



Liquid Chromatography-Tandem Mass Spectrometry Determination and Depletion Profile of Chlortetracycline, Doxycycline, and Oxytetracycline in Broiler Chicken Muscle After Oral Administration

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Abstract

A simple and fast method based on liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) was developed and validated for determination of tetracyclines in broiler chicken muscle. Sample preparation was performed using extraction with acetonitrile, followed by low-temperature purification (at $-20\text{ }^{\circ}\text{C}$) and further concentration. The chromatographic analysis was carried out using a Zorbax SB-C18 column with gradient elution using water and methanol both acidified with 0.025 M of formic acid. Mass spectrometry with electrospray ionization was operated in positive polarity using selected reaction monitoring (SRM) analysis mode, achieving the requirements of four identification points for each compound. Demeclocycline was used as internal standard. The method validation was done according to the criteria of Commission Decision 2002/657/EC. Parameters such as recovery, matrix effects, selectivity/specificity, linearity, precision (intra- and inter-day precision), accuracy, limits of detection (LOD) and quantification (LOQ), decision limit ($CC\alpha$), detection capability ($CC\beta$), and robustness were determined. Intra-day precision values were within the range 2.2–5.8% and inter-day precision was less than 10% for all analytes. Accuracy ranged from 98.2 to 103.2%. The method was successfully applied for depletion studies of chlortetracycline, doxycycline, and oxytetracycline in broiler chicken tissues after multiple oral administrations. After the depletion studies, the present study support more prudent use of CTC, DOX, and OTC for treatment of chickens and suggest a dose of 60 mg kg^{-1} body weight for CTC and OTC and 20 mg kg^{-1} body weight for DOX, orally administrated for five consecutive days.

Keywords LC-MS/MS · Tetracyclines · Depletion · Low-temperature purification · Validation · Withdrawal time

Introduction

In the last years, Brazil has established itself as one of the largest producers of animal protein in the world, mainly based on cattle and poultry farming. The quality of Brazilian poultry products is reflected by increased domestic consumption and high export volumes (Associação Brasileira de Proteína Animal | ABPA Annual Report 2016). The massive production

of poultry generally requires large use of veterinary products such as feed with additives and veterinary drugs. Antibiotics are routinely used in poultry production to enhance growth and feed efficiency through the increase of food conversion ratio and also to prevent and treat diseases. Notwithstanding, this model of production has brought concerns. For instance, it has been demonstrated that the misuse of antibiotics in animal production can lead to the development of antibiotic-resistant bacteria in animals. This resistance could affect human health through the direct or indirect transmission, through the ingestion of contaminated food, or through the environment (Donoghue 2003; Castanon 2007).

Several classes of antibiotics are available for poultry in Brazil, such as tetracyclines, fluoroquinolones, and sulfonamides. Tetracyclines (TCs) are a group of antibiotics widely used in animal industry, both for prophylaxis and for growth promoting. When TCs were administered into food-producing

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animals without the restrict observance of the withdrawal period, residues of these drugs can remain in edible tissues such as muscle, fat, skin, liver, and kidneys. The ingestion of these residues can lead to several adverse effects under human health, such as food-induced allergies and, in long-term exposure, can lead to bacterial resistance development.

TCs are generally administered to poultry by direct addition to feed or water. In the case of poultry, the oral administration of TCs includes all birds of the same husbandry system. In Brazil, the mainly husbandry system for poultry farming is the battery cages system (Gustafson and Kiser 1985; Chopra et al. 1992; Botsoglou and Fletouris 2001; Chopra and Roberts 2001). Notwithstanding, the use of TCs to promote the growth of broiler chickens has been prohibited since 2009 in Brazil. In Europe, cattle and broiler chicken industries voluntarily stop the utilization of all antibiotic as growth promoters (in 1998) followed by swine industry (1999). An act of Europe Union to ban all antibiotics as growth promoters went into effect in 2006 (Granados-Chinchilla and Rodríguez 2017).

TCs are effective against a broad spectrum of Gram-positive and Gram-negative bacteria, *Mycoplasma*, *Chlamydochila*, and *Rickettsia* spp. (Gustafson and Kiser 1985; Botsoglou and Fletouris 2001). These drugs are absorbed moderately well by the digestive system in mammals, but the absorption extension may be lower in birds (Anadón et al. 1994).

To ensure the safe therapeutic use of TCs in food-producing animals, strict regulatory frameworks have been established to control the use of these and other classes of antimicrobials and antibiotics, to minimize the risks to human health associated with consumption of these drug residues (Anadón et al. 2012). Maximum residue limits (MRL) have been established in several countries, and regulatory agencies constantly monitor the food supply to ensure that TC residue concentrations do not exceed the MRL. Brazil and Japan have established MRLs of 200 $\mu\text{g kg}^{-1}$ for chlortetracycline (CTC) and oxytetracycline (OTC) in poultry muscle, whereas the European Union (EU) established an MRL of 100 $\mu\text{g kg}^{-1}$ for both compounds. The MRL for doxycycline (DOX) is 50 $\mu\text{g kg}^{-1}$ in Japan and 100 $\mu\text{g kg}^{-1}$ in Brazil and the EU (The Japan Food Chemical Research Foundation 2017; European Commission 2010; Ministério da Agricultura, Pecuária e Abastecimento 2014).

In order to correctly determine the MRL for a drug, well-designed depletion studies must be performed. Analytical methods with high specificity and low detection limits are necessary for this purpose. Depletion of drugs from food-producing animals must be assessed in order to determine the time needed before the antibiotic disappears from animal tissue and to assess in a definitive way when the treated animal can be safely consumed (Anadón et al. 2008; European Medicines Agency 2017a). To deliver food products that are safe for human consumption, withdrawal times for drugs given to food productions animals must be observed (KuKanich et al. 2005). The withdrawal time is considered as the time

needed after the completion of treatment for tissue concentrations of the drug and any metabolites to decrease to less than the tolerance value or safe concentration for human consumption (Riviere et al. 1998).

The determination of TCs could be an analytical challenge, due to the properties of TCs to chelate metals and its epimerization (Martins et al. 2015). Several analytical methods have been reported for TCs analysis in animal tissues. For screening and semi-quantitative purposes, bioactivity-based methods such as fast antimicrobial screening test (FAST), Premi-Test, and others were described (Hoff et al. 2012), but a quantitative and confirmatory post-analysis is still necessary due to the lack of specificity of these techniques. Recently, for both residues monitoring in food as well as depletion studies, methods based on mass spectrometry have been extensively adopted due their intrinsic high selectivity and the very low detection levels achieved. Regarding depletion studies, some reports describe adequate times to predict that TC concentrations in chicken muscle were below the MRLs established by the EU (100 $\mu\text{g kg}^{-1}$): 5 days for DOX and OTC, and 3 days for CTC (Gustafson and Kiser 1985; Anadón et al. 1994, 2012; Capolongo et al. 2002).

Moreover, sample preparation is critical for TCs analysis. Despite some new protocols recently published (Udalova et al. 2015; Mookantsa et al. 2016), the mainly technique is the use of buffers and solid-phase extraction (SPE) (Zhang et al. 2016; Patyra and Kwiatek 2017).

Despite the widespread use of TCs in Brazil, few studies have investigated residual muscle depletion in broiler chickens and analyzed drug residues by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Gajda et al. 2013; Cornejo et al. 2017b). The objective of this work was the development and validation of a LC-MS/MS method to determine TCs residues in poultry muscle and apply this method to evaluate the rate of depletion of CTC, DOX, and OTC in healthy chicken muscle after multiple oral administrations of these drugs and to compare the results with the established standards of Brazilian, EU, and Japanese laws.

Materials and Methods

Reagents and Chemicals

Analytical standards of oxytetracycline (OTC), chlortetracycline (CTC), doxycycline (DOX), and demeclocycline (DEM, internal standard) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were $\geq 90\%$ of purity. Acetonitrile, methanol, and formic acid (all of HPLC grade) were purchased from J. T. Baker (São Paulo, Brazil). Ultra-pure deionized water was produced by a Milli-Q apparatus (Millipore, Bedford, MA, USA). Disodium ethylenediaminetetraacetate (Na_2EDTA) was

obtained from Merck (Darmstadt, Germany). Nylon filters (Captiva Premium syringe filter nylon 0.45 μm) were obtained from Agilent Technologies (USA).

Animals and Samples

For depletion studies design, an adaptation of Anadón et al. procedure was adopted (Anadón et al. 1994, 2012). Briefly, a total of 168 Cobb 500 broiler chickens were housed in boxes maintained at room temperature with controlled humidity and ad libitum access to water and a drug-free feed to allow the complete elimination of any previous antimicrobial treatment. This preventive withdrawal period was carried out from the day zero until day 27, when the animals were assigned to one of three groups on the basis of individual weight, with 48 animals in each group according to the antibiotic used for the treatment, and with 24 animals in the negative control group. Starting on day 28, each bird received DOX, CTC, or OTC—purchased in local stores—for five consecutive days at doses of 20, 60, and 60 mg kg^{-1} body weight, respectively, which corresponded at recommended treatment doses (Anadón et al. 1994, 2012). The average weight of each group was used to calculate the dosage of each antibiotic given to each broiler chicken. The three drugs were administered orally every 24 h, and food was removed during in the period between 12 h before to 6 h after the TCs administration. The antibiotic solutions for oral administration were prepared daily by dissolving each drug in water and storing protected from light. Micropipettes were used to administer the antibiotic directly into the esophagus.

In agreement with the guidelines of the European Agency for Medicines (European Medicines Agency 2017b), four chickens of each treated group and two chickens of the control group were slaughtered after CO_2 stunning at 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, and 240 h after treatment. Muscle samples (breast) were collected from each animal and individually stored at $-20\text{ }^\circ\text{C}$ until the moment of sample extraction and further chromatographic analysis. The study was undertaken in accordance with ethics requirements and authorized by the official ethical committee of Federal University of Santa Maria, number 061/2014. All efforts were made to minimize suffering of animals. Breast was chosen as target tissue considering that is one of the most consumed meat in Brazil (Bacchi and Spolador 2002). The withdrawal times were calculated using the Withdrawal-Time Calculation Program WT 1.4 software, developed by Dr. P. Hekman and available at www.ema.europa.eu/ema/pages/includes/document/open_document.jsp?webContentId=WC500077921.

Sample Preparation

Each sample of chicken muscle was chopped and homogenized using a food mixer. All apparent fat was previously manually

removed. Aliquots of $2 \pm 0.1\text{ g}$ were exactly weighed into a 50-mL polypropylene centrifuge tube and 400 μL of a 150 mM Na_2EDTA solution was added to each tube, in order to avoid the chelation of TCs. All samples were fortified with the internal standard solution (DEM), except the double blank sample (quality control sample). Matrix-matched calibration curves were prepared with blank samples fortified with DEM and with the pool standard solution containing all analytes (CTC, DOX, and OTC). The curve point corresponding to $0.0 \times \text{MRL}$ was fortified only with DEM. Other calibration curve points were 0.25, 0.50, 1.0, 1.5, and $2.0 \times \text{MRL}$. Before to start the extraction procedures, fortified samples were mixed in a vortex by just few seconds (3–5 s) and were allowed to stand for 10 min in a dark room at room temperature. After that, 800 μL of ultra-pure water was added to each tube and the samples were mixed again using a vortex (10 s) and immediately placed in a refrigerator at $4\text{ }^\circ\text{C}$ for 30 min.

TCs were extracted from the tissue using 6 mL of acidified ACN with 0.025 M formic acid. After the addition of the solvent, samples were mixed in a vortex for 2 min and then placed in an orbital shaker for 10 min at 180 rpm. Following, tubes were centrifuged at $3100 \times g$ for 10 min at $4\text{ }^\circ\text{C}$, and the supernatant was transferred to a 15-mL polypropylene centrifuge tube and then placed in a freezer ($-20\text{ }^\circ\text{C}$) for 1 h. After the low-temperature cleanup, samples were centrifuged again at the same conditions described before. After centrifugation, the upper layer was transferred to a glass tube and the sample was concentrated to dryness in a water bath aided by a gentle nitrogen flow. Dry extracts were reconstituted with 1 mL of water/methanol (70:30) and transferred to a microcentrifuge tube and centrifuged at $20,800 \times g$ for 10 min at $4\text{ }^\circ\text{C}$. Finally, the supernatant was filtered using a nylon filter (0.45 μm), and 5 μL was injected into the LC-MS/MS system for analysis.

Chromatography and Mass Spectrometry Conditions

Analytical determinations were performed using an HPLC Agilent 1200 Series (Agilent Technologies, USA) coupled to a mass spectrometer 4000 QTRAP (Sciex, Canada) equipped with an ESI interface. Instrument control and data processing were performed using the Analyst 1.5.1 software. Chromatographic separation was achieved using a Zorbax SB- C_{18} LC column ($50 \times 4.6\text{ mm}$, 1.8 μm particle diameter), with a C_{18} column guard ($12.5 \times 4.6\text{ mm}$, 5 μm particle diameter), both purchased from Agilent Technologies (USA). Mobile phase was used in gradient mode, composed by a binary system: water (solvent A) and methanol (solvent B), both acidified with 0.025 M of formic acid. The gradient elution program was as follows: 0–1 min, isocratic step with 5% B; 1–7.5 min, gradient to 99% B; 7.5–9 min, isocratic step with 99% B; 10 min, returned to initial composition of 5% B. The flow rate was 1 mL min^{-1} .

The ion source-dependent parameters were optimized using the following flow injection analysis (FIA) approach: curtain gas (CUR) at 15 psi, collision-activated dissociation gas (CAD) was set to medium, source temperature at 650 °C, dry gas 1 (GS1) and dry gas 2 (GS2) at 45 psi, and the ion spray voltage was set to 5000 V. Detection was performed in positive ion mode using selected reaction monitoring (SRM), which monitors two transitions for each analyte. The typical retention times and the optimized compound-dependent parameters of the LC-MS/MS method were showed in Table 1.

Method Validation

The method was validated according to the guidelines proposed by the European Commission Decision 2002/657/EC and the Brazilian Analytical Quality Assurance Manual for veterinary drug residues determination (Brasil. Ministério da Agricultura Pecuária e Abastecimento *n.d.*; Commission of the European Communities 2002). Validation pack includes the following figures of merit: recovery, selectivity/specificity, linearity, precision (expressed as intra- and inter-day precision), accuracy, matrix effects, and method sensitivity according to LOD, LOQ, CC α , CC β , and robustness.

For DOX, Brazil adopts an MRL value of 100 $\mu\text{g kg}^{-1}$. However, considering the fact that TCs exhibit several values of MRL, depending on matrix specie or the analyte, the validation study was performed assuming an action level of 200 $\mu\text{g kg}^{-1}$ for all analytes.

Linearity was evaluated using matrix-matched calibration (MMC): blank samples of chicken muscle were fortified with six concentration levels plus a zero point (20, 50, 100, 200, 300, and 400 $\mu\text{g kg}^{-1}$) for all analytes. Samples were extracted and analyzed by LC-MS/MS. The concentration range covers the MRLs established by Brazilian legislation for the three TCs.

Selectivity/specificity was assessed using chicken muscle samples ($n = 21$) obtained from different producers, in order to identify possible interferences. The use of two transitions for each analyte as the identification points required by European Commission Decision 2002/657/EC was adopted in the presented method to provide a high degree of specificity (Commission of the European Communities 2002).

LOD and LOQ were determined by an experimental approach in which decreasing concentrations of each antibiotic were spiked in blank muscle samples, the samples were extracted using the developed method and then analyzed by LC-MS/MS. LOD and LOQ were firstly estimated using a signal-to-noise ratio (S/N) in which the signal must be 3 and 10 times higher than the noise, respectively. However, these results were considered unrealistic (very low concentrations). Thus, the method chosen to establish LOD and LOQ was the analysis of fortified samples.

Recovery and intra-day precision (repeatability) were evaluated by spiking blank samples at three concentration levels (0.5, 1.0, and 1.5 \times MRL), and analyzing seven replicates for each concentration level in 1 day. Inter-day precision was evaluated by spiking blank samples with the same concentration levels on three different days. The precision values were calculated as coefficients of variation (CV%). The method accuracy was estimated by a recovery test using these same samples at 0.5, 1.0, and 1.5 \times MRL, through the comparison between the theoretical concentrations versus the calculated concentration. The accuracy acceptance limits were between 80 and 110%, as proposed by the European Commission Decision 2002/657/EC for concentrations higher than 10 $\mu\text{g kg}^{-1}$ (Commission of the European Communities 2002).

To evaluate the matrix effects, calibration curves were prepared with and without the presence of matrix, in four replicates. The average response of the analytes from each calibration curve (both in the same concentration range) were compared using the statistical *F* test (Fisher-Snedecor) for variance homogeneity and the *t* test at 95% significance for evaluating the variance and means between the slopes of the calibration curves. To assume that the matrix effect is not significant, there should be no matrix effect at any concentration level of the calibration curve (Ministério da Agricultura, Pecuária e Abastecimento *n.d.*).

The CC α and CC β values were obtained by plotting all data calculated in the precision experiments and applying the calibration curves approach as proposed in the European Commission Decision 2002/657/EC (Commission of the European Communities 2002). In few words, the signal was plotted against the added concentration, and the corresponding concentration at the *y*-intercept plus 1.64 times the standard deviation of the within-laboratory reproducibility give

Table 1 Mass spectrometry conditions and retention times for selected compounds

Analyte	Precursor ion (m/z) [M + H] ⁺	Product ions (m/z) ^a	Declustering potential (V)	Collision energy (V) ^a	Cell exit potential (V) ^a	Retention time (min)
OTC	461.1	426.2/201.1	56	29/59	12/10	5.07
CTC	479.1	444.0/462.0	61	33/30	24/12	5.82
DOX	445.1	428.0/267.0	66	37/49	10/14	6.34
DEM	465.0	448.2/154.0	61	27/43	12/10	5.39

^a Numerical values are given in the order quantifier/qualifier ion

$CC\alpha$ values. $CC\beta$ was calculated by summing the concentration at the $CC\alpha$ and 1.64 times the standard deviation of the within-laboratory reproducibility obtained at the MRL concentration level.

Method robustness was evaluated using Youden's test. In Youden's test, seven method parameters are selected to suffer minor and major changes. Through eight samples analysis, the impact of each factor in the determinations can be statistically assigned. Briefly, blank muscle samples were spiked at the MRL level for each analyte and then analyzed. For each sample, a factor was changed (minor or major changes). Table 2 summarizes the factors chosen and changed ($n = 7$). Three replicates were analyzed in each experiment.

Applicability

Applicability is a very important parameter that is not always included in method development reports. It must be evaluated with real samples, contaminated or not. Through its experimentation, it is possible to check the real feasibility of the method. In our study, for applicability purposes, 65 chicken muscle samples (breast) were obtained from a local slaughterhouse, collected in different days and analyzed.

Drug Quantification in Muscle Depletion Study

Polynomial regression analysis of the logarithmically transformed data was used to calculate CTC, DOX, and OTC depletion. The regression analysis equation of the matrix-matched calibration curves ($r^2 > 0.99$) was calculated at different concentrations to avoid having to extrapolate the results. The concentration range used for the matrix-matched calibration curves were 0, 10, 20, 50, 100, 200, 300, and 400 $\mu\text{g kg}^{-1}$. The concentration range of the curve used for 24 h post-treatment quantification was 0, 50, 100, 200, 300, 400, and 600 $\mu\text{g kg}^{-1}$. This curve was used to quantify the TC residues at 24 h after administration, including a higher point (600 $\mu\text{g kg}^{-1}$) to ensure that all samples fall within the linearity range. In the case of samples until 6 h after drug administration, considering the high concentration of the compounds in these samples, the extracts were diluted before injection.

For these samples, the second concentration range curve was 0, 50, 100, 200, 300, and 400 $\mu\text{g kg}^{-1}$. This matrix-matched calibration curve was also submitted to the same process of dilution applied to samples (1:10 dilution) to maintain the matrix characteristics and the ratio internal standard/analytes standard.

Data Analysis

The depletion results were estimated by polynomial regression analysis of log-transformed concentrations detected in chicken muscle. Each value was the mean \pm SD for four broiler chickens. Statistical analyses were performed using the Statgraphics Centurion statistical program (Statgraphics Centurion 15.1.14, Manugistics Inc., Rockville, MD, USA).

Results and Discussion

Optimization of Analytical Method

An adequate sample preparation method for residues analysis of veterinary drugs in animal products must have consider the time required for analysis, sample throughput, toxicity, extraction efficiency, and costs. For the determination of TC residues, sample preparation is tremendously critical. TCs are amphoteric molecules, which have two ionizable groups. Thus, besides the high affinity by water and polar solvents, TCs are generally in ionic form. The most stable form is the zwitterionic state, when both ionizable groups are charged. TCs are very sensible to degradation under strongly acidic or alkaline mediums, and are leaning to form anhydro-, iso-, or epi-analogues under these conditions. Moreover, TCs also show photodegradation, keto-enol tautomerism, tendency to form chelation complexes with multivalent cations (e.g., metals), binding with proteins, and interactions with charged silanol groups. All these factors can convert any method for TCs determination in a challenging quest. To avoid some of these problems, analysts developed several strategies, such as the addition of EDTA at the beginning of the extraction procedure to minimize the interaction for the TCs with chelating

Table 2 Nominal conditions and variations for muscle samples evaluated in the Youden design

Number	Factors	Nominal condition	Variation
1	Batch of methanol	Batch A	Batch B
2	Additive in mobile phase A	Formic acid 0.1%	Formic acid 0.05%
3	Additive in mobile phase B	Formic acid 0.1%	Formic acid 0.05%
4	Time of low temperature for protein precipitation	- 20 °C at 1 h	- 20 °C at 45 min
5	Column temperature	35 °C	32 °C
6	Source temperature	650 °C	620 °C
7	Flow rate	1 mL min ⁻¹	0.98 mL min ⁻¹

agents, which are abundant in biological samples (Gentili et al. 2005; Bittencourt et al. 2012). To avoid epimerization and other degradation products, HPLC analysis of TCs residues commonly uses buffers as extraction solvent and mobile phase. However, the use of buffers with non-volatile salts is not recommended in mass spectrometry. In the present work, the use of 0.025 M of formic acid provide a slightly acidic condition in which TCs can be analyzed as single peaks without the need to sum parent compounds plus epimers.

The properties of the selected extraction solvent are also crucial. Granelli et al. studied a single extraction of bovine and porcine muscle and kidney samples using methanol (Granelli et al. 2009). Granelli and Branzell showed that methanol and acetonitrile provided the best results for multiresidue analysis in the muscle and kidney (Granelli and Branzell 2007). However, for TCs analysis, methanol should be not the better choice, since TCs can suffer methanolysis. Acetonitrile provides very efficient extraction with high extraction recoveries, minimizes co-extraction of lipids, lowers matrix effects, and is efficient for protein denaturation (ChiaoChan et al. 2010). Thus, for the present method, ACN was chosen as extraction solvent.

After extraction, the application of one or more cleanup techniques is almost always necessary in the analysis of pharmaceuticals residues. Although solid-phase extraction (SPE) is a predominant approach, simple and cheap protocols based on techniques such centrifugation and low-temperature phase separation can achieve similar results (Lopes et al. 2011; Rübensam et al. 2013; Martins et al. 2014, 2015). In the present study, these both techniques were associated and they are effective to obtain the precipitation and removal of matrix proteins and co-extracted compounds. Low-temperature purification (LTP) has been used in ultra-low temperatures, by frozen the aqueous fraction through the immersion of tubes in liquid nitrogen (Lopes et al. 2011; Berendsen et al. 2013). In this technique, the immersion time must be rigorously controlled to avoid the freezing of ACN. Moreover, the use of liquid nitrogen adds complexity and costs to the protocol. Therefore, the LTP was performed by keeping the samples in an ordinary freezer ($-20\text{ }^{\circ}\text{C}$), equipment present in any laboratory. After the LTP, extracts were filtered through a nylon filter ($0.45\text{ }\mu\text{m}$) to remove any suspended particles.

The use of centrifugation is also a critical point: the first centrifugation is performed after the mixing of sample and the extraction solvent and before LTP. If that first centrifugation was not performed, the resulting extracts would be too turbid. After LTP, another centrifugation is necessary to aid the precipitation of proteins and other compounds. Finally, after the sample concentration and reconstitution with solvent, a third centrifugation was used to avoid the presence of particulate material which can remain from the evaporation step.

The use of formic acid as an additive for the mobile phase was chosen not only by an adequate ionization of the analytes

but also by its volatility and the compatibility with TCs. The mobile phase was used in gradient mode to assure an adequate resolution for the target compounds and also to minimize the interferences from the matrix co-eluting compounds (Martins et al. 2015).

The protein precipitation and cleanup steps were effective, which was demonstrated by the lack of significant signal enhancement or suppression and the demonstration of no interference in the MS/MS detection that could affect the determination. It can be assumed that the matrix components responsible for possible interference were effectively removed. However, the matrix effect undergoes variations over time and also depends on the condition of the instrument used. So, it should be constantly evaluated both in the development stage of the method and in the application of this method in routine analysis (Prestes et al. 2009). In our studies of applicability of the method and depletion, we have chosen to analyze the samples using calibration curves in the matrix in order to ensure that there was no interference of the matrix throughout the process.

The results obtained from a MRM chromatogram of blank and spiked muscle samples at the MRL level are shown in Fig. 1. Individual extracted ion chromatograms for TCs are shown in Fig. 2. Several experiments were conducted to evaluate different solvents and additives in the mobile phases. ACN and methanol were evaluated and better peak resolution and sensitivity were obtained when methanol was used as organic solvent. The gradient program was optimized to achieve optimal separation of all compounds in less than 10 min. Parameters such as flow rate, column temperature, and injection volume were optimized. The high percentage of organic solvent in the last minutes of the chromatographic run prevented potential carry-over effects.

For TCs residues analysis in food samples, the sample preparation methods are usually complex. Generally, much attention is given to preliminary sample preparation procedures. In the case of TCs, besides the intrinsic challenges for both cleaning and pre-concentration of substances present in trace level, the specific characteristics of this group of antibiotics lead to laborious protocols. Solid-phase extraction is commonly applied to achieve an adequate extract and several schemes of SPE have been reported for TCs analysis in food samples in recent years, such as new solid phases (Wu et al. 2017), dispersive SPE (Mookantsa et al. 2016), or the use of molecularly imprinted SPE materials (Önal 2011; Feng et al. 2016).

The present sample preparation method is a compromise between simplicity and suitability: although SPE produces more clear extracts; the LTP results in a very adequate cleanup. Furthermore, the absence of SPE is a clear advantage, since SPE requires the full attention of an analyst, unless for fully automated SPE systems, which are expensive and require a detailed optimization. Moreover, at the time of the LTP development, the analyst is free to perform other tasks,

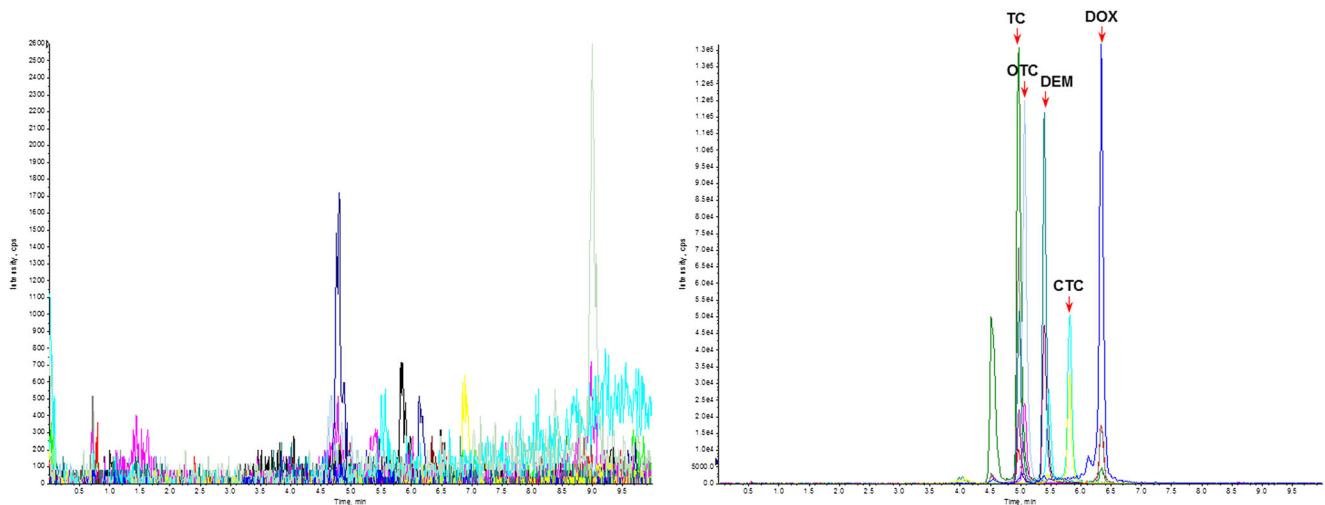


Fig. 1 Selected reaction monitoring (SRM) chromatograms for muscle blank sample (1) and blank sample spiked with tetracyclines at MRL level (2)

such as the preparation of analytical systems and system suitability injections. Recent published methods using SPE for TCs determination associated the use of SPE with previous buffer extractions. For instance, Zhang et al. reported a

multiresidue method for analysis of TCs plus sulfonamides and quinolones. The method is based on a two-step solid-liquid extraction with McIlvaine buffer and phosphate buffer. The combined extracts are applied to an Oasis HLB cartridge.

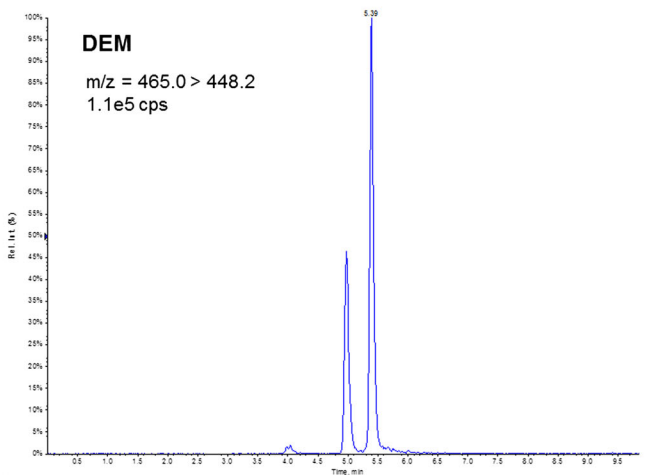
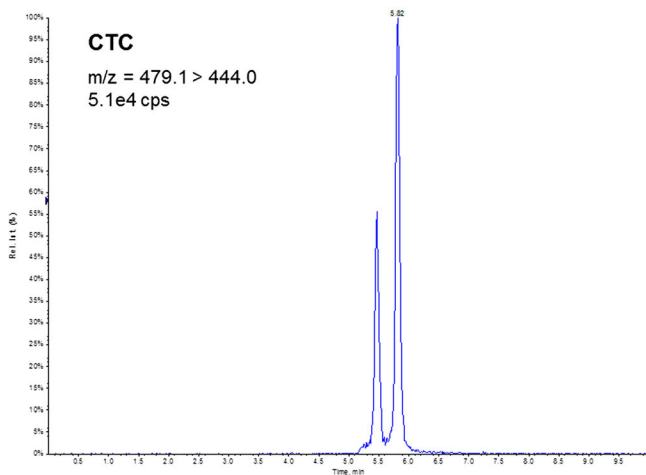
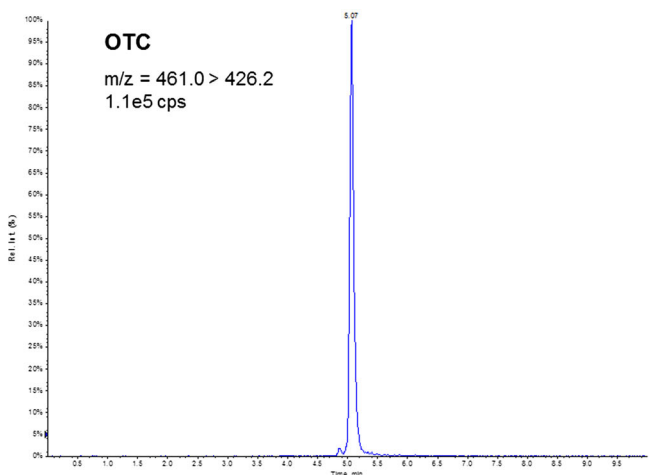
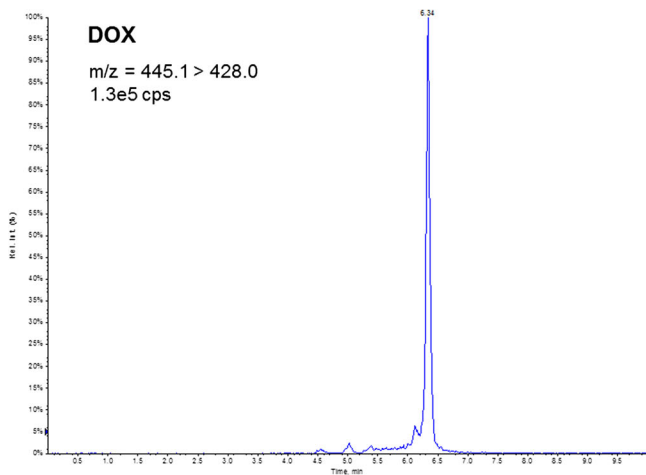


Fig. 2 Extracted ion chromatogram for muscle samples spiked in MRL concentration level for tetracyclines

The eluate is evaporated to dryness, reconstituted, filtered, and injected (Zhang et al. 2016). Pokrant et al. reported a method for chicken muscle analysis, where samples were extracted using 20 mL of EDTA/McIlvaine buffer and cleanup was performed using SPE (C18 cartridges). TCs were eluted with 10 mL of oxalic acid 0.01 M in methanol. Following, extracts were evaporated to dryness and reconstituted with 250 μ L of mobile phase. The resulting extract was then submitted to two sequential centrifugations steps before the injection in order to precipitate and discard particulate matter (Pokrant et al. 2018). The same research group has been reporting some depletion studies of TCs in claws and feathers of poultry, using similar techniques to sample preparation (Cornejo et al. 2017a, b).

Cetinkaya et al. have been reported a method for TCs residues determination in chicken meat using LC-MS/MS, with recoveries ranging from 56.9 to 101.2%. Sample preparation is performed by extraction with methanol followed by an extraction with 1% formic acid and EDTA. After that, samples are centrifuged, filtered, and directly injected into the LC-MS/MS system (Cetinkaya et al. 2012). Although these authors used two solid-liquid extraction steps, there is no need for additional cleanup except centrifugation and filtration.

LTP cleanup was also used by Ahmadi et al. These authors used a method for simultaneous cleanup and pre-concentration of four TCs in poultry tissues based on two phase freezing method: TCs were firstly extracted by Britton-Robinson buffer followed by extraction with ACN: Britton-Robinson buffer: KCl and water. The resulting extract was frozen and the organic phase was removed by centrifugation. The ACN extract is evaporated to dryness and reconstituted with 100 μ L of ACN, before the injection into the HPLC system (Ahmadi et al. 2015). One advantage of our present sample preparation method is the absence of buffers, which requires careful preparation.

Our overall goal was the development and validation of a sample preparation procedure based on a simple and fast solid-liquid extraction, followed by a static and inexpensive low-temperature cleanup step, without the need of SPE, pH adjustments, or ionic exchange.

Method Validation

The calculated $CC\alpha$ and $CC\beta$ values are shown in Table 3. Although these parameters do not include criteria for upper limits, the experimentally determined values were considered satisfactory. The decision limit was determined using batches containing 21 spiked samples, with each batch at a different concentration level, corresponding to 0.5, 1.0, and $0.5 \times$ MRL values for each analyte (Table 3).

The sensitivity of the method was determined by means of LOD and LOQ for each drug. The results obtained are shown in Table 3. The CV% for six replicates in the LOQ value was below 15% for all analytes. Blank poultry muscle samples

($n = 20$) were analyzed to evaluate the presence of interferences. The method was considered as specific for all studied compounds because no significant interference was observed for any analyte.

Linearity was observed for all drugs with determination coefficients (r^2) higher than 0.99. Deviations of individual points from the calibration curve were less than 15%, and residuals were less than 20%. The method showed average recoveries ranging from 98.2–103.2%, demonstrating its accuracy (Table 4). The results for intra-day precision (repeatability) were calculated by means of CV% and ranged between 2.2–5.8%. Inter-day precision (reproducibility) was less than 10% for all analytes. The method was considered reproducible since all CV values were less than 23%, which was the upper limit for the MRL level calculated using the Horwitz equation (Table 4).

No significant analytical differences were observed between the curves prepared in matrix versus those prepared in solvent. This result indicates that there is no significant matrix effect. Notwithstanding, all data was obtained using matrix-matched calibration curves to compensate analytes losses caused throughout the extraction process.

Experiments using the Youden approach were performed to evaluate the method behavior when subjected to minor and major changes: analyses were performed with the analytical conditions at their nominal value and with a slight variation (Table 2). No significant differences were observed between s_{factor} and s_{repro} , indicating that CTC, DOX, and OTC responses were robust for all factors evaluated, with $s_{\text{factor}} < s_{\text{repro}}$, as the following: DOX ($6.48 < 9.51$), CTC ($4.91 < 7.54$), and OTC ($5.35 < 6.11$). The factors which were evaluated were chosen based on the more frequent parameters evaluated for chromatographic methods (Karageorgou and Samanidou 2014).

For CTC, DOX, and OTC depletion studies, the calibration curves of spiked muscle samples were linear ($r^2 > 0.99$) and were used for quantification of the drugs in the samples.

The method applicability for real samples ($n = 65$) was satisfactorily performed. Regarding applicability purpose, chicken muscle samples were obtained from a local slaughterhouse and collected in on different days. Therefore, it was possible

Table 3 MRL, LOD, LOQ, $CC\alpha$, and $CC\beta$ values obtained for the studied analytes

Analyte	MRL ($\mu\text{g kg}^{-1}$)	LOD ($\mu\text{g kg}^{-1}$)	LOQ ($\mu\text{g kg}^{-1}$)	$CC\alpha$ ($\mu\text{g kg}^{-1}$)	$CC\beta$ ($\mu\text{g kg}^{-1}$)
CTC	200	10	20	223.02	246.03
DOX	100 ^a	10	20	223.04	246.07
OTC	200	10	20	220.42	240.84

^a For DOX, Brazil adopts an MRL value of 100 $\mu\text{g kg}^{-1}$, but the validation study was performed assuming an action level of 200 $\mu\text{g kg}^{-1}$ for all analytes

Table 4 Precision and accuracy of CTC, DOX, and OTC in chicken muscle

Analyte	Intra-day precision (CV%)			Inter-day precision (CV%)			Accuracy (%)		
	0.5 × MRL	1.0 × MRL	1.5 × MRL	0.5 × MRL	1.0 × MRL	1.5 × MRL	0.5 × MRL	1.0 × MRL	1.5 × MRL
CTC	2.3	5.8	5.4	2.9	6.5	5.5	98.5	103.2	98.3
DOX	2.2	4.5	5.5	3.2	6.2	5.8	98.4	102.2	98.2
OTC	2.5	3.3	5.6	2.9	4.2	5.6	98.6	102.6	98.3

the evaluation of the method to analyze samples obtained from different batches of broiler chickens, considering the variations, which that possibly may exist in the matrix composition. The results showed that no chicken muscle sample contained CTC, DOX, or OTC residues.

Several multiclass and multiresidue methods using LC-MS/MS have been published recently, covering dozens of veterinary drugs residues (Martins et al. 2015; Moretti et al. 2016). However, a generic sample extraction protocol is commonly a compromise between satisfactory responses and wide scopes. Some authors report the worst recoveries for TCs when generic approaches were applied (Martins et al. 2015; Moretti et al. 2016). Herein, we have chosen to develop, optimize, and validate a specific method for TCs, aiming to be simple and cheap, but showing the necessary fitness to purpose to be used in pharmacokinetics studies.

Depletion Study

Residues were detected in chicken muscle (breast) after oral daily administration. Mean tissue concentrations of CTC, DOX, and OTC were 1239.5, 3420.0, and 1295.0 $\mu\text{g kg}^{-1}$, respectively, at 6 h after the last dose of each drug. The concentrations of all the tested drugs in muscle were initially high and then declined over time. DOX had the highest concentration at 6 h after the last dose, but 48 h after the last dose, its concentration (130.3 $\mu\text{g kg}^{-1}$) was lower than that of OTC (152.8 $\mu\text{g kg}^{-1}$) (Table 5 and Fig. 3).

The mean CTC concentrations were below the LOD of our method (10 $\mu\text{g kg}^{-1}$) at 168 h after the last dose. By contrast, it was still possible to detect low concentrations of DOX (26.4 $\mu\text{g kg}^{-1}$) at 216 h after the last dose and OTC (22.4 $\mu\text{g kg}^{-1}$) at 240 h after the last dose. The greatest decay in the CTC concentration was observed at 6 and 12 h after the last dose (1239.5 to 372.0 $\mu\text{g kg}^{-1}$, respectively).

Doxycycline is the most lipophilic compound and shows the more extent rate of absorption among TCs (Anadón et al. 1994). In the present study, the depletion curves show that concentrations were below the Brazilian MRL at 24 h after the last dose for CTC, at 48 h after the last dose for OTC, and at 72 h after the last dose for DOX.

Previous studies have shown that DOX is readily available for distribution to tissues (Boecker and Estler 1981; Michel et al. 1984; Anadón et al. 1994). In the present study, high DOX concentrations were detected in muscle after oral dosing of 20 mg kg^{-1} daily for five consecutive days, substantial concentrations were still detected at 120 h after the last dose, and low concentrations were detected until 240 h after the last dose. Similar results were reported by Anadón et al., who treated chicken with DOX at 20 mg kg^{-1} daily for 4 days (Anadón et al. 1994). Doxycycline was eliminated slowly from chicken tissues, and the mean concentrations ranging from 28–200 $\mu\text{g kg}^{-1}$ persisted for 5 days after the last treatment dose. Due to its high lipophilicity, DOX was rapidly, but only partially, absorbed after oral administration. This drug has several important advantages over other TC analogs, including essentially complete absorption, good tissue penetration, and slower elimination so that only one dose is needed per day (Anadón et al. 1994).

Table 5 Muscle concentration of CTC, DOX, and OTC for broiler chickens treated orally with each these drugs at the rate of 60, 20, and 60 mg kg^{-1} body weight, respectively, for five consecutive days

Time after the last dose (h)	Residues ($\mu\text{g kg}^{-1}$)		
	CTC	DOX	OTC
6	1239.5 ± 759.3	3420.0 ± 987.4	1295.0 ± 167.4
12	372.0 ± 114.5	2125.0 ± 408.5	1017.8 ± 159.6
24	96.1 ± 42.2	493.0 ± 202.6	326.0 ± 74.3
48	38.8 ± 11.3	130.3 ± 17.5	152.8 ± 25.1
72	38.0 ± 18.1	60.9 ± 12.3	95.5 ± 16.5
96	21.2 ± 5.8	57.5 ± 16.0	46.8 ± 23.8
120	20.2 ± 15.2	45.7 ± 16.0	45.4 ± 2.3
144	< LOQ ^a	27.3 ± 15.1	38.3 ± 7.4
168	< LOD ^b	37.4 ± 19.0	35.9 ± 7.4
192	< LOD ^b	26.9 ± 5.8	25.0 ± 3.8
216	< LOD ^b	26.4 ± 6.3	24.4 ± 1.6
240	< LOD ^b	< LOQ ^a	22.4 ± 5.4

Each value is the mean ± SD for 4 broiler chickens

^a LOQ (limit of quantification) equivalent to 20 $\mu\text{g kg}^{-1}$

^b LOD (limit of detection) equivalent to 10 $\mu\text{g kg}^{-1}$

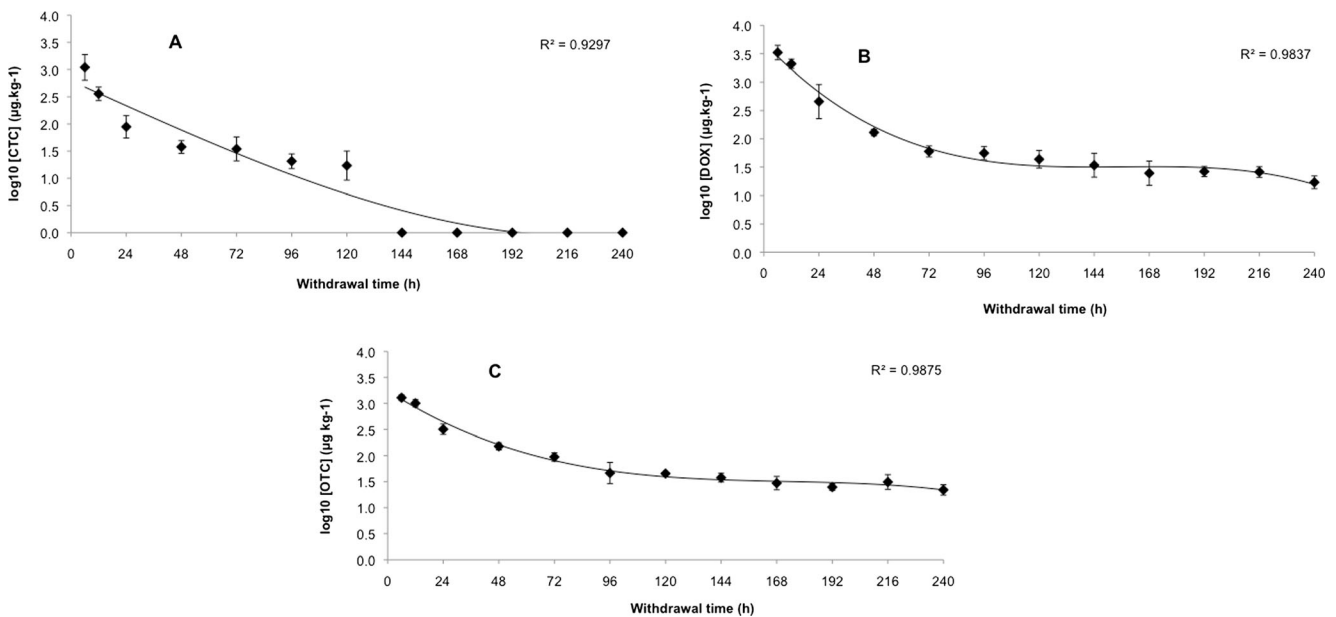


Fig. 3 Mean of concentrations of CTC (a), DOX (b), and OTC (c) in chicken muscle samples. Data represent mean \pm SD values for four broiler chickens

In the present report, CTC concentrations were the lowest of all three tested TCs from the beginning of the depletion study up to 240 h after the last dose. These observations can be explained by the drug pharmacokinetics. In a study of rats who received a single oral dose of 75 mg kg^{-1} body weight of CTC, plasma concentration declined from 2.1 to 0.8 mg L^{-1} at 6 h after the last dose (Berté and Vandoni 1962). The administration of CTC at a dose of 10.8 mg kg^{-1} body weight to broiler chickens by oral gavage resulted in a CTC blood level averaging 130 mg L^{-1} at 2 h and 200 mg L^{-1} at 6 h after dosing. At 24 h after dosing, the blood CTC levels in the majority of animals were less than 0.015 mg L^{-1} (Food and Agriculture Organization of the United Nations 1998).

Gingher (1988) administered 300 mg kg^{-1} CTC to chickens in their feed for 7 days. On the day of the last dose, the liver contained $328 \mu\text{g kg}^{-1}$ CTC and kidney tissues contained $2450 \mu\text{g kg}^{-1}$ CTC (Food and Agriculture Organization of the United Nations). Residues were not detected at 1 day after the last dose. When CTC was not detectable in the liver and kidney, CTC residues were absent from all other edible tissues including muscle (Food and Agriculture Organization of the United Nations 1998).

Considerable research has investigated the presence of OTC residues in eggs (Donoghue and Hairston 1999; Muñoz et al. 2014), milk (Anderson et al. 1995; Dinsmore et al. 1996), as well as fish (Paschoal et al. 2012), calf (Mawhinney et al. 1996), turkey (Capolongo et al. 2002), swine, and sheep tissues (Nouws et al. 1990). However, recent data are not available for OTC residues in chicken tissues. In the present study, OTC residues were detected at a level below the MRL established by Brazilian legislation ($200 \mu\text{g kg}^{-1}$) at 48 h after the last dose. We could detect low OTC concentrations until 240 h

after the last dose. Capolongo et al. administered 400 mg L^{-1} OTC to turkeys for 3 days in their drinking water and measured the residues by liquid chromatography with UV detection. The results showed that OTC residues were detected at very low concentrations in muscle tissue at 1 day after the last dose, and residues were far lower than the EU MRL values at 3 days after the last dose (Capolongo et al. 2002). These results may differ from the earlier study due to the higher sensitivity of our quantitation method (LC-MS/MS), which can detect very low drug concentrations.

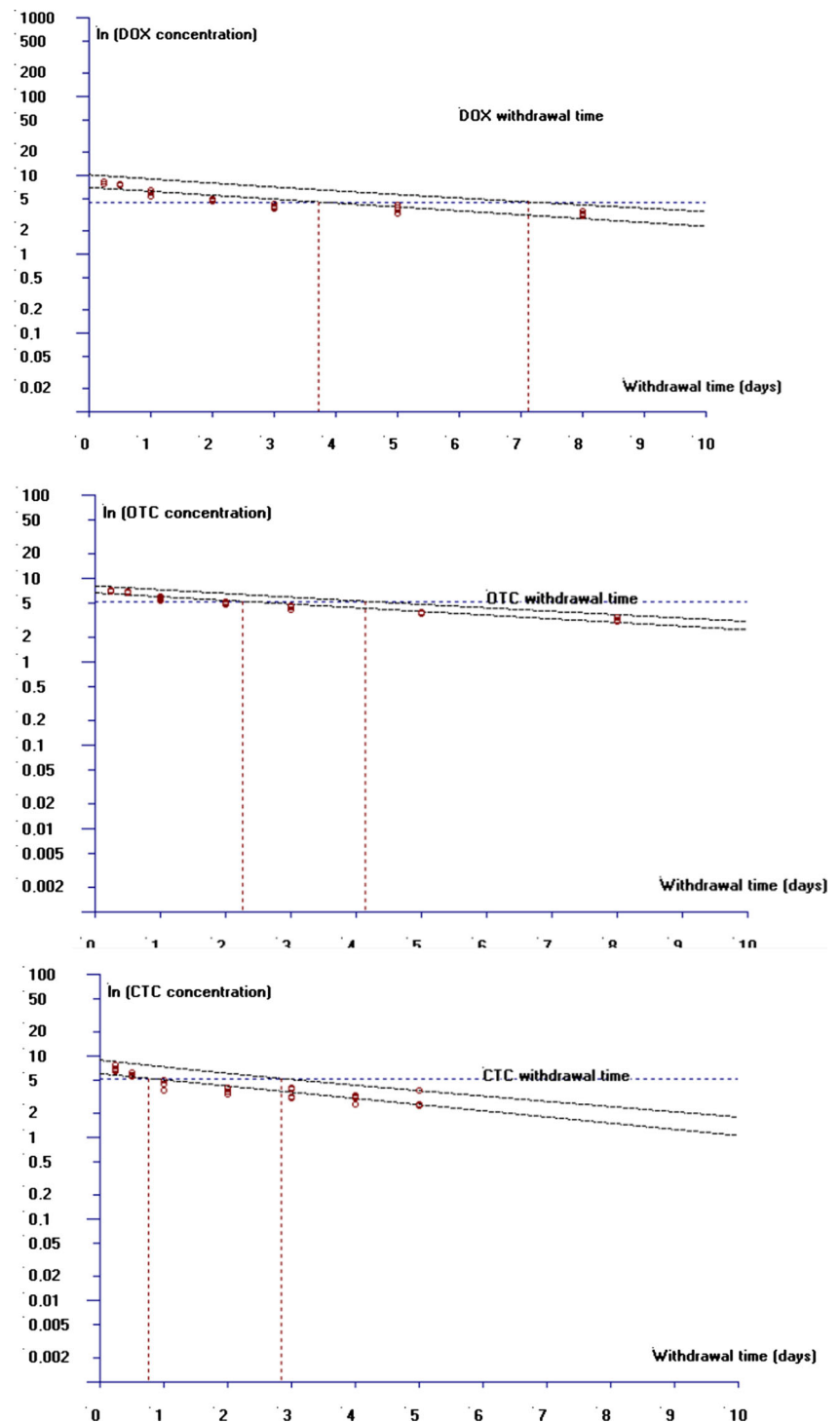
Withdrawal period was calculated using the WT 1.4 software. Those samples which show concentration values below the LOQ were removed from the data set. As the software uses a maximum of seven groups, for DOX and OTC withdrawal time determination, the data from 0.25, 0.5, 1, 2, 3, 5, and 8 days after administration were used. For CTC, considering that samples collected after 6 days show results below the LOD, the seven groups of samples were composed by 0.25, 0.5, 1, 2, 3, 4, and 5 days. Considering a 99% percentile with a 95% confidence level, the results indicated a withdrawal period of 7.189 days for DOX, which was rounded off to 8 days. For OTC, a withdrawal period of 4.19 days (5 days) was estimated and for CTC, 2.88 days (3 days).

Figure 4 shows the plot for withdrawal time determination.

Conclusions

A specific method based on LC-MS/MS for TCs analysis in poultry muscle was developed, optimized, and validated as well as able to provide high-quality results, as required for depletion and other pharmacokinetics studies. The sample

Fig. 4 Plots of withdrawal time determination obtained from the WT1.4 software



preparation protocol was considered simple, fast, and cheap, dispensing the use of laborious techniques such as SPE or pH adjustment.

The present study support more prudent use of CTC, DOX, and OTC for treatment of chickens and suggest a dose of 60 mg kg^{-1} body weight for CTC and OTC and 20 mg kg^{-1} body weight for DOX, orally administrated for five consecutive days. Chlortetracycline residues were under the MRL

considering Brazilian, European, and Japanese laws after 1 day. For DOX, it was found residues below the MRL established by Brazilian and EU laws after 3 days and after 5 days according Japanese laws. For OTC, this period was 2 days according to Brazilian and Japanese laws and 3 days according to EU law. This study presents an analysis of veterinary antibiotic depletion studies in broiler chickens and contributes to food safety for consumers.

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Compliance with Ethical Standards

Conflict of Interest Vanessa G. da Silveira declares that she has no conflict of interest. Maurício S. Oliveira declares that he has no conflict of interest. Carlos A. A. de Almeida declares that he has no conflict of interest. Rodrigo B. Hoff declares that he has no conflict of interest. Carlos A. Mallmann declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants performed by any of the authors. The study with poultry was undertaken in accordance with ethics requirements and authorized by the official ethical committee of Federal University of Santa Maria, number 061/2014. All efforts were made to minimize suffering of animals.

Informed Consent Not applicable.

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