Accepted Manuscript

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PII: S0956-7135(14)00194-7

DOI: 10.1016/j.foodcont.2014.04.011

Reference: JFCO 3795

To appear in: *Food Control*

Received Date: 29 November 2013

Revised Date: 3 April 2014

Accepted Date: 4 April 2014

Please cite this article as: LucatelloL., CagnardiP., CapolongoF., FerraresiC., BernardiF. & MontesissaC., Development and validation OF A LC-MS/MS/MS method for the quantification OF fluoroquinolones IN several matrices from treated turkeys, *Food Control* (2014), doi: 10.1016/j.foodcont.2014.04.011.

DEVELOPMENT AND VALIDATION OF A LC-MS/MS/MS METHOD FOR THE QUANTIFICATION OF FLUOROQUINOLONES IN SEVERAL MATRICES FROM TREATED TURKEYS

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Abstract

The study presents a sensitive and reliable confirmatory method for the extraction, identification, quantification of five fluoroquinolones (FQ) namely enrofloxacin, ciprofloxacin, difloxacin, sarafloxacin and flumequine, in plasma, liver, kidney, muscle, skin + fat, lung and intestinal content from turkeys.

For the extraction and matrix clean-up of FQ residues from all biological matrices, the Quick Easy Cheap Effective Rugged Safe (QuEChERS) methodology was adopted; only for plasma samples acetonitrile was used.

The analyses were performed by liquid chromatography with mass spectrometry detection (LC-MS). LC separation was performed on a C18 Kinetex column (100 x 2.1 mm, 2.6 μm, Phenomenex, CA, USA) with gradient elution using ammonium acetate solution (10 mM, pH 2.5) and methanol containing 0.1% formic acid. Mass spectrometric identification was done using a LTQ XL ion trap (Thermo Fisher Scientific, CA, USA), with a heated electrospray ionization probe, in positive ion mode.

The method was validated according to the European Legislation (decision 2002/657/EC) and EMA guideline (EMA/CVMP/VICH/463202/2009); selectivity, linearity response, trueness (in terms of recovery), precision (within-day repeatability and within-laboratory reproducibility), limit of detection, limit of quantification, decision limits, detection capability, absolute recovery and robustness were evaluated using turkey blank matrices. All data were within the required limits established for confirmatory methods except for flumequine which presented a recovery value slightly higher than 110% in muscle and intestinal content. For all FQs, all the extraction rates were greater than 70% and limits of quantification ranged from 1.2 μg kg^{-1} to 118.8 μg kg^{-1}.

This fast and robust method was suitable for the identification and quantification of FQ residues in tissues, plasma and intestinal content as confirmed by data obtained from incurred samples of turkeys treated at farm for therapeutic purposes.

Keywords:

Fluoroquinolone; residue depletion; pharmacokinetics; turkey; QuEChERS; LC-MS.

Abbreviations: FQ, fluoroquinolone; ENRO, enrofloxacin; CIPRO, ciprofloxacin; DIFLO, difloxacin; SARA, sarafloxacin; FLUME, flumequine; NOR, norfloxacin; IS, internal standard, MRL, maximum residual limit.

1. Introduction

In EU, fluoroquinolones (FQs) have been authorized for several veterinary species for the treatment of gastrointestinal and respiratory infections caused by gram positive and negative bacteria (Webber, & Piddock, 2001; Barnes, Nolan, & Vaillancourt, 2008, Riviere, & Papich, 2009).

In USA, in 2005, ENRO was banned in poultry due to the widespread of resistance in *Campylobacter spp*, a commensal microorganism for poultry but a pathogen for human (FDA, 2005). In EU, the drug is still authorized and largely used in poultry (EMA, 2006), despite monitoring plans indicate the increase of resistant microorganisms in poultry farms (Walsh, & Fanning, 2008; EFSA, 2010; Russo et al., 2012; Piccirillo Dotto, Salata, & Giacomelli, 2013).

Recently, in the North East of Italy, from the surveillance of medication protocols in poultry farms, resulted that pulsed water medication was more frequently used than the authorized continuous water medication to treat the birds in the sheds. When different dosage, treatment interval or administration route are adopted, the residue monitoring on animal carcasses becomes determinant to guarantee food safety and high through-put analytical methods are required to process large numbers of samples. Moreover, to ensure a prudent use of antimicrobial drugs in veterinary medicine, the restriction on drug usage in food producing animals cannot be sufficient and the optimal dosage regimen to minimize bacterial resistance should always be assessed for an effective treatment (Aliabadi, & Lees, 2000; McKellar, Sanchez Bruni, & Jones, 2004; Martinez,

McDermott, & Walzer, 2006). In this context, it is very important to have a selective, sensitive and rapid method for the determination of FQ concentrations in food-producing animals.

An important and fundamental step for all analytical procedures is the sample preparation, especially when complex matrix as animal tissues composed of lipids, carbohydrates, proteins, vitamins, phenolic compounds and organic acids are used.

Several extraction strategies were described in the literature for FQs detection in food of animal origin: solid phase extraction (SPE) (Toussaint, Chedin, Bordin, & Rodriguez, 2005; Verdon, Couedor, Roudaut, & Sandérs, 2005; Garcés, Zerzanová, Kucera, Barrón, & Barbosa, 2006; Hermo, Barrón, & Barbosa, 2006), liquid to liquid extraction (LLE) (García, Sarabia, Ortiz, & Aldama, 2005), solid-phase micro-extraction (SPME) (Huang, Lin, Yu, & Feng, 2006) and supercritical fluid extraction (SFE) (Shim, Lee, Kim, Lee, & Kim, 2003), mostly laborious and time consuming techniques with poor extraction efficiency and relatively low recoveries (Huan et al., 2012).

Recently, more innovative FQ extraction tecnique from different matrices, were applied: pressurized liquid extraction (PLE) from enfant food product (Rodríguez, Navarro-Villoslada, Moreno-Bondi, & Marazuela, 2010), microwave assisted extraction (MAE) with *in situ* LLE clean-up from chicken breast muscle (Xu et al., 2011), accelerated solvent extraction (ASE) from muscle, liver, kidney of swine, bovine, chicken and fish (Huan et al., 2012), dispersive liquid-liquid microextraction (DLLME) from chicken liver (Moema, Nindi, & Dube, 2012) or fish muscle (Tsai et al., 2009) and molecularly imprinted polymers (MIP) from chicken muscle or eggs (Qiao, & Sun, 2010; Blasco, & Picò, 2012) and QuEChERS technology.

The QuEChERS (QUick, Easy, CHeap, Effective, Rugged and Safe) extraction and clean-up approach, attracted great interest in the last few years because it allowed to reduce and simplify the time needed to complete the processes; initially applied to the analysis of pesticides (Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003) was subsequently extended to veterinary drug residues extraction from different biological matrices (Stubbings, & Bigwood, 2009; Lopes, Reyes, Romero-González, Frenich, & Vidal, 2012).

QuEChERS technique was adopted for the extraction of FQs from milk (Lombardo-Agüí, Gámiz-Gracia, Cruces-Blanco, & García-Campaña, 2011; Karageorgou, Myridakis, Stephanou, & Samanidou, 2013), eggs (Garrido Frenich, Aguilera-Luiz Mdel, Martínez Vidal, & Romero-González, 2010; Capriotti, Cavaliere, Piovesana, Samperi, & Laganà, 2012), honey (Lombardo-Agüí, García-Campaña, Gámiz-Gracia, & Cruces-Blanco, 2012; Wang, & Leung, 2012), chicken muscle (Lopes, Reyes, Romero-González, Frenich, & Vidal, 2012), bovine muscle and swine muscle (Nakajima et al. 2012).

The objective of the study was to optimize and validate a fast, simple, sensitive, and specific LC–MS/MS/MS method suitable for the detection of a wide range of concentrations of FQs as those occurring in pharmacokinetic and residue depletion studies from several matrices. In the present study, five FQs (enrofloxacin, ENRO; ciprofloxacin, CIPRO; difloxacin, DIFLO; sarafloxacin, SARA; flumequine, FLUME) were extracted from plasma, lung, intestinal content, muscle, liver, kidney, skin + fat from turkeys, applying one single LLE to plasma samples and QuEChERS clean-up procedure to the other matrices.

For the validation purposes, all the five FQs above reported were used and the biological matrices were obtained from healthy never treated turkeys; all the incurred samples were obtained from turkeys experimentally administered with ENRO and FLUME *via* pulsed medicated water as reported in previous studies by Ferraresi et al. (2013) and Cagnardi et al. (2014).

2. Experimental

2.1 Animals and treatments

The study was conducted according to Italian law (D.L. 116/1992) and was ethically approved by the Italian Health Ministry (Animal Welfare Unit, 2009R4KM4F_002).

Thirty-two female turkeys (breed B.U.T.6) 63-79 days old, weighing about 4-6 kg and determined to be healthy by a thorough physical examination, were used. Turkeys were randomly assigned to 4 groups of 8 animals to be subjected to treatments with the FQs: groups 1 and 3 were repeatedly

120 treated for 5 days via drinking water in a 10-h pulsed scheme administration with ENRO (Baytril
121 oral solution 10%, BAYER, Milano, Italy) at the dose of 20 mg kg^{-1} b.w. while groups 2 and 4 were
122 treated for 5 days via drinking water in a 10-h pulsed scheme administration with FLUME
123 (Flumechina 40% DOXAL) at the dose of 30 mg kg^{-1} b.w. (Ferraresi et al., 2013; Cagnardi et al.,
124 2014). The doses selected were double the recommended doses of ENRO (10 mg kg^{-1} b.w.) and
125 FLUME (15 mg kg^{-1} b.w.) in poultry. Plasma and tissue samples used as blank matrices were
126 collected from healthy, never treated animals from an organic farm.
127 For groups 1 (ENRO) and 2 (FLUME), blood samples were collected on days 1 and 5, immediately
128 before the treatment, at 1, 3, 6, 9 h during the 10-h treatment, and at 1, 2, 4, 8, 14 h after the
129 withdrawal of medicated water. Plasma was separated by centrifugation at 2000 rpm for 10 minutes
130 and stored at -20°C pending analysis. Three and 5 days after the last treatment, turkeys of group 1
131 and 2 respectively, were sacrificed and liver, kidney, muscle (breast), skin + fat, were collected and
132 stored at -80°C before analysis.
133 Animals of groups 3 and 4 were sacrificed 24 h after the last treatment and lung and intestinal
134 content were collected and stored at -80°C before analysis.
135
136 ***2.2 Chemical and reagents***
137 Enrofloxacin (ENRO, purity: 99.0 %), ciprofloxacin (CIPRO, purity: 99.9 %), difloxacin (DIFLO,
138 purity: 99.8 %), sarafloxacin (SARA, purity: 97.2 %) flumequine (FLUME, purity: 99.7%) and
139 norfloxacin (internal standard, NOR, purity: 99.7 %) were obtained from Sigma-Aldrich
140 (Steinheim, Germay).
141 Acetonitrile (ACN) and methanol (MeOH) were from Carlo Erba Reagents. Formic acid (FA, 98%),
142 ammonium acetate (98%), potassium phosphate monobasic KH_2PO_4 were from Sigma-Aldrich
143 (Steinheim, Germay). All reagents were of analytical grade. Ultrapure water generated by the Milli-
144 Q system (Millipore) was used.

SampliQ Quick Easy Cheap Effective Rugged Safe (QuEChERS) EN buffered extraction kits and SampliQ QuEChERS dispersive-SPE 2ml tube for drug residue in meat (containing 25 mg of C18 and 150 mg of anhydrous $MgSO_4$) were used for the analysis of FQs in turkey matrices (liver, kidney, muscle, skin + fat, lung, intestinal content) and were purchased by Agilent (Santa Clara, CA, USA). Phenex-RC (Regenerated Cellulose) syringe filters 0.22 μm (Phenomenex, Torrance, CA, USA) were used to filter the extracts before the injection in the LC-MS system.

2.3 Standards and stock solutions

Individual stock solutions of ENRO, CIPRO, DIFLO, SARA, FLUME, NOR (IS) were prepared at a concentration of 1000 μg ml^{-1} by dissolving the proper quantity of each compound, exactly weighted, in methanol with 10 % (v/v) of NaOH into volumetric flasks. These solutions were stored at 4°C in amber glass and prepared fresh every 6 months.

Working solutions (containing all FQs except of the IS) used to spike blank samples of turkey, were prepared by appropriate dilutions of the concentrated stock standard solutions with mobile phase (10 mM ammonium acetate pH 2.5 : 0.1% formic acid in methanol, 80:20).

From IS stock solution, different dilutions were prepared to spike matrices: IS at 3 μg ml^{-1} for plasma, IS at 160 μg ml^{-1} for kidney and liver, IS at 100 μg ml^{-1} for muscle, lung, skin + fat and intestinal content.

2.4 Instrumentation

All analyses were performed by liquid chromatography with mass spectrometry detection (LC-MS). The chromatographic separation was achieved using an Accela 600 HPLC pump with CTC automatic injector (Thermo Fischer Scientific, San Jose, CA, USA) equipped with a C-18 Kinetex (100 x 2.1 mm, 2.6 μm) analytical column by Phenomenex (Torrance, CA, USA).

The mass detection was achieved with a LTQ XL ion trap (Thermo Fischer Scientific, San Jose, CA, USA), equipped with a heated electrospray ionization (HESI-II) probe.

171 The system was controlled by the X-calibur software (version 2.1), that was also used for the data
172 acquisition and analysis.
173
174 *2.4.1 Chromatographic and mass spectrometric conditions*
175 Gradient elution was applied using a 10 mM ammonium acetate adjusted at pH 2.5 with formic acid
176 as solvent A and methanol with 0.1% formic acid (v/v) as solvent B. The mobile phase composition
177 (A:B, v/v) was: 80:20 at 0 min, 50:50 at 10 min, 10:90 at 13 min and kept unchanged until 14 min,
178 0:100 from 14.50 min to 16 min and 80:20 from 17 min to 20 min to re-equilibrate the system. The
179 sample trays was maintained at 4°C and the flow rate was set on 200 µl min^{-1}.
180 Standard solutions at 1 µg ml^{-1} of each FQ were infused directly via syringe pump with 20 µl min^{-1}
181 flow rate to the mass spectrometer in order to find fragmentation patterns, tuning parameters, and
182 MS^3 parameters for each analyte. Precursor ions, product ions, collision energies and retention
183 times are shown in Table 1.
184 Due to the presence of the amino group in most FQs that is easily protonated in acidic medium, the
185 ESI source was used in positive mode. The mass analyser was set on the full scan monitoring mode.
186 The following optimum tuning parameters were common for all FQs: sheath gas flow 40 arbitrary
187 units, auxiliary gas flow 5 arbitrary units; ion spray voltage 3.5 kV; capillary temperature 300 °C;
188 capillary voltage 26 V; tube lens 80 V.
189 Retention time windows for each analyte were checked daily with a mixture of the five FQs in
190 mobile phase. Confirmation was achieved by examination of the relative ion intensities of two
191 major MS^3 product ions.
192
193 ***2.5 Sample preparation***
194 The plasma samples purification was performed as reported by Ferraresi et al. (2013) whereas
195 QuEChERS technology, which consists of two steps, a salting-out extraction and a dispersive SPE

196 clean-up, was adopted and used for the extraction of FQs from all turkey tissues (Núñez, Gallart-
197 Ayala, Martins, & Lucci, 2012; Stubbings, & Bigwood, 2009).
198 Before proceeding with the extraction, IS solution (10 µl) was added to plasma samples to obtain IS
199 at 150 µg l^{-1} final concentration.
200 Turkey matrices (liver, muscle, kidney, skin + fat, lung, intestinal content) were first chopped into
201 small pieces and homogenized; 2 g of samples (1 g for intestinal content) were placed into 50 ml
202 centrifuge tubes and added with 50 µl of the different IS solutions reported above (see Section 2.3),
203 to obtain IS final concentration at 4 µg g^{-1} in liver and kidney and at 2.5 µg g^{-1} in muscle, lung, skin
204 + fat, and intestinal content, respectively.
205 A 8 ml volume of 30 mM of KH_2PO_4 buffer pH 7.0 were added and the tubes were agitated for 1
206 min. To each tube, a 10 ml volume of 5% formic acid in ACN was added and the tubes were shaken
207 for other 1 min. Then, an Agilent SampliQ QuEChERS EN extraction salt packet was added to each
208 tube and the sample tubes were capped tightly and shaken vigorously for 3 min. After centrifugation
209 at 4000 rpm for 5 min, a 1 ml aliquot of the upper ACN layer was transferred into an Agilent
210 SampliQ QuEChERS dispersive-SPE 2ml tube and the samples were vortexed for 1 min and
211 centrifuged at 13000 rpm for 5 min with a micro-centrifuge.
212 The supernatant (700 µl) was transferred to a 15 ml tube and evaporated to dryness under a stream
213 of air at 50°C with a TurboVap evaporator (Zymarck, Hopkinton, MA, USA). The residue obtained
214 was dissolved in 700 µl of mobile phase (10 mM ammonium acetate pH 2.5 : 0.1% formic acid in
215 methanol, 80:20), vortex mixed, sonicated for 10 min and filtered through a Phenex-RC
216 (Regenerated Cellulose) syringe filter 0.22 µm (Phenomenex, Torrance, CA, USA) before LC-
217 MS/MS/MS analysis.
218 Fluoroquinolone concentrations of all incurred and spiked sample were quantified with a daily
219 calibration curve prepared in matrix.
220
221 ***2.6 Method validation***

Prior to application to incurred samples, the method was validated according to the European Commission Decision 2002/657/EC for the residue depletion study in liver, kidney, skin + fat and muscle, and to the EMA guidelines (EMA/CVMP/VICH/463202/2009) for the pharmacokinetic and distribution study in plasma, lung and intestinal content. Blank biological matrices from different untreated turkeys were used.

Aliquots of blank samples (200 µl for plasma, 2 g for liver, kidney, lung, muscle, skin + fat and 1 g for intestinal content) were transferred into a polypropylene tubes and spiked with 50 µl of IS and with proper amounts of working solutions of FQs to obtain fortified samples at different concentrations (for intestinal content, working solutions containing only ENRO, CIPRO and FLUME were used). The mixtures were shaken and then the samples were allowed to stand in the dark for 30 min at room temperature to permit the interaction between FQs and tissues before proceeding with the extraction described in Section 2.5.

The following analytical performance parameters were assessed: specificity, linearity response, trueness, precision (within-day repeatability and within-laboratory reproducibility), limit of detection and quantification, decision limits, detection capability, matrix effect, absolute recovery and robustness.

Confirmation of the identities of the FQs was carried out by comparison of the chromatographic peak area of two prominent product ions in MS^3, with the calibration standard at comparable concentrations. Identification was considered reliable if the ratio was within the criteria laid down in the European Commission Decision.

2.6.1 Specificity

To verify specificity, a representative number of blank biological matrix samples of different origin (n = 10-20) were analyzed to check the absence of potential matrix interference peaks at the retention time of the target FQs.

248 *2.6.2 Linearity*

249 Method linearity was evaluated by preparing six different calibration curves on six different days by
250 spiking each of the seven biological matrices from untreated turkeys (blank samples) with different
251 FQ mixed standard solutions, before proceeding with the extraction. Final concentrations of FQs
252 were different in plasma, lung and intestinal content: 2.5, 5, 10, 25, 50, 100, 200 μg l^{-1}, 6.2, 12.5,
253 25, 50, 125, 250, 500, 1000 μg kg^{-1} and 12.5, 25, 50, 100, 250, 500, 1000, 2000 μg kg^{-1},
254 respectively.
255 For each FQ, a different range of concentrations in liver, kidney, muscle and skin + fat, was adopted
256 and final concentrations were reported in Table S1 (see Supplementary data).
257 Calibration lines were constructed by plotting the ratio of the standard area to internal standard area
258 versus the added concentrations and carrying out linear regression analysis. The linearity was
259 considered acceptable when the coefficient of correlation was above 0.990 and the evaluation of
260 residual was lower than 20%.
261
262 *2.6.3 Limit of detection (LOD) and limit of quantification (LOQ)*
263 For plasma, lung and intestinal content, limit of detection (LOD) and limit of quantification (LOQ)
264 were determined as follows: LOD = 3.3 x SD/S; LOQ = 10 x SD/S, where SD is the standard
265 deviation of y-intercepts and S is the average slope obtained from the different calibration curves
266 prepared for each matrix (Ribani, Collins, & Bottoli, 2007). For kidney, muscle, liver and skin + fat
267 LOD values were determined as described above, whereas LOQ for each FQ was defined as the
268 smallest measured content of the identified analyte that can be quantified with an acceptable
269 precision and trueness (EMA/CVMP/VICH/463202/2009) in agreement with the limits reported by
270 European Commission Decision.
271
272 *2.6.4 Decision limit (CCα) and detection capability (CCβ)*

The Commission of the European Communities, to ensure food safety, has established MRLs legally permitted and accepted in liver, kidney, muscle, skin + fat for ENRO, CIPRO, DIFLO, FLUME (Council Regulation 2377/90/EEC). For these FQs, the decision limit (CCα) and detection capability (CCβ) were calculated. As no MRL has been set for SARA in muscle and kidney, CCα and CCβ for this FQ were calculated only for liver and skin+fat.

These values were determined by analyzing blank samples fortified around the permitted limit in equidistant steps (the calibration curve procedure). CCα was calculated as the mean measured concentration at the MRL of each compound plus 1.64 times the standard deviation of the within-laboratory reproducibility at this concentration; CCβ was calculated as CCα plus 1.64 times the standard deviation of the within-laboratory reproducibility at CCα (Verdon, Hurtaud-Pessel, & Sanders, 2006).

2.6.5 Precision and trueness

Precision and trueness of the method were determined by performing tests on three sets of blank samples fortified with FQs at three different concentrations (six replicates each): for plasma, lung and intestinal content, the levels considered were 2.5, 10, 50 μg l^{-1}, 12.5, 50, 500 μg kg^{-1} and 25, 100, 1000 μg kg^{-1} respectively. The matrices liver, kidney, muscle and skin + fat, for which an MRL has been set (see Table 3), were fortified with FQ concentrations at 0.5, 1, 1.5 times each respective MRLs. Blank samples of muscle and kidney were fortified with SARA at 5, 10, 15 μg kg^{-1} and 50, 100, 150 μg kg^{-1} respectively.

For each matrix, samples were analyzed on three different days in the same laboratory, with the same instrument but by three different operators, corresponding to a total number of 54 samples.

The precision of the method has been calculated either in terms of within-day repeatability, the variability of independent test results obtained on the same day, with the same method on identical test items in the same laboratory by the same operator using the same equipment, or in terms of within-laboratory reproducibility, the variability of independent test results obtained by different

operators in different times as unique difference from above (Karageorgou, Myridakis, Stephanou, & Samanidou, 2013; Muscarella, Lo Magro, Palermo, & Centonze, 2007). For the matrix intestinal content, due to the limited availability of blank material, only within-day repeatability was evaluated.

Precision was expressed in terms of imprecision and calculated as the variation coefficient (CV %) of measured concentrations at each level: CV % = (standard deviation/mean measured concentration) x 100. The CV % values for repeatability are acceptable if they are below two third of the value calculated from the Horwitz equation, whereas for reproducibility, they are acceptable if they are below the values calculated from the Horwitz equation (23% if concentration is between 100 and 1000 μg kg^{-1} and 16% if the concentrations are higher than 1000 μg kg^{-1}). The Horwitz equation is not applicable to concentrations below 120 μg kg^{-1}, and the values of repeatability and within-laboratory reproducibility are considered acceptable if they are below 14.7% and 22% respectively, as suggested by Thompson (Thompson, M., 2000).

The trueness, as no certified reference materials for FQs in the turkey tissues are available, was evaluated by the recovery of the known amount of FQs added to the blank matrices. It was calculated by dividing the mean measured value by the fortification level and multiply by 100 to express the result as a percentage. According to 2002/657/EC, the trueness should be between 70 and 100% for fortification levels between 1.0 and 10.0 μg kg^{-1}, and between 80 and 110% for fortification levels ≥ 10.0 μg kg^{-1}.

2.6.6 Absolute recovery and matrix effect

The absolute recovery of all analytes from all biological matrices was determined by comparing the analytical results of extracted FQs from fortified samples (FQs and IS were added before the extraction procedure) with unextracted standards added at the same concentrations in blank extracts representing 100% recovery.

Matrix effects were evaluated by calculating the peak area of the analytes in the presence of matrix (analytes added to blank matrix after extraction), to the peak area in absence of matrix (pure solution of the analyte at the same concentration).

Absolute recovery and matrix effect for each analyte were evaluated at three different levels (the same concentrations considered for the evaluation of precision and trueness), depending on the target biological matrix and FQ (n = 6). Three sets of samples were used for determination, one consisting of neat standards (set 1), one prepared in a blank matrix extract and spiked after extraction (set 2) and one spiked before extraction (set 3). Absolute recovery (REC %) and matrix effect (ME) were calculated using the formulas:

REC (%)= set 3_{area} /set 2 $_{area}$ x 100; ME = (set 2 $_{area}$ / IS $_{area}$)/(set 1 $_{area}$ / IS $_{area}$)

2.6.7 Robustness

The robustness of the method was assessed according to the Youden and Steiner approach (Youden & Steiner, 1975). For this purpose, seven reasonable variables were chosen in the sample preparation procedure (volume and pH of dilution buffer; shaking, centrifugation and sonication time; formic acid percentage in acetonitrile and evaporation temperature of the final extract) and slightly modified with respect to the standard procedure. For each factor two different conditions were adopted. Eight experiments were carried out for the evaluation of the seven selected factors by using eight spiked turkey liver samples at the MRL. The effect of each factor was calculated by subtracting the mean result obtained with the variable at high level and the mean result achieved with the factor at low level. The standard deviation of the differences has been calculated and compared with the values obtained under within-laboratory reproducibility conditions.

3. Results and discussion

3.1 Method validation

The specificity was assessed by comparing the chromatograms of blank samples with those of the corresponding spiked samples to test for endogenous interference; no significant endogenous interferent peaks were evident at the retention time of the five FQs.

The linearity of the calibrations curves in matrix was checked at 6 different days after calculating slopes and intercepts of each individual curve. Good linearity was observed within the concentrations range for all FQs in all matrices since the calculated determination coefficients R^2 was always > 0.99 (Table 3) and residual in the range 10-20%. The slopes of the different calibration curves did not vary considerably and the intercepts were near to theoretical zero value, demonstrating good constancy of the measuring system.

The LOQs for all FQs in plasma, lung and intestinal content were set according to method sensitivity and by far lower than the FQs concentrations in matrices from treated turkeys, confirming the method suitability for distribution study.

The LOQ set in liver, muscle, kidney, skin + fat for all FQs, is significantly lower than the respective half MRL: the values were from 5 to 16 times below these limits (Table S2, Supplementary data). Considering the aim of this work and the MRL in these matrices, the LOQs were considered acceptable although, based on the performance of the analytical method used and on the basis of signal-to-noise ratio, it was possible to define even lower LOQ values.

In Table S2 of Supplementary data, the CCα values with an error of 5 % (probability of false non-compliance ≤ 5 %) and the CCβ values with an error of 5% (probability of falsely compliant samples ≤ 5 %) are reported. The decision limit (CCα) and detection capability (CCβ) take into account the variability of the method and the statistical risk of making a wrong decision, and allow the assessment of the critical concentrations above which the method reliably distinguishes and quantifies a substance (European Decision no. 657/2002/EC). These parameters were established for ENRO, CIPRO, DIFLO, FLUME, in liver, kidney, muscle and skin + fat; for SARA, CCα and CCβ values were calculated only for liver and skin + fat, because there is no fixed MRL in kidney and muscle.

For each matrix, the precision of the method was evaluated at three different levels of fortification by calculating the CV % of the FQ concentrations under within-day repeatability conditions (calculated from six replicated samples analyzed on one day), and under within-laboratory reproducibility conditions (calculated from batches of 18 samples analyzed on three different days by different operators). The results, listed in Tables 5, 6 and 7, reveal that all CV % values, for within-day repeatability and within-laboratory reproducibility, were acceptable, ranging from 1.1 to 14.2% and from 1.3 to 13.1% respectively, for all concentrations.

The trueness of the developed method, expressed as relative recovery, ranged from 86.1 - 106.9 % for all FQs (Tables 5, 6, 7) in agreement with the limits reported by Commission Decision 2002/657/EC. The only exception was FLUME in muscle with a recovery of 111.7 % and 113.1% at 1 and 1.5 MRL respectively, and of 111.3% at 25 $\mu g\ kg^{-1}$ in intestinal content; thus, an overestimation of this FQ in muscle and intestinal content could be expected.

The QuEChERS-based extraction procedure adopted for FQs recovery, from different matrices, did not require further clean-up step. For all analytes, the absolute recovery ranged from 69.1 % to 112.8 %, with CV % lower than 14.4%, all of this confirms the good reproducibility of the method.

The matrix effects ranged from 0.70 to 1.50, indicating that the analytes are only slightly influenced by the matrix of the tissues and by plasma extract as a consequence of optimized samples clean-up procedures, optimized chromatography conditions and dilution of extracts that allowed to minimize the matrix effect due to the different biological matrices considered in this study.

Results of robustness test indicated that the method was not affected by slight variations of some critical factors in the sample preparation procedure and can be considered acceptably robust.

3.2 Analysis of samples from treated turkeys

The validated method allowed to detect ENRO, CIPRO and FLUME concentrations in plasma and in the biological matrices obtained from turkeys orally treated via 10-h pulsed medicated water for 5 consecutive days with ENRO and FLUME.

ENRO and CIPRO were determined separately but, for pharmacokinetic analysis, tissue distribution and depletion study, the sum of ENRO + CIPRO was always considered.

The plasma concentration-time profiles of ENRO and FLUME at day 5 of pulsed administration are reported in Fig. 1. The FQ distribution in target tissues reported in Fig. 2 confirmed the ability of FQs to diffuse freely in lungs reaching concentration higher than in plasma together with the importance of biliary elimination route for ENRO and FLUME as indicated by the great concentrations of the two FQs in intestinal content at the last day of treatment.

ENRO concentrations in muscle, kidney and skin + fat at 3 days after treatment, were always lower than the corresponding MRL and, in several turkeys, lower than the LOQ values (Fig. 3).

With the exception of skin + fat, no large variability of ENRO concentrations was observed in the different tissues from treated birds. As reported by San Martín, Cornejo, Iragüen, Hidalgo, & Anadón (2007), quinolones accumulate in follicles and feathers can become a long lasting reservoir; thus, the variability observed in skin + fat can be related to the accidental occurrence of a few small feathers.

No figures are reported for flumequine as after 5 days of withdrawal time, its concentrations were always lower than LOQ (average concentration: 10.8 μg kg^{-1}) despite the double dosage administered with medicated water.

4. Conclusions

A LC-MS/MS/MS method was developed and validated for rapid and simultaneous determination of the five FQs ENRO, CIPRO, DIFLO, SARA and FLUME in incurred plasma, liver, kidney, muscle, skin + fat, lung and intestinal content from treated turkeys.

For the first time, the QuEChERS technology was successfully applied for the extraction of FQs from matrices such as the lung, skin + fat, kidney and intestinal content.

The method proved to be simple, fast, efficient, stable, precise, accurate and robust, providing good validation parameters, such as linearity, limits of quantification, precision, trueness and recovery in all the matrices considered.

The applicability of the method and its good performances were confirmed in all the different approach of the study, plasma kinetics, target tissue distribution and residue depletion in liver, kidney, muscle, skin + fat, thus making an effective and reliable determination of the target FQs in real samples.

Acknowledgements

The study was supported by MIUR PRIN 2009 R4KM4F grant to CM.

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TABLE CAPTIONS:

Table 1: Instrument acquisition data for the analysis of FQs by LC-MS/MS/MS; [a] product ion used for quantification; Rt: retention time.

Table 2: Linearity evaluation and sensitivity data for the FQs detected in this study in the different biological matrices (plasma, liver, kidney, muscle, skin + fat, lung and intestinal content): linear determination coefficient (R^2), limit of detection (LOD) and limit of quantification (LOQ).

Table 3: Validation results obtained from plasma and lung (C_N: Nominal Concentration; CV = Coefficient of Variation; REC: absolute recovery; ME: matrix effect).

Table 4: Validation results obtained from liver, kidney and muscle (C_N: Nominal Concentration; CV = Coefficient of Variation; REC: absolute recovery; ME: matrix effect).

Table 5: Validation results obtained from skin + fat and intestinal content (C_N: Nominal Concentration; CV = Coefficient of Variation; REC: absolute recovery; ME: matrix effect).

SUPPLEMENTARY DATA-TABLE CAPTIONS:

Table S1: Concentrations of FQs considered for the evaluation of linearity in the different tissues and MRL values established for each analyte (there is no MRL for SARA in kidney and muscle).

Table S2: MRL of FQs established in liver, kidney, muscle and skin + fat and CCα and CCβ calculated expressed in μg kg^{-1} (there is no MRL for SARA in kidney and muscle).

Table 1

Analyte	Precursor ion (m/z)	**Fragmentation pattern**	**C**ollision energy (%)	Rt (min)
Enrofloxacin	360	360 > 316	46	
		316 > 288, **245**[a]	23	5.6
Ciprofloxacin	332	332 > 288	22	
		288 > **268**[a]**,** 245	30	5.3
Difloxacin	400	400 > 356	30	
		356 > 336**, 299**[a]	20	6.2
Sarafloxacin	386	386 > 342	30	
		342 > **322**[a]**,** 299	30	6.6
Flumequine	262	262 > 244	40	
		244 > **202**[a]**,** 176	25	12.5
Norfloxacin (IS)	320	320 > 276	36	
		276 > **256**[a]**,** 233	30	4.9

Table 2

Plasma			
analyte	R^2	LOD (µg l^{-1})	LOQ (µg l^{-1})
ENRO	0.9999	0.8	2.5
CIPRO	0.9998	0.5	1.4
DIFLO	0.9995	1.5	4.6
SARA	0.9998	0.6	1.8
FLUME	0.9996	0.9	2.5

Liver			
analyte	R^2	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
ENRO	0.9999	2.6	12.5
CIPRO	0.9998	5.7	12.5
DIFLO	0.9993	43.8	118.8
SARA	0.9997	3.3	6.3
FLUME	0.9997	29.3	50.0

Kidney			
analyte	R^2	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
ENRO	0.9999	9.8	18.8
CIPRO	0.9999	4.1	18.8
DIFLO	0.9999	9.7	37.5
SARA	0.9998	1.7	6.3
FLUME	0.9996	25.1	62.5

Muscle			
analyte	R^2	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
ENRO	0.9995	5.2	12.5
CIPRO	0.9995	2.0	12.5
DIFLO	0.9990	13.0	37.5
SARA	0.9992	0.5	1.3
FLUME	0.9988	8.3	50.0

Skin + fat			
analyte	R^2	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
ENRO	0.9942	4.8	12.5
CIPRO	0.9998	8.8	12.5
DIFLO	0.9969	16.7	50.0
SARA	0.9972	0.9	1.2
FLUME	0.9978	22.7	31.2

Lung			
analyte	R^2	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
ENRO	0.9998	2.7	8.2
CIPRO	0.9996	2.9	8.9
DIFLO	0.9997	2.2	6.8
SARA	0.9998	0.9	2.7
FLUME	0.9997	1.8	5.4

Intestinal content			
analyte	R^2	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
ENRO	0.9997	3.1	9.5
CIPRO	0.9997	1.1	3.4
FLUME	0.9994	4.5	13.8

Table 3

PLASMA						
analyte	**C_N µg l^{-1}**	TRUENESS (%)	Within-day Repeatability (CV %)	Within-Laboratory Reproducibility (CV %)	REC% ± SD	ME
ENRO	**2.5**	96.0	3.4	10.6	101.6 ± 5.9	$1.5 \pm 4.0 \times 10^{-2}$
	10	99.0	3.9	5.5	108.5 ± 6.1	$0.9 \pm 2.0 \times 10^{-2}$
	50	103.8	5.5	7.1	97.3 ± 2.6	$0.9 \pm 5.0 \times 10^{-2}$
CIPRO	**2.5**	92.0	8.8	12.6	99.5 ± 7.9	$1.0 \pm 1.0 \times 10^{-2}$
	10	98.0	6.6	7.2	103.7 ± 14.3	$1.0 \pm 3.0 \times 10^{-2}$
	50	100.6	6.7	5.7	110.1 ± 11.2	$0.9 \pm 3.0 \times 10^{-2}$
DIFLO	**2.5**	96.0	2.2	10.3	105.6 ± 10.9	$1.2 \pm 5.0 \times 10^{-2}$
	10	101.0	10.5	9.8	101.7 ± 11.0	$0.9 \pm 3.0 \times 10^{-2}$
	50	103.4	2.9	10.1	102.9 ± 10.9	$0.9 \pm 2.0 \times 10^{-2}$
SARA	**2.5**	100.0	8.7	8.0	101.0 ± 9.2	$1.0 \pm 1.0 \times 10^{-2}$
	10	99.0	7.1	7.2	102.0 ± 5.6	$0.9 \pm 6.0 \times 10^{-2}$
	50	96.6	3.6	5.3	99.0 ± 10.0	$0.9 \pm 5.0 \times 10^{-2}$
FLUME	**2.5**	104.0	4.7	12.2	112.8 ± 1.9	$1.2 \pm 5.0 \times 10^{-2}$
	10	104.0	2.7	5.9	105.6 ± 14.4	$0.9 \pm 2.0 \times 10^{-2}$
	50	96.8	3.0	6.2	112.2 ± 10.9	$0.9 \pm 3.0 \times 10^{-2}$
LUNG						
analyte	**C_N µg kg^{-1}**	TRUENESS (%)	Within-day Repeatability (CV %)	Within-Laboratory Reproducibility (CV %)	REC% ± SD	ME
ENRO	**12.5**	96.8	7.9	12.9	95.2 ± 10.5	$1.3 \pm 9.0 \times 10^{-2}$
	50	100.4	4.4	7.1	87.9 ± 5.2	$1.0 \pm 1.2 \times 10^{-1}$
	500	96.2	7.9	5.6	87.4 ± 6.6	$1.3 \pm 5.0 \times 10^{-2}$
CIPRO	**12.5**	94.4	2.7	10.2	70.6 ± 5.4	$1.3 \pm 2.1 \times 10^{-1}$
	50	97.6	5.6	5.5	69.1 ± 3.7	$1.1 \pm 1.5 \times 10^{-1}$
	500	98.2	2.6	3.4	77.9 ± 11.8	$1.4 \pm 7.0 \times 10^{-2}$
DIFLO	**12.5**	103.2	4.7	13.1	102.4 ± 9.5	$1.3 \pm 5.0 \times 10^{-2}$
	50	104.2	5.1	5.5	88.8 ± 6.6	$1.0 \pm 1.0 \times 10^{-1}$
	500	95.8	3.6	5.2	95.7 ± 10.5	$1.1 \pm 6.0 \times 10^{-2}$
SARA	**12.5**	93.6	5.9	6.6	92.3 ± 5.8	$1.2 \pm 3.0 \times 10^{-2}$
	50	99.4	3.9	7.3	86.6 ± 6.2	$1.1 \pm 1.3 \times 10^{-1}$
	500	99.5	4.5	4.9	91.4 ± 9.4	$1.1 \pm 5.0 \times 10^{-2}$
FLUME	**12.5**	104.8	6.3	9.7	89.4 ± 9.0	$1.1 \pm 6.0 \times 10^{-2}$
	50	101.6	8.5	11.2	78.9 ± 6.2	$1.0 \pm 1.3 \times 10^{-2}$
	500	96.5	5.1	6.0	89.8 ± 8.9	$1.0 \pm 3.0 \times 10^{-2}$

Table 4

LIVER						
analyte	**C_N µg kg^{-1}**	TRUENESS (%)	Within-day Repeatability (CV %)	Within-Laboratory Reproducibility (CV %)	REC% ± SD	ME
ENRO	**100**	94.3	3.6	4.6	82.9 ± 4.8	0.9 ± 3.0 x 10^{-2}
	200	97.3	7.9	6.5	87.1 ± 4.6	0.9 ± 3.0 x 10^{-2}
	300	100.3	3.2	3.8	100.0 ± 4.8	1.1 ± 1.6 x 10^{-1}
CIPRO	**100**	94.2	6.9	6.9	70.0 ± 6.5	1.0 ± 2.0 x 10^{-2}
	200	98.9	5.7	7.8	75.1 ± 2.0	1.1 ± 5.0 x 10^{-2}
	300	98.4	5.2	6.0	84.2 ± 3.7	1.2 ± 1.5 x 10^{-1}
DIFLO	**950**	97.7	2.2	2.9	86.8 ± 3.8	0.8 ± 8.0 x 10^{-2}
	1900	106.9	2.7	4.7	84.8 ± 3.3	1.0 ± 5.0 x 10^{-2}
	2850	98.6	3.0	3.9	93.1 ± 4.3	1.2 ± 1.3 x 10^{-1}
SARA	**50**	96.2	10.3	8.1	84.4 ± 7.5	0.7 ± 1.0 x 10^{-2}
	100	104.1	6.2	6.5	88.5 ± 1.7	0.9 ± 4.0 x 10^{-2}
	150	100.9	4.6	3.4	105.7 ± 2.8	1.1 ± 8.0 x 10^{-2}
FLUME	**400**	91.9	4.7	7.3	95.0 ± 2.2	0.7 ± 4.0 x 10^{-2}
	800	98.9	2.6	5.5	97.1 ± 3.3	0.8 ± 2.0 x 10^{-2}
	1200	102.1	2.4	2.6	102.5 ± 4.4	0.9 ± 5.0 x 10^{-2}
KIDNEY						
analyte	**C_N µg kg^{-1}**	TRUENESS (%)	Within-day Repeatability (CV %)	Within-Laboratory Reproducibility (CV %)	REC% ± SD	ME
ENRO	**150**	98.7	1.8	2.9	94.7 ± 3.4	1.0 ± 3.0 x 10^{-2}
	300	96.2	1.2	2.4	100.8 ± 11.8	0.9 ± 3.0 x 10^{-2}
	450	98.3	1.2	1.3	106.1 ± 9.8	0.9 ± 2.0 x 10^{-2}
CIPRO	**150**	99.6	1.6	4.5	81.3 ± 7.3	0.8 ± 4.0 x 10^{-2}
	300	100.3	1.7	5.0	85.9 ± 8.7	0.8 ± 2.0 x 10^{-2}
	450	100.4	1.1	2.3	88.8 ± 8.1	0.7 ± 4.0 x 10^{-2}
DIFLO	**300**	99.6	2.1	2.7	99.9 ± 5.7	0.9 ± 5.0 x 10^{-2}
	600	99.7	1.9	1.9	102.5 ± 6.7	0.9 ± 4.0 x 10^{-2}
	900	101.3	1.8	1.8	108.8 ± 7.2	0.8 ± 3.0 x 10^{-2}
SARA	**50**	99.8	5.2	6.3	108.1 ± 6.5	1.0 ± 8.0 x 10^{-2}
	100	101.4	2.0	3.1	103.2 ± 9.1	1.0 ± 8.0 x 10^{-2}
	150	100.1	1.8	2.4	101.2 ± 5.9	0.9 ± 6.0 x 10^{-2}
FLUME	**500**	97.9	2.0	3.4	102.6 ± 2.6	0.9 ± 4.0 x 10^{-2}
	1000	97.8	2.2	2.8	102.5 ± 7.8	1.1 ± 8.0 x 10^{-2}
	1500	98.0	1.1	3.2	106.2 ±7.3	0.9 ± 7.0 x 10^{-2}
MUSCLE						
analyte	**C_N µg kg^{-1}**	TRUENESS (%)	Within-day Repeatability (CV %)	Within-Laboratory Reproducibility (CV %)	REC% ± SD	ME
ENRO	**50**	90.0	10.0	6.9	92.9 ± 2.7	0.9 ± 2.0 x 10^{-2}
	100	103.4	3.7	7.0	99.6 ± 2.3	1.1 ± 5.0 x 10^{-2}
	150	104.3	4.7	4.8	98.8 ± 3.0	1.1 ± 5.0 x 10^{-2}
CIPRO	**50**	92.4	4.7	5.2	83.0 ± 5.0	0.9 ± 1.0 x 10^{-2}
	100	100.7	4.0	5.6	80.4 ± 3.2	1.2 ± 4.0 x 10^{-2}
	150	100.9	4.3	5.6	81.3 ± 3.9	1.2 ± 4.0 x 10^{-2}
DIFLO	**150**	86.1	5.8	7.0	97.9 ± 1.2	0.9 ± 2.0 x 10^{-2}
	300	102.8	5.1	7.7	105.0 ± 1.7	1.1 ± 3.0 x 10^{-2}
	450	105.0	3.7	9.3	102.3 ± 4.2	1.1 ± 6.0 x 10^{-2}
SARA	**5**	92.0	12.8	10.3	98.1 ± 11.3	0.9 ± 6.0 x 10^{-2}
	10	99.0	9.8	9.9	99.9 ± 7.1	1.1 ± 9.0 x 10^{-2}
	15	100.7	7.3	9.9	104.5 ± 6.8	1.0 ± 5.0 x 10^{-2}
FLUME	**200**	95.7	6.9	7.5	104.9 ± 2.3	0.9 ± 6.0 x 10^{-2}
	400	111.7	6.9	6.5	109.0 ± 1.9	1.2 ± 6.0 x 10^{-2}
	600	113.1	4.5	4.8	105.8 ± 2.7	1.1 ± 2.0 x 10^{-2}

Table 5

SKIN + FAT						
analyte	**C_N µg l^{-1}**	TRUENESS (%)	Within-day Repeatability (CV %)	Within-Laboratory Reproducibility (CV %)	REC% ± SD	ME
ENRO	**50**	102.2	2.5	10.9	109.6 ± 10.1	$0.9 \pm 5.0 \times 10^{-2}$
	100	102.8	5.2	8.2	97.1 ± 4.9	$0.9 \pm 3.0 \times 10^{-2}$
	150	101.7	5.9	6.3	99.9 ± 3.6	$0.9 \pm 2.0 \times 10^{-2}$
CIPRO	**50**	99.6	3.8	8.4	102.1 ± 2.4	$0.8 \pm 1.0 \times 10^{-2}$
	100	100.7	8.1	5.7	84.2 ± 8.8	$0.9 \pm 4.0 \times 10^{-2}$
	150	102.8	9.6	8.9	82.1 ± 5.5	$0.9 \pm 3.0 \times 10^{-2}$
DIFLO	**200**	100.4	3.6	5.2	111.7 ± 6.3	$0.8 \pm 7.0 \times 10^{-2}$
	400	97.3	2.5	2.9	101.7 ± 7.9	$0.9 \pm 6.0 \times 10^{-2}$
	600	95.8	2.6	2.6	95.2 ± 7.7	$1.0 \pm 6.0 \times 10^{-2}$
SARA	**5**	91.0	14.2	11.0	110.6 ± 4.2	$0.9 \pm 6.0 \times 10^{-2}$
	10	102.3	9.3	6.8	107.7 ± 4.9	$0.8 \pm 6.0 \times 10^{-2}$
	15	102.8	9.5	7.4	98.1 ± 5.6	$0.9 \pm 7.0 \times 10^{-2}$
FLUME	**200**	101.7	2.4	4.4	110.5 ± 4.4	$0.9 \pm 6.0 \times 10^{-2}$)
	400	99.1	5.1	4.8	103.1 ± 13.3	$0.9 \pm 4.0 \times 10^{-2}$
	600	105.5	1.7	5.0	99.1 ± 3.2	$1.0 \pm 6.0 \times 10^{-2}$
INTESTINAL CONTENT						
analyte	**C_N µg l^{-1}**	TRUENESS (%)	Within-day Repeatability (CV %)	REC% ± SD	ME	
ENRO	**25**	104.2	2.4	96.7 ± 11.3	$1.1 \pm 3.0 \times 10^{-2}$	
	100	102.6	3.6	85.2 ± 2.2	$1.2 \pm 6.0 \times 10^{-2}$	
	1000	97.5	3.3	87.4 ± 5.2	$1.1 \pm 2.4 \times 10^{-1}$	
CIPRO	**25**	99.6	7.0	79.3 ± 6.5	$1.0 \pm 1.0 \times 10^{-2}$	
	100	102.6	1.9	100.2 ± 2.2	$0.9 \pm 1.0 \times 10^{-1}$	
	1000	97.4	2.1	99.3 ± 4.9	$0.8 \pm 1.3 \times 10^{-1}$	
FLUME	**25**	111.3	6.8	80.6 ± 2,4	$1.0 \pm 1.0 \times 10^{-1}$	
	100	106.5	2.8	92.7 ± 1.7	$0.9 \pm 1.6 \times 10^{-1}$	
	1000	97.7	3.0	94.5 ± 5.4	$0.9 \pm 1.6 \times 10^{-1}$	

SUPPLEMENTARY DATA

Table S1

LIVER		
analyte	Concentrations (μg kg^{-1})	**MRL (μg kg^{-1})**
ENRO	12.5, 25, 50, 125, 250, 500, 1000, 2000	**200**
CIPRO	12.5, 25, 50, 125, 250, 500, 1000, 2000	**200**
DIFLO	118.8, 237.5, 475, 1187.5, 2375, 4750, 9500, 19000	**1900**
SARA	6.2, 12.5, 25, 62.5, 125, 250, 500, 1000	**100**
FLUME	50, 100, 200, 500, 1000, 2000, 4000, 8000	**800**
KIDNEY		
analyte	Concentrations (μg kg^{-1})	**MRL (μg kg^{-1})**
ENRO	18.8, 37.5, 75, 187.5, 375, 750, 1500, 3000	**300**
CIPRO	18.8, 37.5, 75, 187.5, 375, 750, 1500, 3000	**300**
DIFLO	37.5, 75, 150, 375, 750, 1500, 3000, 6000	**600**
SARA	6.2, 12.5, 25, 62.5, 125, 250, 500, 1000	**--**
FLUME	62.5, 125, 250, 625, 1250, 2500, 5000, 10000	**1000**
MUSCLE		
analyte	Concentrations (μg kg^{-1})	**MRL (μg kg^{-1})**
ENRO	12.5, 25, 50, 125, 250, 500, 1000	**100**
CIPRO	12.5, 25, 50, 125, 250, 500, 1000	**100**
DIFLO	37.5, 75, 150, 375, 750, 1500, 3000	**300**
SARA	1.2, 2.5, 5, 10, 25, 50, 100	**--**
FLUME	50, 100, 200, 500, 1000, 2000, 4000	**400**
SKIN + FAT		
analyte	Concentrations (μg kg^{-1})	**MRL (μg kg^{-1})**
ENRO	12.5, 25, 50, 125, 250, 500, 1000	**100**
CIPRO	12.5, 25, 50, 125, 250, 500, 1000	**100**
DIFLO	50, 100, 200, 500, 1000, 2000, 4000	**400**
SARA	1.2, 2.5, 5, 12.5, 25, 50, 100	**10**
FLUME	31.2, 62.5, 125, 312.5, 625, 1250, 2500	**250**

Table S2

LIVER				KIDNEY			
analyte	**MRL (μg kg^{-1})**	**CCα**	**CCβ**	**analyte**	**MRL (μg kg^{-1})**	**CCα**	**CCβ**
ENRO	**200**	226.4	252.7	**ENRO**	**300**	313.2	326.3
CIPRO	**200**	232.5	265.5	**CIPRO**	**300**	320.0	339.6
DIFLO	**1900**	2067.4	2234.7	**DIFLO**	**600**	624.2	648.4
SARA	**100**	109.4	118.8	**SARA**	**--**	--	--
FLUME	**800**	908.2	1016.4	**FLUME**	**1000**	1040.2	1080.4
MUSCLE				**SKIN + FAT**			
analyte	**MRL (μg kg^{-1})**	**CCα**	**CCβ**	**analyte**	**MRL (μg kg^{-1})**	**CCα**	**CCβ**
ENRO	**100**	114.8	129.5	**ENRO**	**100**	124.2	125.1
CIPRO	**100**	108.0	115.7	**CIPRO**	**100**	109.0	118.0
DIFLO	**300**	343.9	387.8	**DIFLO**	**400**	439.1	478.1
SARA	**--**	--	--	**SARA**	**10**	12.2	14.2
FLUME	**400**	465.9	531.8	**FLUME**	**250**	305.8	361.6

FIGURE CAPTIONS:

Figure 1: ENRO (+ CIPRO) and FLUME plasma concentration–time profiles at the 5th day of 10-h oral pulsed administration. Mean values (± SD) of 8 turkeys.

Figure 2: ENRO, CIPRO and FLUME concentrations in plasma, lung and intestinal content after oral pulsed administration for 5 days. Mean values (± SD) of 8 turkeys sacrificed at 24 h after the last treatment.

Figure 3: ENRO concentrations in muscle, kidney, liver and skin + fat from 8 turkeys (T1-T8) after oral pulsed administration for 5 days. Animals were sacrificed after three days from the end of treatment. CIPRO concentrations higher than LOD (5.7 μg kg^{-1}) were detected only in liver.

Figure 1

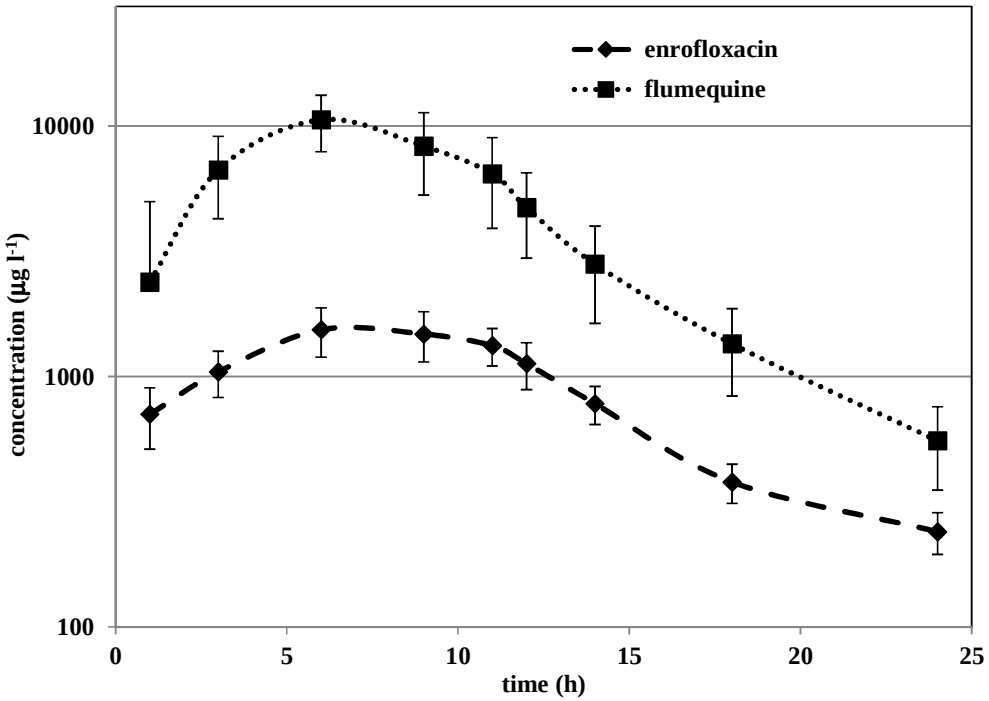


Figure 2

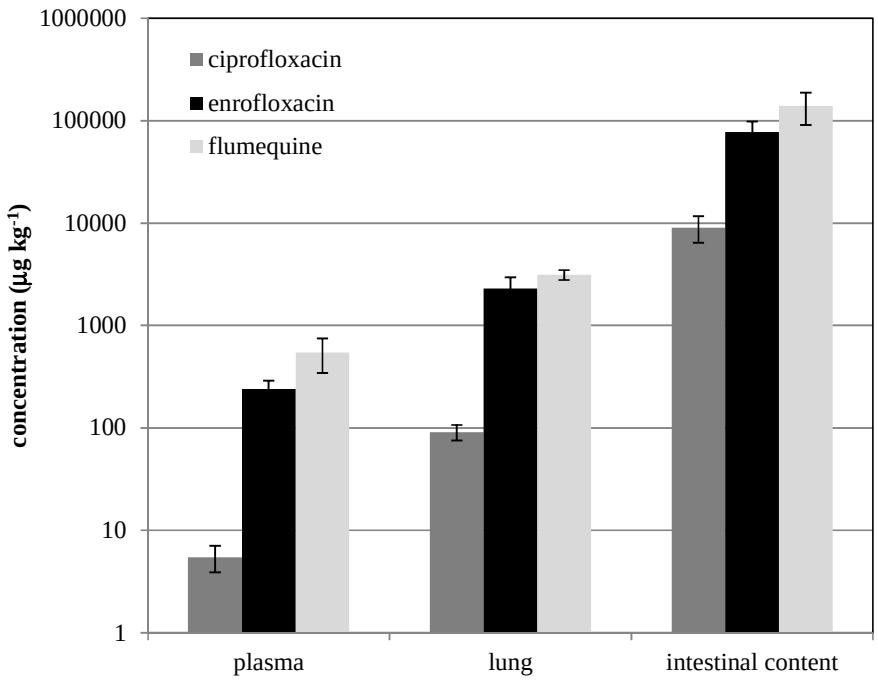
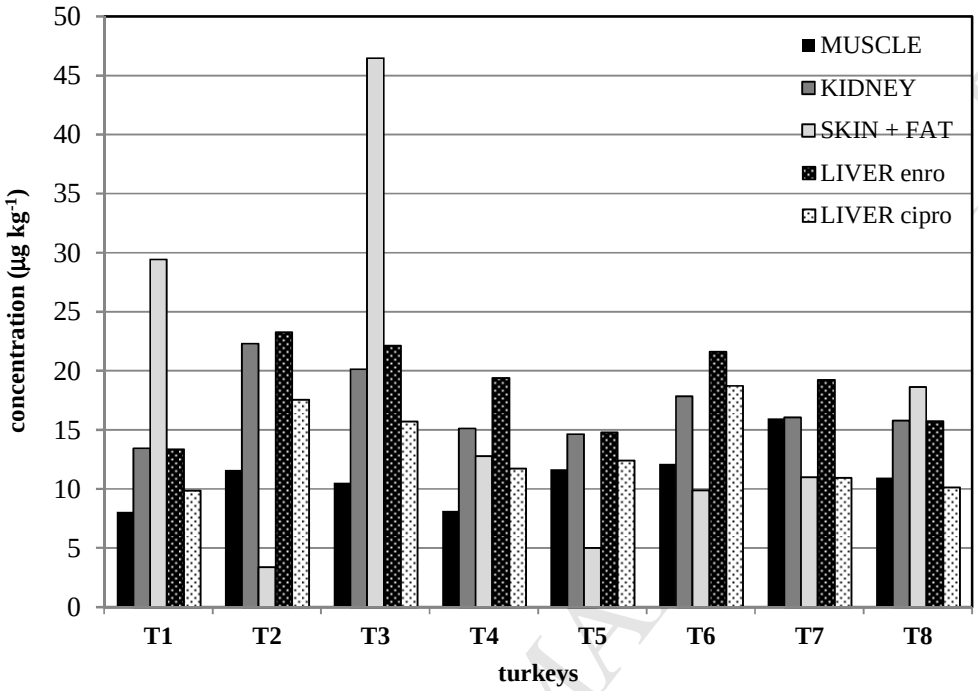


Figure 3



HIGHLIGHTS

- A LC-MS method was developed for five fluoroquinolones quantification.
- The method was validated in seven matrices (tissues and fluids) from turkeys.
- The fluoroquinolones were detectable in a wide range of concentrations.
- The method was successfully applied to plasma samples for pharmacokinetic study.
- Residue distribution and depletion were evaluated for two fluoroquinolones.