MS system. The analytical curve was performed with blank tissue samples prepared as described above and spiked with the appropriate standard solutions.

For LLE extraction, 1 g of muscle was homogenized in 15 mL tube with 3 mL of 24.5 mM TFA solution. Then, 5 mL of dichloromethane was added within the homogenate, shaken for 5 min and centrifuged at 1500 g for 10 min. The solvent layer was transferred to a fresh tube and re-extracted with 3 mL of dichloromethane by shaking for another 5 min and centrifuged. Solvent layers were combined and filtered to clean the fraction, and 5 mL of the extract was then evaporated at 60 °C. A 2000 µL aliquot of mobile phase was added to the tubes to re-suspend the extract. This volume was filtered through a 0.45 µm nylon membrane, and 500 µL of the filtrate was injected into the LC-MS/MS.

Analytical Validation

The analytical method was validated using CIP-d8 as an internal standard. The following parameters were evaluated: extraction efficiency, linearity, selectivity, intra-(using fortified and incurred samples) and inter-day precisions, matrix effects, accuracy, decision limits, detection capabilities, detection limits and quantification limits.

Selectivity was determined by comparing five chromatograms of muscle, plasma, skin/fat, liver and kidney samples, either as blanks or when spiked with 5 ng mL^{-1} of ENR and CIP.

Three types of quality controls (QCs) were prepared and utilized for the analytical validation procedure: (a) the ENR and CIP analytes were dissolved in the mobile phase; (b): ENR and CIP were dissolved in the fortified extract and (c) ENR and CIP were added in the fortified matrix.

The MRL values of 100 ng g^{-1} (muscle, plasma and skin/fat), 200 ng g^{-1}, (liver) and 300 ng g^{-1} (kidney) for ENR and CIP were considered to establish the concentration levels of these FQ in the analytical curves. The analytical curves were prepared with blank samples spiked by adding the appropriate stock solutions for each analyte. The calibration standard mixtures thus had the following final concentrations: 1, 2, 5, 10, 20, 50, 75 and 150 ng mL^{-1} (ENR/CIP) for plasma and skin/fat; 2, 5, 10, 50,100, 150, 200 and 350 ng mL^{-1} (ENR) and 2, 5, 8, 10, 30, 50, 80 (CIP) for muscle; 5, 10, 25, 50,100, 250, 300 and 500 ng mL^{-1} (ENR/CIP) for kidney and liver. These solutions were prepared daily from the stock solutions by serial dilutions. The concentration of the IS in all calibration standard mixtures and final sample solutions was 80 ng mL^{-1}.

The matrix effect was investigated at four different levels of ENR and CIP in muscle, kidney, liver, and skin/fat tissue and plasma QC samples. Concentration levels of 6, 100 and 2600 ng mL^{-1} of ENR and CIP were used for plasma and skin/fat; 3, 45 and 4000 ng g^{-1} (ENR) and 3, 45 and 200 ng g^{-1} (CIP) for muscle; 6, 290

and 3000 ng g^{-1} (ENR and CIP) for kidney, and 6, 290 and 4000 pg ng^{-1} (ENR and CIP) for liver. Matrix effects were determined by comparing the peak areas of the QC samples prepared in mobile phase with ENR and CIP in muscle, kidney, liver, skin/fat tissue and plasma when spiked at the same nominal concentrations following the extraction procedure.

Solutions at the same concentrations as the QC were prepared for extraction efficiency, but the comparison was performed by comparing the peak areas of the QC prepared in mobile phase with ENR and CIP in muscle, kidney, liver, skin/fat tissue and plasma chickens samples QCs extracts spiked at the same nominal concentrations before the extraction procedure.

In this study, the QC samples were fortified prior to the extraction process, and the concentration levels were obtained by comparing the peak area of each compound with their respective analytical curve. The accuracy was determined via the recuperation assay, as no certified reference material was available to determine the true accuracy. The accuracy of the method was defined by the measured concentration based on a percentage of the expected concentration. Analysis of the four levels of QC samples was performed on the same day by injection of three samples for intra-day variation assessment. Inter-day assay variation was assessed by the injection of three samples of each concentration on three different days. The percentage relative standard deviation (% RSD) of the regressed (measured) concentrations was used to report precision.

The limit of detection (LOD), limit of quantification (LOQ) and signal-to-noise (S/N) values were obtained using data from five injections of muscle, kidney, liver, skin/fat tissue and plasma chickens samples extracts spiked with 5 ng mL^{-1} of ENR and CIP.

Twenty different samples were spiked at MRL for both ENR and CIP for muscle, kidney, liver, skin/fat tissue, and plasma samples. These values were used to calculate the decision limit (CCα) and the detection capability (CCβ). CCα is the lowest concentration at which a method can discriminate with a statistical certainty of 1-α (α = 1% in the case of banned compound). At CCα, a sample contains the target analyte with a probability of 0.99. CCβ is the concentration at which truly contaminated samples can be detected by the method with a statistical certainty of 1-β.

Results and Discussion

Extraction protocols in the literature were taken as references for extracting ENR and CIP chicken tissue with good selectivity, recovery and quickness. Most studies showed LLE (liquid-liquid extraction) using sample amounts of 1-5 g and high volume of chloride solvents such as dichloromethane and chloroform.[21-22] The SPE (solid phase extraction) by ENR and CIP extraction usually included solvent evaporation steps.[23-24] Methods with HPLC systems with UV or DAD detection use buffers incompatible with mass spectrometry.[25-26] Therefore, we propose the use of of dichloromethane with a sample amount lower than 1 g, and the use of SPE to elute the analytes to the mobile phase without evaporation, which is faster and compatible with the high selectivity of the LC-MS/MS system.

The LC-MS/MS experiments for method development and analytical validation have been performed at the ThoMSon Mass Spectrometry Laboratory, at the University of Campinas. We tested two different extraction protocols performed via liquid-liquid extraction (LLE) or solid phase extraction (SPE). **Figure 1** shows chromatograms of samples extracted either by LLE or SPE. Note that the chromatogram of the sample extracted with LLE (**Fig 1A**) had much more interferences and higher baseline noise compared to SPE extraction (**Fig 1B**), which resulted in much better S/N and selectivity. Although LLE is cheaper, SPE extraction/clean-up was faster and far superior.

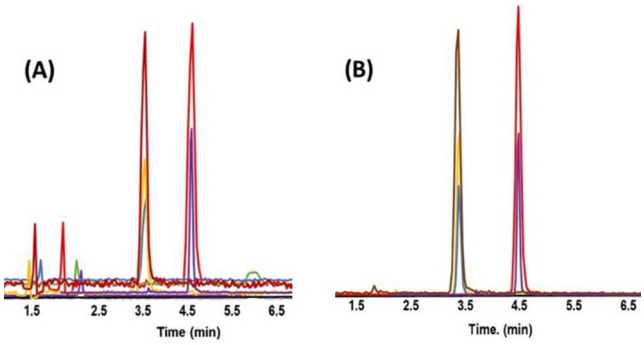


Figure 1. LC-MS/MS chromatograms after (A) LLE or (B) SPE for muscle samples spiked with 100 ng g^{-1} of ENR/CIP and 80 ng g^{-1} of CIP-d8 (IS). Peaks at 3.5 min for CIP (blue and orange line), CIP-d8 (brown), and 4.5 min for ENR (red and purple).

The ionization and fragmentation parameters were optimized for each compound individually. MRM was used to enhance the sensitivity and selectivity of the determination. Consequently, for identification and confirmation, two transitions were selected for each used antibiotic, as determined by direct infusion followed by ESI-MS/MS via collision-induced dissociation (CID). ESI of ENR in the positive ion mode detects its protonated molecule $[M + H]^+$ of *m/z* 360. Its CID **Scheme 1S** (supplementary material) shows CO_2 loss to form the fragment ion of *m/z* 316, C_4H_9N loss from the piperazine ring to form the fragment ion of *m/z* 245 (quantifier ion) and loss of a neutral molecule of C_2H_4N composition to form the fragment ion of *m/z* 203 (qualifier ion).

The protonated CIP **Scheme 2S** and CIP-d8 **Scheme 3S** (supplementary material) as well as a rationale for their fragmentation are also shown.

Note that, likewise, CID of $[M + H]^+$ of *m/z* 340 for CIP-d8 also showed CO_2 loss to form the respective fragment ion of *m/z* 296 (IS fragment).

Good chromatographic separation between ENR and CIP was achieved using ACN:H_2O (60:40 v/v and 0.1% formic acid) as mobile phase and a pentafluorophenyl (PFP) column. However, serious peak tailing was initially observed (**Fig 2A**), although

near perfectly symmetrical chromatographic peaks were obtained when 5 mM EDTA was added into the mobile phase used for sample dilution (**Fig 2B**). EDTA addition was performed according to Kim and collaborators 2013, who added 10 mM EDTA into the mobile phase used to elute ENR and CIP. [27]

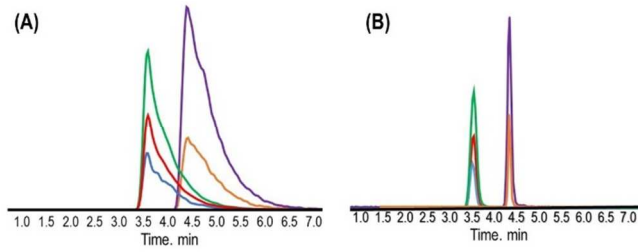
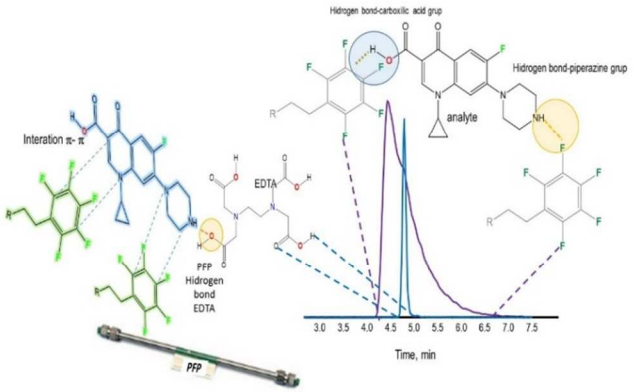


Figure 2. LC-MS/MS chromatograms of a muscle sample spiked with 100 ng g^{-1} of ENR (4.30 min), CIP (3.50 min) and 80 ng g^{-1} of CIP-d8 (3.50 min). (A) Without and (B) with addition of 5 Mm EDTA solution into the mobile phase.

Although EDTA seems not to alter ESI (+) ionization efficiency for the two FQ, it should act as a salt sequester improving LC separation and ESI performance.

We believe that peak tailing results from strong interactions between analytes and the stationary phase (PFP). These interactions can occur by π-π interactions (weak) and/or hydrogen bonding (stronger). Without EDTA, peak tailing results from strong hydrogen bonding between PFP and ENR and CIP (**Scheme 1**). However, when EDTA is added as an organic modifier, it establishes strong H-bonds with the PFP stationary phase, hence ENR and CIP interacts with PFP only via weaker π-π interactions.



Scheme 1. Interactions between analytes and the stationary phase (PFP). π-π interactions (weak) and hydrogen bonding (stronger).

The extraction efficiency for ENR and CIP was investigated at all concentration levels; they ranged from 95% to 102% (ENR) and 88% to 99% (CIP) for plasma, from 90% to 99% (ENR) 95% and 101% (CIP) for muscle, from 94% to 98% for (ENR) and 93% and 101% for skin/fat, from 98% to 103% (ENR) and 97% to 101% for kidney and 93 to 99% (ENR) and 90% to 97% (CIP) for liver. These results showed that SPE extraction followed by the proposed LC-MS/MS method is efficient and can be used in this validation.

Analytical curves were obtained from the peak area ratio (y) of the analyte to the internal standard against the concentration of the analyte (x) using MultiQuant Software Version 1.3.1 (AB Sciex). The correlation coefficient values (R2 > 0.999) indicated appropriate correlations between the investigated compound concentrations and their peak area within the test ranges (**Table 2**). Concentrations of ENR and CIP higher than 8 µg mL^{-1} caused column carry-over. The adequate work range to obtain good method performance for both ENR and CIP was 0.05-3000 ng g^{-1}. LOD were calculated based on at least 3 times the signal-to-noise (S/N) values, and LOQ were calculated based on 10 times the S/N values. LOD and LOQ were found to be 0.15 and 1.0 ng g^{-1} for ENR and CIP, in plasma and skin/fat. For muscle, LOD and LOQ were 0.15 and 2.0 ng g^{-1} and for kidney and liver 0.25 and 5 ng g^{-1} for ENR and CIP, respectively.

Matrix effects for plasma were 17, 12 and 7 % for ENR and 16, 15 and 6 % for CIP; for skin/fat were 14, 10 and 7 % for ENR and 16, 15 and 6 % for CIP; for muscle were 30, 22 and 10 % for ENR and 28, 20 and 8 % for CIP; for kidney were 38, 30 and 18 % for ENR and 34, 27 and 15 % for CIP and for liver were 36, 30 and 16 % for ENR and 35, 30 and 9 % for CIP.

Table 2. Retention time (RT), correlation coefficient (R^2), analytical curve equation and analytical curve range for ENR and CIP determination in different biological tissues:

Tissue	*analite*	*RT*	*R^2*	*Equation*	*Curve Range* ng g^{-1} / ng mL^{-1}
Plasma	ENR	4.30±0.02	0.99865	**y**=0.00328**x** + 0.06223	1-150
	CIP	3.50±0.02	0.99975	**y**=0.00236**x** + 0.0843	1-150
Muscle	ENR	4.30±0.02	0.99907	**y**=0.00267**x** + (-) 0.01333	2-350
	CIP	3.50±0.02	0.99965	**y**=0.00468**x** + 0.04345	2-100
Kidney	ENR	4.30±0.02	0.99988	**y**=0.00188**x** + (-) 0.03567	5-500
	CIP	3.50±0.02	0.99934	**y**=0.00289**x** + 0.03223	5-500
Liver	ENR	4.30±0.02	0.99911	**y**=0.00234**x**+ 0.04678	5-500
	CIP	3.50±0.02	0.99922	**y**=0.00376**x** + 0.0363	5-500
Skin/fat	ENR	4.30±0.02	0.99883	**y**=0.00431**x**+ 0.07443	1-150
	CIP	3.50±0.02	0.99897	**y**=0.00317**x** + 0.0654	1-150

When using ESI (+), the presence of matrix components (salts, proteins, lipids, carbohydrates, etc.) that affect the ionization of the target analytes may pose a significant problem, by either reducing or enhancing the analytes response.[23] Quantification using the matrix analytical curves mode is strongly recommended when ion signal suppression is observed in the samples. Nevertheless, the matrix effect values reported for other extraction methods of FQ are higher than those obtained in this study.

No significant degradation (less than 0.5%) of ENR and CIP was observed in chicken muscle, kidney, liver, skin/fat tissues and plasma samples that have been stored in the auto sampler at 10 ºC for 24 h. Nonetheless, at room temperature the degradation was higher than 10% after 24 h. The standard solution (150 ng mL^{-1}) diluted in ACN with 0.1% formic acid degraded by 5% in four

weeks stored at –20 ºC. The calibration mixtures diluted in the mobile phase degraded by 5% for CIP and 10% for ENR within 72 h when stored at 10 ºC. These results indicate that ENR and CIP are stable under the analytical method conditions.

The recoveries were investigated at four concentration levels, and they ranged from 93% to 111% (ENR) and 102% to 107% (CIP) for plasma, from 99% to 113% (ENR) 97% and 107% (CIP) for muscle, from 98% to 103% for (ENR) and 98% and 108% for skin/fat, from 100% to 114% (ENR) and 99% to 115% for kidney and from 98 to 113% (ENR) and 101% to 108% (CIP) for liver. **Table 1S** for ENR and **table 2S** for CIP (supplementary material) report intra-day and inter-day variation data (RSD) of chicken tissue samples.

Based on decision, some new performance characteristics such as CCα and CCβ were also introduced to interpret analytical results regarding the legal noncompliance of the tested samples. **Table 3** shows CCα and CCβ values for ENR and CIP in each matrix.

Table 3. CCα and CCβ value for ENR+CIP sum.

Tissue	MRL ng g^{-1}	CCα ng g^{-1}	CCβ ng g^{-1}
Plasma	(*)	98*	104*
Skin/fat	100	102	109
Muscle	100	105	110
Kidney	300	298	307
Liver	200	205	211

(*) used 100 ng mL^{-1} for plasma statistical performance and ng mL^{-1} unit of measure.

The method developed in this study was applied to monitor ENR and CIP in chicken tissues for ten days following administration of ENR. FQs are known to be quickly absorbed, with a bioavailability of approximately 50–60%. [28-29] Metabolism and distribution to tissues are extensive, [30-32] and elimination half-lives are generally between 3 and 8 h, depending on the avian animal species. [33-34] Elimination pathways of FQs have not been explicitly studied in avian species, but residues of parent FQ and metabolites have been found in both liver and kidney following oral administration to chickens. [35] Based on the values measured in the tissue samples (**Table 4**), elimination of ENR was primarily via the renal and hepatic system (~80%) and bile/faeces (~20%) [36-37] and its concentration in muscle, plasma and skin/fat decreased as a function of time after ENR ingestion. Results also show that our analytical method is characterized by low LOQ values, thus allowing for the detection of lower concentration levels of ENR/CIP in muscle, liver, kidney, skin/fat and plasma.

Conclusions

A LC-MS/MS method displaying high chemical specificity and sensitivity has been developed and validated to quantitate the two most common FQ, ENR and CIP, in five different poultry matrices (plasma, muscle, skin/fat, kidney and liver). For most analytical methodologies for ENR and CIP quantification in food products based on HPLC with UV, FLD or MS detection, sample amount is greater than 1 g. Therefore, sample preparation occurs with a large expenditure of solvent evaporation by using C18 columns that cause the co-elution of analytes of similar chemical properties. The present method addresses all matrices necessary for the study of chicken quality with the chemical specificity proportionated by tandem mass spectrometry (MS/MS) and to the faster sample workflow by using SPE. Good recoveries, reproducibility and accuracy were demonstrated during analytical validation. The analytical performance of the method was validated using the high quality FDA guidelines and EC. The detection and quantification limits were found to be low enough to determine ENR and CIP residues in chicken tissues below the permissible established MRLs. This method seems, therefore, to be applicable in the efforts to restrict ENR and CIP use in poultry and the resulting bacteria-resistant strains that can pose risks to human health.

Table 4. ENR and its active metabolite CIP in chicken tissues. Administration ENR at a dose 10 mg mL^{-1} of 10% solution Baytril® mixed in feed during 10 days (mean n=3 ± SD) for muscle, liver, kidney, skin/fat and plasma sample.

Time (day)	Muscle (ng g^{-1})		Liver (ng g^{-1})		Kidney (ng g^{-1})		Skin/fat (ng g^{-1})		Plasma (ng mL^{-1})	
	ENR	CIP	ENR	CIP	ENR	CIP	ENR	CIP	ENR	CIP
1	173±3.51	9.91±0.39	534±4.16	589±7.09	229±2.65	71.5±0.75	103±2.10	12.1±0.66	402±1.10	87.9±0.66
2	32.4±0.23	2.20±0.11	113±8.02	129±0.58	102±2.08	44.5±1.12	28.5±0.88	4.11±0.32	128±0.88	32.6±0.18
3	22.5±0.40	1.37±0.04	58.0±3.80	67.8±0.44	26.8±0.76	9.31±0.32	17.8±0.32	2.44±0.24	88.3±0.55	12.3±0.40
7	5.38±0.20	3.39±0.08	19.5±1.22	3.39±0.08	12.2±0.82	1.36±0.10	7.25±0.44	2.98±0.32	15.3±0.34	2.23±0.12

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Notes

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