# Determination of five antimicrobial families in droppings of therapeutically treated broiler chicken by high-performance liquid chromatography-tandem mass spectrometry

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**ABSTRACT** Antimicrobials are currently used in poultry for disease treatment. However, their excretion in bird feces may contaminate the environment. Considering this, the objective of this work was to quantify antimicrobials residues concentrations in therapeutically treated broiler chicken droppings throughout the post-treatment period. For this aim a multiresidue method using high-performance liquid chromatography-tandem mass spectrometry **(HPLC-MS/MS)** was validated. Forty-eight male broiler chickens were distributed and treated with commercial formulations of 5 different antimicrobials. Results showed that oxytetracycline and 4-epi-oxytetracycline, presented the highest concentrations during all sampling period, detecting concentrations of 1471.41 $\mu$g kg$^{-1}$ at the last sampling point (day 22 post-treatment). Florfenicol, tylosin, enrofloxacin, and ciprofloxacin were eliminated and detected in treated chicken droppings until d 18 post-treatment. Sulfachloropyridazine decrease gradually during post-treatment period until day 30. Results demonstrate that studied antimicrobials in treated chicken droppings were eliminated for prolonged periods, therefore becoming a significant route of residues dissemination into the environment.

**Key words:** antimicrobial, chicken droppings, therapeutically treated broiler, HPLC-MS/MS, multiresidue detection

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## INTRODUCTION

In recent years, the poultry industry has expanded due to population growth and increased individual consumption, as chicken meat has a high protein content at a low price. However, this intensive production goes hand in hand with the use of antimicrobials to treat different diseases that can affect the birds during the farming process. Although, the use of these compounds is regulated in most of the countries, it must be considered that still in some large poultry producing countries such as China and Brazil; antimicrobials are still licensed for use as growth promoters (Roth et al., 2019).

Among the different families of antimicrobials used in poultry production are tetracyclines, macrolides, quinolones, phenicols, and sulfonamides (Sumano López et al., 2010). Generally, these drugs are administered to the entire flock through food or drinking water, and are applied to treat various pathologies, including intestinal infections such as colibacillosis, necrotic enteritis and other diseases generally caused by *E. coli, Salmonella* spp. or *Clostridium* spp., which represent an important concern in the industry as these generate enormous economic losses (Roth et al., 2019). However, residues of the drugs can persist in different products of animal origin. For this reason and to avoid adverse effects in the population, different international organizations have established maximum residue limits **(MRL)** to monitor the levels of these drugs in products of animal origin (FAO, 2018; Commission Regulation EU, 2010) and



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thus prevent concentrations above permissible levels from being transferred to consumers through the food chain (Love et al., 2012).

Inedible by-products are not controlled or monitored for any veterinary drug residue, as these are not intended for direct consumption. In the poultry industry, one of the main by-products obtained from the production of poultry is poultry litter, a mixture of food waste, animal waste such as feathers, litter material and mainly bird droppings (Dalolio et al., 2017; Ghirardini et al., 2020). This by-product is produced in large volumes, for example in the United States and Brazil, a production of more than 14 and 8 to 10 million tons per year has been described, respectively (Dalolio et al., 2017; Yang et al., 2019).

Poultry litter is generally used as a low-cost organic fertilizer, as it is an important source of nitrogen, phosphorus and trace elements for crop production, and has been shown to be effective in improving physical and biological fertility of crops (Bolan et al., 2010; Cheng and Jiang, 2014; Pizarro et al., 2019). In some countries it is also used as an input in the formulation of diets of other productive species, as it constitutes a good source of protein, energy and minerals (Cornejo et al., 2019) especially for cattle, which due to its digestive characteristics, can make a more efficient use of nutrients (Cabrera-Núñez et al., 2018 ). However, in the absence of any control of antimicrobial presence, these by-products can contribute to the re-entry of antimicrobial residues through the food chain, and their transfer and dissemination to the environment. The application of chicken manure containing antibiotics is considered to be one of the major sources of contamination and transfer of these compounds to the environment (Muhammad et al., 2020). This is due to the fact that once antibiotics are administered, they can be excreted in large quantities through urine and feces, and it has been reported that between 17 and 90% of them are excreted in their nonmetabolized form or as active metabolites (Massé et al., 2014).

This issue has gained relevance in recent years and different studies have determined the presence of antimicrobial residues in the manure of productive animals and poultry droppings (Berendsen et al., 2015; Yévenes et al., 2018). It has also been described that antimicrobials and their metabolites are strongly adsorbed in feces due to chemical interaction with metals and organic substances, forming complexes with soluble organic compounds that remain stable during storage (Massé et al., 2014). However, the probability of entry of these drugs to environmental reservoirs differs according to the compound and the animal species that excretes it (Spielmeyer, 2018; Jansen et al., 2019).

The application of manure as fertilizer on soils constitutes a massive entry route for these residues to the environment, as a significant fraction becomes mobile with water, polluting the surrounding environment and surface and groundwater through runoff and leaching processes, thus becoming important reservoirs of antibiotics (Tong et al., 2011; Massé et al., 2014; Slana et al., 2014; Albero et al., 2018; Conde et al., 2018).

Depending on the physicochemical properties of antibiotics, soil characteristics and environmental conditions, antibiotics can be retained in the soil or uptake by vegetables and even toward the fruits (Wang et al., 2006; Wang and Yates, 2008; Kang et al., 2013; Pan and Chu et al., 2017). Likewise, in the aquatic environment, various manifestations of toxicity have been reported in different aquatic species (Isidori et al., 2005; Park and Choi, 2008; Daghrir and Drogui, 2013; Ortiz et al., 2014; de Vasconcelos et al., 2017)

For this reason, it is important to determine the elimination of antimicrobial residues in therapeutically treated bird droppings and thus assess whether this by-product is a potential route of transfer and dissemination of antibiotics to the environment. In addition, the use of noninvasive matrices can be a useful tool for monitoring the use of antimicrobials on farms (Nebot et al., 2012).

Different studies have demonstrated the presence of antimicrobial residues in poultry feces (Slana et al., 2017; Berendsen et al., 2018; Cornejo et al., 2018; Yévenes et al., 2018). However, we are not aware of any study that quantify and project the concentrations that may persist in this matrix after the antimicrobial treatment is applied to these animals during the farming process. Therefore, the objective of this work was to quantify the concentrations of the most widely used antimicrobials in the poultry industry, by simultaneously detecting them in therapeutically treated broiler chicken droppings during the post-treatment period. For this and in order to analyze the persistence of antimicrobial of tetracycline, macrolide, fluoroquinolones, phenicols and sulfonamides families, a multiresidue method by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was validated.

## MATERIAL AND METHODS

### *Certified Standards, Reagents, and Solvents*

Analysis and quantification of analytes in droppings was performed using certified standards with a purity greater than 90%. Tetracycline **(TC)** hydrochloride, 4-epi-tetracycline **(4-epi-TC)** hydrochloride, oxytetracycline **(OTC)** hydrochloride, 4-epi-oxytetracycline **(4-epi-OTC)**, chlortetracycline **(CTC)** hydrochloride, 4-epi-chlortetracycline **(4-epi-CTC)** hydrochloride, tylosin **(TYL)** tartrate, enrofloxacin **(EFX)**, ciprofloxacin **(CFX)** hydrochloride, flumequine **(FLU)**, florfenicol **(FF)**, sulfachloropyridazine **(SCP),** and sulfadiazine **(SDZ)**, were used for the HPLC-MS/MS analysis. All standards were manufactured by Toronto Research Chemicals (Toronto, Canada), Dr. Ehrenstorfer Gmbh (Augsburg, Germany) and Sigma Aldrich (Merck KgaA, Darmstadt, Germany).

The following certified reagents were used as internal standards: isotopic tetracycline d-6 **(TC-D6)**, certified purity 80%, manufactured by Toronto Research Chemicals (Toronto, Canada); enrofloxacin D5 hydrochloride **(EFX-D5)**, certified purity 94%, manufactured by Dr. Ehrenstorfer Gmbh (Augsburg, Germany); sulfamethazine-phenyl-13C6 hemihydrate **(SMZ13C6)**, certified purity 99.8%; chloramphenicol D5 **(CAF-D5)**, certified purity 98.95% and erythromycin-N-methyl-13C, d3 **(ETM13C-D3)**, certified purity 98%, manufactured by Sigma Aldrich (Merck KgaA, Darmstadt, Germany).

A stock solution was prepared for each of the analytes of interest and internal standards at a concentration of 1,000 $\mu$g mL$^{-1}$ in methanol. Two intermediate or working solutions at 1,000 ng mL$^{-1}$ in methanol were prepared from the stock solutions, which contained the mix of analytes and the internal standards, respectively, and were used for spiking samples.

Solvents used for the analysis were water, methanol and acetonitrile, from the LiChroslv line, liquid chromatography grade (Merck KgaA, Darmstadt, Germany). EDTA-McIlvaine buffer was prepared with citric acid monohydrate, disodium hydrogen phosphate dihydrate and ethylenedinitrilotretraacetic acid (EDTA) disodium salt, manufactured by Merck KgaA (Darmstadt, Germany).

## Extraction of Antimicrobial Residues from Chicken Droppings

The procedure was based on an analytical methodology previously published by Berendsen et al. (2015) and was optimized to detect and quantify multiple antimicrobials in animal waste by means of high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS) after cleaning by solid phase extraction.

To extract the different analytes from the droppings matrix, samples were homogenized and placed in 50 mL polypropylene tubes (1 ± 0.01 g). As solvent for extraction, 8 mL of EDTA-McIlvaine buffer (pH 4.0 ± 0.1) and 2 mL of acetonitrile were used. Samples were shaken in a Multi Reax agitator (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) for 10 min, subsequently centrifuged in an Eppendorf Centrifuge 5804 centrifuge (Merck KGaA, Darmstadt, Germany) at 3,234 g for 10 min, and filtered through glass microfiber filters without binding agents, grade GF/A (1.6 $\mu$m) (MERCK KGaA, Darmstadt, Germany). For the clean-up, SUPEL-SELECT HLB 200 mg/6 mL solid phase extraction columns (Waters Corp., Milford, MA) were used, which were previously conditioned with 5 mL of methanol and 5 mL of water, both LC-MS grade.

Then, columns were washed with 5 mL of LC-MS grade water and dried with a manifold pump for later elution with 10 mL of LC-MS grade methanol. Finally, the eluate was dried under a gentle nitrogen flow in a water bath at 40−50°C, in an automated solvent evaporation system (TurboVap LV, Biotage, Uppsala, Sweden). The samples were reconstituted with 200 $\mu$L of methanol and 300 $\mu$L of LC grade water, shacked and sonicated for 5 min, then centrifuged at 17,136 g for 5 min. Finally, the supernatant was filtered through a 0.22 $\mu$m Millex syringe filters (MERCK KGaA, Darmstadt, Germany).

## Instrumental Analysis

Samples were analyzed using an Agilent 1290 series Liquid Chromatograph (Santa Clara, CA) coupled to a triple quadrupole tandem mass spectrometer, in multiple reaction monitoring mode via an electrospray interface. In particular, an AB Sciex API 5500 mass spectrometer (AB Sciex LLC, Framingham, MA) was used. This device through polarization switching was operated in positive ionization mode for the analytes TC, 4-epi-TC, OTC, 4-epi-OTC, CTC, 4-epi-CTC, TYL, EFX, CFX, FLU, SCP, SDZ, SMZ13C6, EFX-D5, ETM13C-D3, TC-D6 and in negative ionization mode for the FF and CAF-D5 analytes. The specifications of the mass spectrometer and the liquid chromatograph are described in Supplementary Table 1 while Supplementary Table 2 shows the specific mass spectrometer conditions for the analytes.

## Analytical Methodology Validation

In order for this methodology to be valid for the determination of antimicrobial residues from broiler chicken droppings, and that the results obtained from experimental samples are reliable and reproducible; a validation of the analytical methodology was carried out according to an internal validation protocol, which was developed following the recommendations from Commission Decision 2002/657/EC (Commission Decision, 2002) and the guideline VICH topic GL49 (EMA, 2015). In accordance with this internal validation protocol, following parameters were evaluated to demonstrate that the analytical method reliably and accurately met the criteria applicable to performance characteristics.

***Specificity*** The specificity of the method was determined by analyzing 21 samples from different sources, in order to determine the presence of interferents in the retention time of the analytes of interest.

***Detection Range*** The limit of detection **(LOD)** and limit of quantification **(LOQ)** were determined in 2 steps; first, a preliminary estimation of LOD and LOQ was performed to verify the existence of a linear relationship between concentration and instrument response. These values were determined as instrumental LOD and LOQ. Subsequently, the LOD and LOQ for each analyte were determined in a fortified matrix. The criteria for establishing the LOD were to achieve a signal-to-noise ratio greater than 3:1; while, for determining the LOQ, a signal-to-noise ratio higher than 10:1.

***Linearity of Calibration Curves*** To determine the linear response of the matrix calibration curves, we

performed a linear regression analysis of the area ratio and target levels. For this, an analysis was performed on different days of 3 calibration curves in samples free of antibiotic residues, spiked at different levels including the zero; the concentration corresponded at 12.5, 25, 50, 75, and 100 $\mu$g kg$^{-1}$.

***Recovery and Precision*** Recovery was determined by analyzing blank spiked samples at 25, 50, and 75 $\mu$g kg$^{-1}$. The concentration present in each sample was determined after each analysis, and the resulting concentrations were used to calculate the recovery percentage using the following equation:

$$Recovery(\%) = (quantifiedconcentration * 100) / spikedlevel.$$

Precision was evaluated by analysis of repeatability and intralaboratory reproducibility. For the determination of repeatability, independent tests were carried out by the same operator using the same method, same solvent and test equipment in the same laboratory. To determine the proximity of the agreement of the results from these independent samples analyzed under the same conditions, we worked with 18 blank samples that were spiked at 3 different concentrations (0.5, 1 and 1.5 times the established limit of 50 $\mu$g kg$^{-1}$), with 6 replicates for each concentration.

On the other hand, intralaboratory reproducibility was determined by the same analysis, using the same working concentrations and number of replicates. However, the analyzes were performed under different laboratory conditions (different reagent lots, ambient temperatures, days, and operators).

The precision of the method was determined by calculating the relative standard deviation **(RSD)** of the concentrations detected for each spike level, in the intralaboratory repeatability and reproducibility tests.

The following analytes were included in the internal validation plan: TC, 4-epi-TC, OTC, 4-epi-OTC, CTC, 4-epi-CTC, TYL, EFX, CFX, FLU, FF, SCP and SDZ). On the other hand, the following standards labeled with stable isotopes corresponding to the nuclear component were used as internal standards: CAF-D5, EFX-D5, ETM13C-D3, SMZ13C6 and TC-D6.

## Experimental Animals

The depletion study was performed with Ross 308 genetics male broiler chickens (Ross, Aviagen Inc., Huntsville, AL). These commercial hybrids have a good growth rate, good feed conversion, yield and robustness, where at 42 d of life, present a body live weight of approximately 2.9 kg with an average daily gain of 100 g (Ross, 2021), which is why they were chosen for this study.

The birds were raised from their first day of life in conditioned pens and under controlled environmental conditions of temperature (25 ± 5°C) and humidity (50 −60%), according to their life stage requirements (Ross, 2018). Throughout the experiment the birds had free access to water and food. The latter was formulated according to the nutritional requirements of the birds.

For the maintenance of the birds, the recommendations of the national animal welfare regulations of Law No. 20,380 "On Protection of Animals" (MINISTERIO, 2009) and of Directive 2010/63/EU related to the protection of animals used for scientific purposes (European Parliament and the Council of the European Union, 2018) were followed. For the slaughter of the birds, Regulation (EC) No. 1099/2009 on the protection of animals at the time of slaughter was respected (European Commission, 2009). Furthermore, the study was approved by the Institutional Committee for the Care and Use of Animals (**CICUA**, by its Spanish acronym) of the University of Chile (Certificate No. 19276-VET-UCH). All the biosecurity measures, the work with the birds and the analysis of the experimental samples, were carried out with the approval of the Biosecurity Committee of the Faculty of Veterinary and Livestock Sciences (**FAVET**, by its Spanish acronym) of the University of Chile (Certificate No. 145).

## Pharmaceutical Formulations for In vivo Study

Five pharmaceutical formulations, which representing different families of antimicrobials, were used to treat the experimental animals. Specifically, for the study, oral formulations of 10% OTC, 10% TYL, 20% EFX, 2% FF, and 10% SCP were used. The products had different established withdrawal period for muscle of 7 d for OTC, 5 d for TYL, 10 d for EFX and FF, and 30 d for SCP. All these formulations are authorized for use in broiler chickens, which are registered and authorized at the national level by the Agricultural and Livestock Service (SAG by its Spanish acronym) (SAG, 2020).

## Treatment and Sample Collection

Forty-eight birds were distributed into 6 groups, so that each experimental group consisted of 8 birds. The number of individuals was determined according to the recommendations of the VICH topic GL48 (EMA, 2015).

Groups A, B, C, D and E corresponded to the groups of birds that were treated with OTC, TYL, EFX, FF and SCP, respectively. The treatment was carried out orally using a No. 6 Levin gastric tube, to ensure the complete intake of the therapeutic dose of each antimicrobial and thus reduce variability due to consumption. Group A was treated with 10% OTC, with a dose of 80 mg kg$^{-1}$ every 24 h for 10 consecutive d. Group B was treated with 10% TYL, with a dose of 35 mg kg$^{-1}$ every 24 h for 7 consecutive d. Group C was treated with 20% EFX, with a dose of 10 mg kg$^{-1}$ every 24 h for 7 consecutive d. Group D was treated with 2% FF, with a dose of 15 mg kg$^{-1}$ every 12 h for 4 consecutive d, and group E was treated with 10% SCP, with a dose of 30 mg kg$^{-1}$ every 24 h for 5 consecutive d.

On the other hand, the sixth group, called group F, corresponded to the control group and consisted of 8 birds kept under the same conditions, but without antimicrobial treatment.

After treatment, each experimental group was sampled. The samples were obtained on d 5, 7, 10, 14, 18, and 22 post-treatment. For SCP analysis, 2 additional samples were taken on d 30 and 34 post-treatment, as the withdrawal period of this pharmaceutical formulation was much longer than the other antimicrobials analyzed.

Cloaca samples were collected individually with a torula, homogenized and stored at -20°C in sterile 50 mL polypropylene tubes for further processing, extraction and chromatographic analysis.

### Quantification of Residues from Experimental Samples

After analysis and detection of the samples through the multiresidue method, the concentrations of OTC, 4-epi-OTC, TYL, EFX, CFX, FF and SCP were determined using the line equation obtained from the regression analysis of the calibration curves of spiked samples, carried out together with each sampling. The samples used to construct the curves were free of residues and were spiked at different and equidistant concentrations to avoid extrapolations for the quantification of the different analytes. For the quantification, only those curves that presented a coefficient of determination $R^2$ greater than 0.98 were considered.

### Statistical Analysis and Depletion Study

To determine whether there were differences between antimicrobials and days post-treatment, an analysis of variance (ANOVA) and Fisher's least significant difference **(LSD)** test were performed. Antimicrobial concentrations in droppings were expressed as natural logarithm **(LN)** and corresponded to the dependent variable. The days post-treatment **(DPT)**, antimicrobial **(AB)** and the interaction between both were also considered as factors. The statistical software Infostat version 2020I was used. In all experiments, differences were considered statistically significant when the associated probability level (*P-value*) was less than 0.05.

In addition, and to determine and extrapolate the depletion of residues, a regression analysis was performed with the concentrations detected vs. the time of analysis, following the recommendations of the Guidance: Approach harmonization of withdrawal periods and Guideline on determination of withdrawal periods for edible tissues (EMEA, 1996; EMA, 2018). According to these guidelines the depletion of the residues was determined with a one-sided upper tolerance limit with a 95% of confidence according to Stange equation.

## RESULTS

### Multiresidue Methodology Validation

In the specificity analyzes, no interferents were observed in the retention times for each analyte studied from the samples of chicken droppings free of antimicrobial residues. The retention times for each analyte are described in

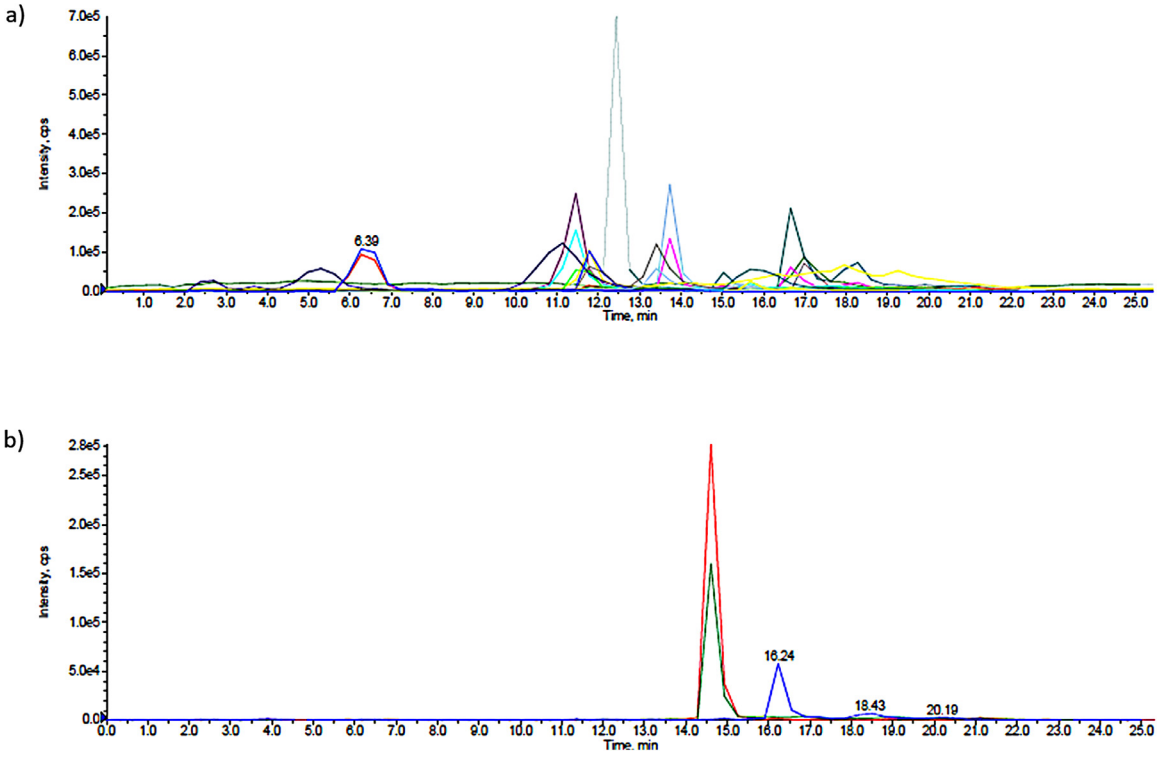


**Figure 1.** (A) Representative chromatogram of droppings blank sample spiked with 50 $\mu$g kg$^{-1}$, in positive mode. (B) Representative chromatogram of droppings blank sample spiked with 50 $\mu$g kg$^{-1}$, in negative mode.

**Table 1.** Limit of detection and limit of quantification for each studied analyte.

| Analyte | IDL[1] ($\mu$g kg$^{-1}$) | IQL[2] ($\mu$g kg$^{-1}$) | LOD[3] ($\mu$g kg$^{-1}$) | LOQ[4] ($\mu$g kg$^{-1}$) |
|---|---|---|---|---|
| Florfenicol | 3.3 | 11.1 | 11.2 | 33.5 |
| Tylosin | 3.4 | 11.4 | 7.3 | 21.9 |
| Enrofloxacin | 2.0 | 6.7 | 10.7 | 32.0 |
| Ciprofloxacin | 3.1 | 10.4 | 5.8 | 17.5 |
| Flumequine | 2.9 | 9.7 | 11.7 | 35.1 |
| Sulfachloropyridazine | 3.0 | 10.1 | 7.4 | 22.2 |
| Sulfadiazine | 2.8 | 9.5 | 12.3 | 36.8 |
| Tetracycline | 3.4 | 11.4 | 11.9 | 35.8 |
| 4-epi-tetracycline | 2.9 | 9.7 | 12.0 | 36.0 |
| Oxytetracycline | 2.7 | 8.9 | 12.1 | 36.4 |
| 4-epi-oxytetracycline | 2.3 | 7.8 | 12.2 | 36.5 |
| Chlortetracycline | 3.3 | 11.1 | 12.5 | 37.4 |
| 4-epi-chlortetracycline | 2.9 | 9.6 | 11.7 | 35.1 |

[1]Instrumental Limit of detection.
[2]Instrumental Limit of quantification.
[3]Limit of detection in matrix.
[4]Limit of quantification in matrix.

Supplementary Table 3. Supplementary Figures 1 and 2 show the chromatograms of the analyzed analytes from the injection of certified purity standards, using the conditions established for the analytical method in the API 5500 spectrometer (ABSciex). Figure 1 show a representative chromatogram (positive and negative mode, respectively) of a sample spiked with 50 $\mu$g kg$^{-1}$.

The instrumental and spiked matrix LOD and LOQ for each analyte are shown in Table 1. LODs for the analytes in the droppings matrix were equal to or less than 12.5 $\mu$g kg$^{-1}$ (Table 1). The LOQ presented values between 17.5 and 37.4 $\mu$g kg$^{-1}$ (Table 1). From these concentrations, experimental samples could be reliably and accurately quantified.

The curves analyzed presented a linear response in the area ratio vs. concentration regression analysis, with an $R^2$ greater than 0.99 for all the analytes of interest, as shown in Table 2.

The recovery of the analytes met the minimum acceptance criteria according to the internal validation protocol, where the recovery percentage for the working concentrations used had to be between 90 and 110%. The recovery ranges obtained in the study ranged from 91.9% to 108.1%, with the lowest and highest recovery value for CFX. Regarding the precision of the method, the results for the repeatability test presented a lower RSD than the results obtained for intralaboratory reproducibility, and these values did not exceed 23% of variation. The intralaboratory repeatability and reproducibility values are shown in Table 2.

## Quantification of Antimicrobials in Droppings

The quantified concentrations for the different antimicrobials in the droppings of therapeutically treated birds are shown in Table 3.

A tendency to increase in concentration was observed for OTC and its epimer throughout the post-treatment period. For this antimicrobial, the value of the last sampling point was approximately 69% higher than the concentrations detected in the first sampling point, corresponding to d 5 post-treatment. This coincides with the pharmacokinetic characteristics of OTC, which presents an accumulation in adipose tissue due to its great fat solubility (Vicente and Pérez-Trallero, 2010).

A similar behavior was observed for TYL, EFX and CFX. However, the concentrations detected were much lower than those of OTC and its epimer, and the highest standard deviation for these analytes was observed in the fifth sampling point, corresponding to 22 d post-treatment (Table 3 and Figure 2).

The concentrations detected for FF remained constant during the analysis period and although an increase in concentrations was observed with the passage of days after treatment, this increase was more marked in the second sampling point, where 274.07 $\mu$g kg$^{-1}$ were detected (vs. 134.70 $\mu$g kg$^{-1}$ detected on the first sampling point).

On the other hand, a decrease in the elimination of SCP was observed throughout the samplings. In the first sampling point, 516.61 $\mu$g kg$^{-1}$ were quantified in the matrix, and on the penultimate sampling point (d 30 post-treatment, corresponding to the withdrawal period established for the pharmaceutical formulation for muscle), 34.04 $\mu$g kg$^{-1}$ were detected and quantified, which is equivalent to a 93.4% decrease in residues. On the last sampling point, corresponding to d 34 post-treatment, it was only possible to detect traces of this antimicrobial, which were found below the LOD of the analytical methodology.

According to the results obtained from the ANOVA model, the AB factor and the AB-DPT interaction were significant ($P$-$value < 0.05$), but the DPT effect was not. The model explained 73% of the variation observed in LN. In the Fisher's LSD, there were significant differences for the AB factor between all the means for each antibiotic used, with TYL being the antibiotic with the lowest mean and OTC and its epimer the antibiotic with the highest mean. Furthermore, significant differences were observed between all means according to the interaction between the DPT and AB factors, the lowest mean being for TYL at 14 d post-treatment and the highest mean for OTC and its epimer at 14 d post-treatment.

## Projection of Antimicrobials Persistence in Broiler Chicken Droppings

The results were graphed on a semilogarithmic scale of concentration vs. time and a linear regression analysis was performed considering a confidence level of 95%. Data that were below the LOQ of the analytical methodology were set to one-half of the LOQ.

For the SCP, TYL, EFX and CFX, a projection was made and through this analysis we determined the time in which the concentrations would be equal to or lower than the 50 $\mu$g kg$^{-1}$ established limit and the LOQ

**Table 2.** Validation parameters of analytical methodology for antibiotic detection from chicken droppings.

| Analyte | Spike level ($\mu$g kg$^{-1}$) | Recovery[a] (%) | RSD$_r$[b] (%) | RSD$_{RL}$[c] (%) | Linearity[d] ($R^2 \pm$ SD) |
|---|---|---|---|---|---|
| Florfenicol | 25 | 97.5 | 3.7 | 13.0 | 0.996 ± 0.002 |
| | 50 | 102.5 | 3.9 | 12.4 | |
| | 75 | 99.2 | 1.2 | 4.3 | |
| Tylosin | 25 | 106.2 | 3.6 | 12.8 | 0.995 ± 0.003 |
| | 50 | 93.8 | 3.8 | 14.5 | |
| | 75 | 102.1 | 1.2 | 4.5 | |
| Enrofloxacin | 25 | 97.4 | 2.2 | 12.2 | 0.996 ± 0.005 |
| | 50 | 102.6 | 2.1 | 11.6 | |
| | 75 | 99.1 | 0.7 | 4.0 | |
| Ciprofloxacin | 25 | 91.9 | 3.1 | 10.1 | 0.996 ± 0.003 |
| | 50 | 108.1 | 2.8 | 8.6 | |
| | 75 | 97.3 | 1.0 | 3.2 | |
| Flumequine | 25 | 97.6 | 2.0 | 21.5 | 0.996 ± 0.004 |
| | 50 | 102.4 | 2.1 | 20.5 | |
| | 75 | 99.2 | 0.7 | 7.1 | |
| Sulfachloropyridazine | 25 | 97.3 | 3.7 | 9.7 | 0.999 ± 0.001 |
| | 50 | 102.7 | 3.5 | 9.2 | |
| | 75 | 99.1 | 1.2 | 3.2 | |
| Sulfadiazine | 25 | 94.0 | 4.9 | 10.4 | 0.996 ± 0.004 |
| | 50 | 106.0 | 4.8 | 9.3 | |
| | 75 | 98.0 | 1.6 | 3.3 | |
| Tetracycline | 25 | 100.4 | 3.9 | 7.0 | 0.997 ± 0.002 |
| | 50 | 99.6 | 3.9 | 7.1 | |
| | 75 | 100.1 | 1.3 | 2.3 | |
| 4-epi-tetracycline | 25 | 99.7 | 4.0 | 21.2 | 0.999 ± 0.000 |
| | 50 | 100.3 | 4.1 | 21.1 | |
| | 75 | 99.9 | 1.3 | 7.1 | |

| Analyte | Spike level ($\mu$g kg$^{-1}$) | Recovery[1] (%) | RSD$_r$[2] (%) | RSD$_{RL}$[3] (%) | Linearity[4] ($R^2 \pm$ SD) |
|---|---|---|---|---|---|
| Oxytetracycline | 25 | 104.3 | 3.3 | 4.7 | 0.995 ± 0.004 |
| | 50 | 95.7 | 3.2 | 5.2 | |
| | 75 | 101.4 | 1.1 | 1.6 | |
| 4-epi-oxytetracycline | 25 | 98.4 | 3.3 | 21.3 | 0.997 ± 0.003 |
| | 50 | 101.6 | 3.2 | 20.6 | |
| | 75 | 99.5 | 1.1 | 7.0 | |
| Chlortetracycline | 25 | 100.8 | 2.7 | 14.8 | 0.995 ± 0.003 |
| | 50 | 99.2 | 2.8 | 15.1 | |
| | 75 | 100.3 | 0.9 | 5.0 | |
| 4-epi-chlortetracycline | 25 | 103.6 | 4.2 | 7.9 | 0.996 ± 0.002 |
| | 50 | 96.4 | 4.4 | 8.5 | |
| | 75 | 101.2 | 1.4 | 2.7 | |

[1]Recovery percentage (%) from spiked matrix.
[2]Relative Standard Deviation of repeatability.
[3]Relative Standard Deviation of intralaboratory reproducibility.
[4]Linearity of 3 calibration curves in matrix spiked at 12.5, 25, 50 and 100 $\mu$g kg$^{-1}$ ($R^2$: coefficient of determination ± Standard Deviation).

established for each analyte, with 95% confidence. As the values obtained were a fraction of a day, the depletion period was considered as the value rounded to the next unit.

The projected days for the depletion of SCP were 42.70 and 49.91, considering the cut-off point established in the study (50 $\mu$g kg$^{-1}$) and the LOQ of the analytical methodology (22.2 $\mu$g kg$^{-1}$), respectively, with 95% confidence (Figure 3).

For EFX and its active metabolite, the projected days for depletion were 49.44 and 55.76, considering as cut-off point the established limit and the LOQ of the analytical methodology, respectively, with 95% confidence (Figure 4).

The days projected for the depletion of TYL were 100.78 and 131.51, considering as cut-off point the established limit and the LOQ of the analytical methodology, respectively, with 95% confidence (Figure 5).

On the other hand, for OTC and its epimer, a projection was not possible because the concentrations tended to increase after treatment. In the case of FF, the projection could not be carried out either because its concentrations remained above 100 $\mu$g kg$^{-1}$ during the first 4 sampling points in little variable ranges and only at the fifth sampling point, corresponding to 22 d post-treatment, the quantified concentrations were under the LOD (11.2 $\mu$g kg$^{-1}$) of the analytical methodology. Therefore, when projecting the results by linear regression, the depletion period of FF residues was overestimated.

## DISCUSSION

Poultry droppings are the main component of the poultry litter. This by-product is produced in high volumes and is used to feed other productive species or as an organic fertilizer (Slana et al., 2014). The results of this study show that antibiotic residues remain in droppings for long periods and their excretion can even increase during the post-treatment period. This

**Table 3.** Average concentration ($\mu$g kg$^{-1}$) of oxytetracycline plus 4-epi-oxytetracycline, enrofloxacin plus ciprofloxacin, tylosin, florfenicol and sulfachloropyridazine at different d post-treatment in droppings of treated broiler chickens.

| | | | Average concentration ($\mu$g kg$^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|
| Sampling point | Post-treatment day | Day of life of the birds | OTC + 4-epi-OTC[1] | EFX + CFX[2] | TYL[3] | FF[4] | SCP[5] |
| 1 | 5 | 25 | 872.04 | 113.54 | 104.66 | 134.70 | 516.61 |
| 2 | 10 | 30 | 754.15 | 68.23 | 66.50 | 274.07 | 141.56 |
| 3 | 14 | 34 | 2058.97 | 283.78 | 37.70 | 126.43 | 171.17 |
| 4 | 18 | 38 | 1481.77 | 75.69 | 71.84 | 156.55 | 319.84 |
| 5 | 22 | 42 | 1471.41 | <LOQ[6] | ND[7] | <LOD[8] | 20.99 |
| 6 | 30 | 46 | - | - | - | - | 34.04 |
| 7 | 34 | 50 | - | - | - | - | <LOD[9] |

[1]Oxytetracycline plus 4-epi-oxytetracycline.
[2]Enrofloxacin plus ciprofloxacin.
[3]Tylosin.
[4]Florfenicol.
[5]Sulfachloropyridazine.
[6]Enrofloxacin: 32 $\mu$g kg$^{-1}$, only one sample quantified above the Limit of quantification of 38.02 $\mu$g kg$^{-1}$ for enrofloxacin and 20.98 $\mu$g kg$^{-1}$ for ciprofloxacin).
[7]Non detected.
[8]Limit of detection in matrix for florfenicol: 11.2 $\mu$g kg$^{-1}$.
[9]Limit of detection in matrix for sulfachloropyridazine: 7.4 $\mu$g kg$^{-1}$.

coincides with the research of Spielmeyer (2018), who indicated that antibiotic residues were present in the excreta of different animals including poultry, and that the percentage of the active ingredient ranged from <5% to 90% depending on the substance used days after the end of treatment, which did not correlate with the concentrations of residues that could be detected in edible tissues.

Also, multiresidue methods have gained relevance in recent years because they allow the simultaneous detection of different analytes from the same sample, being very useful for detecting residues of importance for public and animal health (Nebot et al., 2012). Currently, analytical methods using HPLC-MS/MS for the detection of antimicrobial residues from animal waste have been developed by different authors (Jansen et al., 2019; Bajkacz, et al., 2020; Patyra et al., 2020;Zheng et al., 2021). However, in the present study, a multiresidue analytical methodology based on the one published by Berendsen et al., (2015) was optimized and validated for the detection of different analytes in droppings of broiler chickens.

Through the use of the validated multiresidue method, this study showed that antibiotic residues belonging to the families of tetracyclines, sulfonamides, quinolones, macrolides and phenicols, persist in the

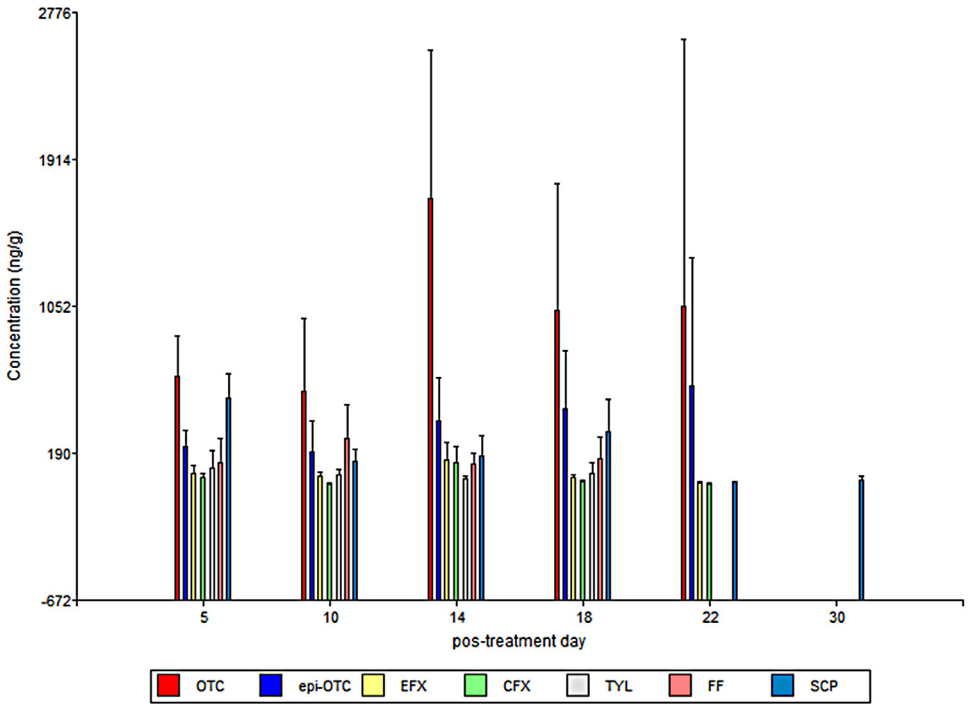


**Figure 2.** Concentrations of oxytetracycline, 4-epi-oxytetracycline, enrofloxacin, ciprofloxacin, florfenicol, tylosin and sulfachloropyridazine detected in broiler chicken droppings during the post-treatment period, after the administration of antimicrobials in therapeutic doses. Error bars represent the standard deviation.

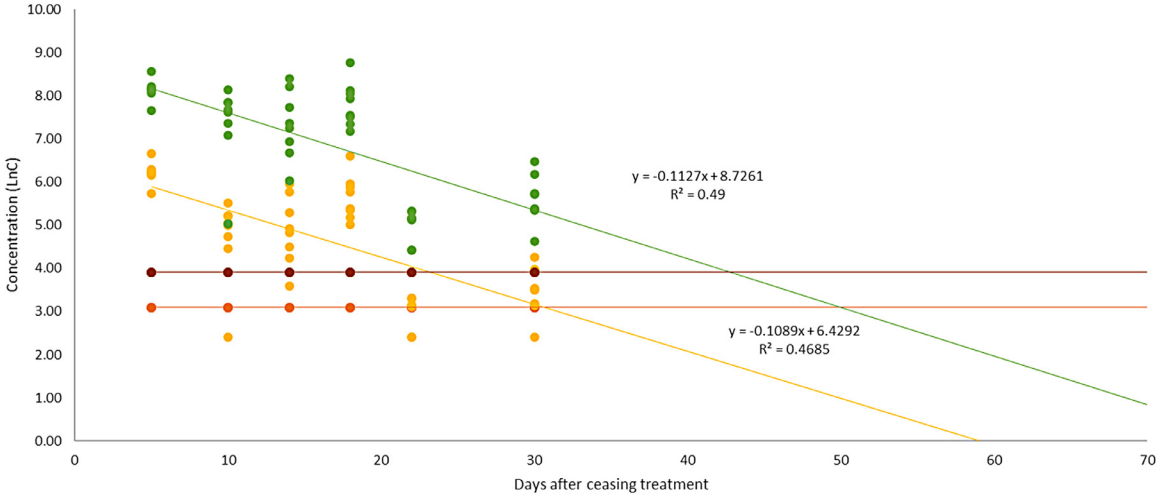


**Figure 3.** Projection of sulfachloropyridazine concentrations detected in broiler chicken droppings during the post-treatment period. Green: concentrations in LN considering 95% confidence; yellow: concentrations in LN; orange tree: LOQ of the analytical methodology for sulfachloropyridazine (22.2 $\mu$g kg$^{-1}$); dark red: established limit of 50 $\mu$g kg$^{-1}$.

droppings of antibiotic-treated birds for periods longer than withdrawal times and even longer beyond the slaughter of the birds. They can therefore persist in the litter or manure and spread to the environment or to other animals as part of the diet. Therefore, poultry litter represents a risk and an unknown route for the reentry of antibiotic residues through the food chain and their transfer to the environment (Slana et al., 2017). It is important to consider that the persistence of these drugs varies according to the species, Berendsen et al. (2018) evaluated the persistence of a wide range of antibiotics during the storage of calf, pig and broiler chicken droppings, and found that tetracyclines were moderately persistent or very persistent in calf and broiler chicken manure and slightly less persistent in pig manure. In contrast, sulfonamides showed a high withdrawal rate in most manure samples, with a half-life between 0.2 and 30 d overall (Berendsen et al., 2018).

Previous studies have analyzed antimicrobial depletion in broiler chicken droppings (Cornejo et al., 2018; Yévenes et al., 2018). Yévenes et al. (2018) indicate that residues of FF, SCP, CTC and 4-epi-CTC persist in broiler chicken droppings and could be a risk to public health. The authors reported that the longest excretion period was obtained for CTC, a tetracycline, for which the highest concentrations were also detected after the end of the treatment with therapeutic doses. In the present study, the highest concentrations were also observed for an antibiotic of the tetracycline family, OTC and its epimer, which even exceeded the concentrations of the other antimicrobials by more than 10 times at the same post-treatment day. Additionally, an increase of excretion of OTC and its epimer was observed throughout

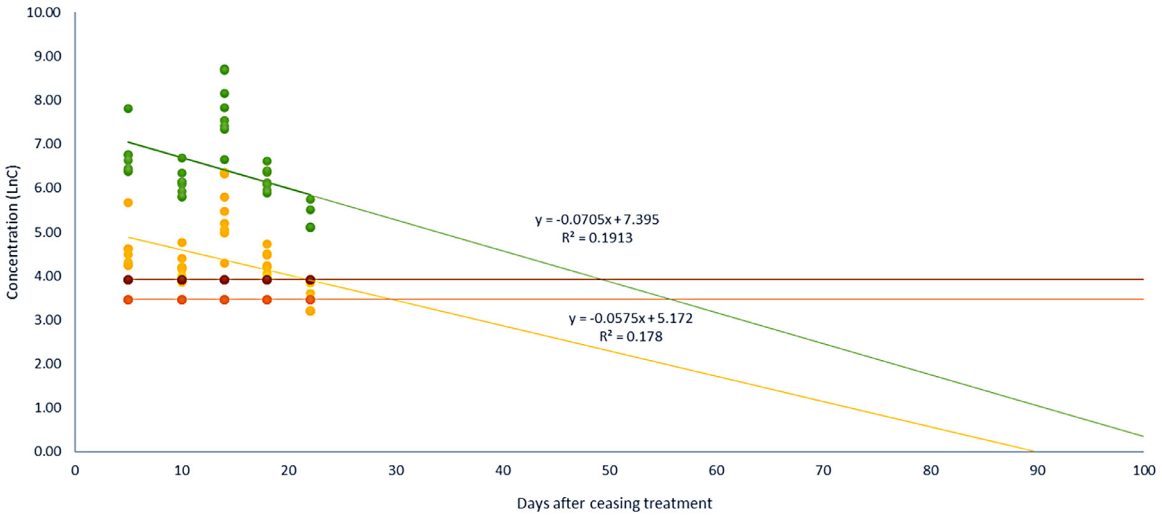


**Figure 4.** Projection of enrofloxacin and its metabolite concentrations detected in broiler chicken droppings during the post-treatment period. Green: concentrations in LN considering 95% confidence; yellow: concentrations in LN; orange tree: LOQ of the analytical methodology for enrofloxacin (32 $\mu$g kg$^{-1}$); dark red: established limit of 50 $\mu$g kg$^{-1}$.

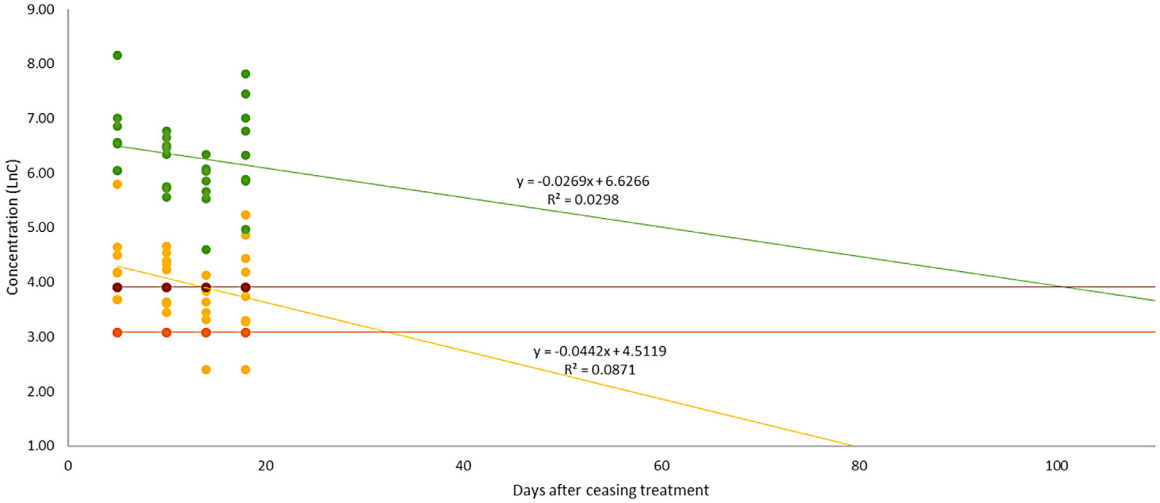


**Figure 5.** Projection of tylosin concentrations detected in broiler chicken droppings during the post-treatment period. Green: concentrations in LN considering 95% confidence; yellow: concentrations in LN; orange tree: LOQ of the analytical methodology for tylosin (21.9 $\mu$g kg$^{-1}$); dark red: established limit of 50 $\mu$g kg$^{-1}$.

the samplings, which may be due to the fact that these antimicrobials are absorbed quickly and completely in the gastrointestinal tract, presenting a variable binding to plasma proteins. In addition, they are distributed throughout all tissues and have a high affinity for bone tissue. They are partially metabolized throughout the body, being eliminated through urine and feces (Vicente and Pérez-Trallero, 2010). These characteristics could explain why the concentrations detected would not correlate with those of edible tissues measured in other studies, in which a depletion of residues is observed in muscle, liver and even feathers (Cornejo et al., 2017).

Detected concentrations of FF were higher than 100 $\mu$g kg$^{-1}$ on almost all samplings, and only on the last sampling point (d 22 post-treatment) were traces found, below the LOD. In previous studies by Yévenes et al. (2018) the concentrations of this antibiotic decreased more rapidly, being below the LOD (50 $\mu$g kg$^{-1}$) on d 15 post-treatment.

For TYL, an alternation of decrease and increase of concentrations was observed. TYL absorption is relatively poor orally (Gutiérrez et al., 2018), which would explain why high concentrations were detected on the first sampling point (d 5 post-treatment). Then, there was a decreasing trend in concentration on the second and third sampling points and then a slight increase on the fourth sampling point. However, at 22 d post-treatment, it was only possible to detect TYL residues in one of the samples below the LOQ (32 $\mu$g kg$^{-1}$), which was registered as not detected. Fluctuations in TYL concentrations during d 14 and 18 posttreatment (Table 3) could be related with the metabolism and recirculation of this antimicrobial from other tissues of the bird, such as liver or fat, because like OTC, TYL is a lipophilic antibiotic (Ozdemir et al., 2018).

EFX and its metabolite CFX showed a marked increase on the third sampling point (d 14 post-treatment), increasing to more than double the concentrations obtained on the first sampling point (d 5 post-treatment). Unlike our results, Slana et al. (2014) studied the EFX pattern and indicated that after the end of the treatment, from d 8 onward, no metabolites were observed in the excreta; however, EFX was continuously excreted until the end of the observation. The increased concentration of EFX and CFX in droppings can be attributed to metabolization and recirculation to other tissues and compartments of the bird as muscle and feathers (Martin et al., 2007; Sampaio de Assis et al., 2016). Peng et al. (2016), analyzed CFX in layer chicken manure and concluded in their risk assessment study that CFX in this waste will not cause environmental risk after a withdrawal period of 28 d. Nevertheless, in our study we estimate through a linear regression analysis that at 56 d the concentration of both analytes, EFX and CFX will be below the 32 $\mu$g kg$^{-1}$ (LOQ in matrix of EFX), considering a confidence of 95%.

On the other hand, SCP showed a decrease during the post-treatment period, these results are consistent with those described by Yévenes et al. (2018).

Due to the decrease in the detected concentrations of SCP, TYL, EFX and CFX, a projection was carried out using linear regression, with 95% confidence and considering the LOQ of the analytical methodology as the cut-off point. It was obtained that at d 50, 132 and 56 post-treatment, the concentrations of SCP, TYL and EFX +CFX would be equal to or less than 22.2, 21.9, and 32 $\mu$g kg$^{-1}$, respectively. It was not possible to project the depletion of FF and OTC residues because they did not show a progressive decrease, but rather decreased and increased throughout the sampling points.

The present research show through a controlled study, that different antimicrobials commonly used in poultry industry were eliminated in high concentrations and for a prolonged period through chicken droppings, treated with therapeutic doses of pharmaceutical formulations. Thus, our results demonstrate that this matrix should be considered as a possible

route of antibiotic transfer to the environment such as water and agricultural soil.

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## DISCLOSURES

The authors declare no conflicts of interest.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2021.101313.

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