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Article

In-House Validation of HPLC-MS/MS Methods for Detection and Quantification of Tetracyclines in Edible Tissues and Feathers of Broiler Chickens

Ekaterina V. Pokrant,[a] Aldo E. Maddaleno,[b] Carolina E. Araya,[b] Betty V. San Martín[b] and Javiera Cornejo,[a]*

[a]*Preventive Medicine Department and* [b]*Laboratory of Veterinary Pharmacology, Faculty of Veterinary and Animal Sciences, University of Chile, Av. Santa Rosa 11735 La Pintana, 8820808 Santiago, Chile*

For the detection of tetraciclines in feathers, muscle and liver, an internal protocol was designed for in-house validation of two methods, through high performance liquid chromatography with tandem mass spectrometric (HPLC-MS/MS) techniques. This protocol was based on the recommendation of the decision 2002/657/EC from the European Community as well as the food and drug administration (FDA) VICHGL49. Limit of detection (LOD) was set at 20 μg kg^{-1} and the limit of quantification (LOQ) ranged from 21.5 to 24.2, 21.2 to 21.6, and 25.0 to 27.7 μg kg^{-1} in feahers, muscle and liver samples, respectively, for all analytes. The calibrations curves show a coefficient of determination (R^2) above 0.98, 0.99 and 0.96 for feathers, muscle and liver samples, respectively. Analyte recovery ranged from 92 to 108%. In conclusion, these methods can be deemed accurate and reliable, and their validation is a fundamental step to be performed in depletion studies on these matrices.

Keywords: in-house validation, HPLC-MS/MS, tetracyclines, feathers, broiler chickens

Introduction

Antimicrobials are the main therapeutic tool currently being used to control infectious pathologies from bacterial origin, thus they are used to improve the efficiency of farming systems of animals destined for human consumption. Within the poultry industry, for example, tetracyclines are widely used due to its broad spectrum activity. These antimicrobials are naturally synthesized by *Streptomyces* spp. and were discovered in 1948 by Benjamin Duggar. They are active against Gram positive and Gram negative bacteria, either aerobic or anaerobic, as well as *Spirochaetes*, *Actinomyces*, *Rickettsia*, *Chlamydia*, *Mycoplasma* and even some protozoa. Such a broad spectrum makes them especially valuable for the treatment of respiratory and digestive diseases.[1,2]

Chlortetracycline (CTC) and oxytetracycline (OTC) were the first members of this family and their bioavailability varies from 30% for CTC to 60% for OTC, approximately. On oral administration, they are best absorbed during fasting and worst when ingested concurrently with calcium salts or aluminium hydroxide as these salts exhibit chelating properties. CTC and OTC are widely distributed throughout the body and are excreted in urine and prostatic fluid.[3]

Anadón *et al.*[4] determined that, following oral administration to broiler chickens, CTC has a bioavailability of 19.12 ± 3.31, 18.44 ± 2.77, and 17.76 ± 1.48%, after the first, fifth and final dose of the therapeutic regime. Meanwhile, following administration of a dose of 15 mg kg^{-1} to broiler chickens. Ziółkowsky *et al.*,[5] calculated a bioavailability of 76.88 ± 12.90, 92.20 ± 10.53, and 12.13 ± 4.56%, for intramuscular, subcutaneous and oral routes, respectively.

They also accumulate in reticuloendothelial cells from liver, spleen, bone marrow, bone, dentine and tooth enamel due to their keto-enol functional group, which allows them to chelate divalent cations.[6,7] As they can also accumulate in human skin and nails,[8] it can also be expected for them to bioaccumulate in other complementary structures from bird's integumentary system such as feathers, which are also built mainly from keratin.[3]

In this regard, some studies have shown that antimicrobials can accumulate in bird feathers at higher concentrations and for longer periods of time than in edible tissues, even after finishing the therapy and respecting withdrawal periods

*e-mail: jacornej@uchile.cl

that have been established for a given formulation. San Martin *et al*.,[9] found that feathers had higher concentrations of enrofloxacin and its metabolite ciprofloxacin than muscle, liver and kidney tissues from birds that had been treated with enrofloxacin. Later on, Cornejo *et al*.,[10] found higher antimicrobial concentration levels in feather matrix than in liver and muscle samples, after the calculated withdrawal period for their formulation had elapsed. Likewise, they found that flumequine was eliminated from feathers more slowly than from other tissues. On top of that, Cornejo *et al*.[11] showed that enrofloxacin and ciprofloxacin were transferred to feathers and that their concentrations remained higher, and for longer periods of time, than those found in edible tissues. These authors detected a 100 μg kg^{-1} concentration level even 9 days after treatment had elapsed. At this point in time, edible tissues had no detectable residue levels (with a detection limit of 1 μg kg^{-1}).

Meanwhile, Heinrich *et al*.[12] and Berendsen *et al*.[13] studied bioaccumulation of ceftiofur and oxytetracycline in chicken feathers and found evidence of residues of these antimicrobials in them even when withdrawal periods were respected. More importantly, these residues were found even when drug concentrations in muscle and liver samples were either below maximum residue limits (MLR) or not detectable.

In regards to the current situation of OTC and CTC MRLs for chicken tissues, the European Union (EU) has established values of 100, 300, and 600 μg kg^{-1} in muscle, liver and kidney, respectively.[14] Alternatively, the Codex Alimentarius has set limits of 200, 600 and 1200 μg kg^{-1} for muscles, liver and kidney samples, respectively.[15] Meanwhile, United States of America (USA) regulations specify that allowed concentrations for those tissues are 2000, 6000 and 12000 μg kg^{-1} for the same tissues, respectively.[16] Contrarily, for the by-products of the poultry industry like feathers, no MRLs have been determined for them.

However, based on the aforementioned information it can be concluded that antimicrobials are being transferred to feathers, and therefore represent a public health risk as they are a by-product that returns to the food chain in diets for other animals, particularly fish.[17] These diets include feathers in their formulation as a low cost source of aminoacids.[18]

Consequently, to safeguard food safety from farm to table, antimicrobials such as tetracyclines need to be studied regarding their behavior in feathers as it relates to their concentration levels in edible tissues. However, pursuing this goal necessarily implies implementing and validating analytical methods. These methods must be able of confidently quantifying precise concentration levels of tetracycline in feather, muscle and liver samples.

However, different high resolution liquid chromatography (HPLC) methods have been reported to detect tetracyclines in edible tissues, such as muscle and liver in broiler chicken.[19-21] For the feathers matrix, an analytical methodology for the detection of OTC was described by Berendsen *et al*.[13]

Several institutions and organisms have established directives for the validation of analytical methods such as the Codex Alimentarius by FAO, VICH GL 49 by the Food and Drug Administration (FDA)[22] and the 2002/657/CE decision by the European Commission.[23]

Based on these international guidelines, this study developed an internal protocol to validate analytical methodologies of HPLC coupled to mass spectroscopy detection (HPLC-MS/MS). The method simultaneously detects and quantifies OTC, CTC and their active metabolites 4-epi-OTC and 4-epi-CTC in feather, muscle and liver samples from broiler chicken.

Experimental

Standard solutions

Primary stock solutions for each analyte were prepared at a concentration of 1,000 ng mL^{-1} by dissolving oxytetracycline hydrochloride, chlortetracycline hydrochloride, 4-epi-oxytetracycline and 4-epi-chlortetracycline in methanol. All these analytes were manufactured by Dr Ehrenstorfer GmbH brand (Augsburg, Germany) and the standard had a 95.6% purity grade.

For the internal standard (IS), a primary stock solution at a concentration of 500 ng mL^{-1} was prepared by dissolving deuterated tetracycline (TC-d_6) in methanol. This standard was of 80% purity grade and it was manufactured by Toronto Research Chemicals (Toronto, Canada).

To spike blank samples, working solutions of OTC, CTC, 4-epi-OTC and 4-epi-CTC had a concentration of 2.5 ng mL^{-1}, while TC-d_6 working solution had a concentration of 20 ng mL^{-1}. All these solutions were then stored at −80 °C.

Chemicals and reagent

For the extraction, an ethylenediaminetetraacetic acid (EDTA)-McIlvaine buffer solution (pH 4 ± 0.1) was prepared by mixing 500 mL of solution A (14.2 g of disodium hydrogen phosphate dihydrate in 500 mL of water) with 312.5 mL of solution B (10.5 g of citric acid monohydrate in 500 mL of water), and then adding 3.72 g of EDTA to complete the solution.

Methanol, acetonitrile and water solvents were of HPLC grade, while disodium hydrogen phosphate dihydrate, citric acid monohydrate and ethylenediaminetetraacetic acid reagents were of analytical grade quality.

Instrumentation

The equipment used for detection of the analytes was an Agilent series 1200 liquid chromatograph with a quaternary pump, an autosampler and a column oven coupled to an AB Sciex API 4000 mass triple quadrupole spectrometer. The analytical column was the Sunfire C18 (3.5 × 2.1 × 150 mm) model and it was manufactured by Waters® (Milford, Massachusetts, USA).

Chromatographic separation was performed by using a mobile phase gradient of 0.1% formic acid in water for phase A followed by 0.1% formic acid in methanol for phase B. Flow rate was set at 0.2 mL min^{-1}, injection volume was set at 25 µL and column temperature was set at 30 °C. The Analyst V.1.6.2 software was used for sample integration.

Experimental animals

Experimental animals were kept in agreement with the animal welfare guidelines approved by the Bioethics Committee of the Veterinary Sciences Faculty, University of Chile, and the recommendations of the European Council Directive 2010/63.[24] Additionally, animals were monitored by a veterinarian specialized in avian medicine throughout the whole experiment. The birds were sacrificed under the animal welfare rules of the European Council Directive 1099/2009.[25]

Twenty male broiler chickens (Ross 308 genetic) were kept from 1-day-old onwards in individual cages under controlled environmental conditions (25 ± 5 °C of temperature, 50-60% relative humidity), and these cages were provided with an elevated wire floor in order to prevent birds from contaminating their feathers with faeces. Additionally and prior to beginning the experiment, birds had *ad libitum* access to water, and non-medicated feed whose ingredients were all analysed by HPLC-MS/MS to determine if they were free of tetracycline residues.

After slaughter, 500 g of muscle tissue, approximately, were sampled from each chicken, specifically from the pectoral girdle and the pelvic limb muscles. In order to have an appropriate amount of feathers and liver for sample preparation, these matrices were collected completely from each animal.

Sample processing

The muscle and liver samples were directly ground in an industrial food processor before proceeding to extract analytes from them. Feather samples though, had to be washed off to avoid contamination and then were cryogenically treated with liquid nitrogen before they could be ground. In the case of feathers, the R4 table top cutter food processor model (Robot Coupe®) was used to grind all samples.

Implementation and optimization of analytical methodologies

The extraction procedure for feather samples was based on an analytical methodology described by Berendsen *et al*.[13] For muscle and liver samples, extraction was based on methods described by Reveurs and Díaz,[26] Khong *et al*.[27] and Castellari *et al*.[28] The modifications to these methods allowed their adaption to differences arising from matrix composition and were standardized as a standardized operation procedure (SOP).

Extraction procedure for feathers

For the analysis, 5 ± 0.05 g of samples were weighed in within 50 mL polypropylene centrifuge tubes and then fortified with oxytetracycline hydrochloride, chlortetracycline hydrochloride, 4-epi-oxytetracycline, 4-epi-chlortetracycline standards as well as with TC-d_6 as an IS.

The extraction procedure began by adding 20 mL of acetone and 20 mL of EDTA/McIlvaine to each tube. Each tube was then agitated for 30 min and sonicated for 5 min before centrifugation at 1,800 g for 15 min. The resulting supernatant was filtered through a 0.22 µm polyvinylidene difluoride (PVDF) membrane filter (Millipore®). This filtrate was transferred to another 50 mL polypropylene tube and centrifuged again at 1,800 g for 5 min. Next, 10 mL of EDTA/McIlvaine solution were added to this supernatant and mildly shaked by hand.

This supernatant was then passed through an OASIS HLB® solid phase extraction (SPE) column (manufactured by Waters®) at a flux of 1 mL min^{-1}. This column had been previously conditioned with 4 mL of methanol, 4 mL of HPLC water and 4 mL of EDTA/McIlvaine buffer. Afterwards, a vacuum pump was used for 10 min to dry the column off before eluting it with 3 mL of methanol (1 mL min^{-1}). Subsequently, each sample was evaporated under a mild nitrogen flux at a temperature of 40-50 °C and reconstituted with 250 µL of mobile phase solution of oxalic acid 0.01 M and acetonitrile (pH 2.2 ± 0.2).

The resulting solutions were mixed in a vortex for 5 min, sonicated for 5 min, and centrifuged for 5 min at 1,800 g. Each centrifuged sample was then transferred to an Eppendorf tube for a final centrifugation at 1,700 g for 10 min. Finally, these fully processed samples were transferred to a glass vial and ready to be analyzed chromatographically.

Extraction procedure for muscle and liver

For muscle and liver samples, a similar sequence of steps to what was described for feather samples was followed during the extraction procedure, though there were a few differences that are detailed below.

In this case, 10 ± 0.1 g of muscle and liver samples were weighed in within 50 mL polypropylene centrifuge tubes and then fortified with oxytetracycline hydrochloride, chlortetracycline hydrochloride, 4-epi-oxytetracycline, 4-epi-chlortetracycline standards as well as with TC-d_6 as an IS.

Analyte extraction for muscle and liver tissue samples began by adding 20 mL of EDTA/McIlvaine to each tube. Each sample was then homogenized in a tube shaker for 5 min and sonicated for 5 min before centrifugation at 1,800 g for 15 min. The resulting supernatant was filtered through glass wool and collected in another 50 mL centrifuge tube. Afterwards and only in the case of the liver samples, 15 mL of hexane were added to all tubes containing this matrix to clean and remove fat, followed by another centrifugation at 1,800 g for 5 min. For the cleaning up, the filtrate was passed through a Sep-Pak® C18 (manufactured by Waters®) SPE column at a flux of 1 mL min^{-1}. This column had been previously conditioned with 5 mL of HPLC water and 5 mL of HPLC acetonitrile. Afterwards, this column was dried off for 10 min in a vacuum pump and eluted with 10 mL of oxalic acid 0.01 M in methanol (1 mL min^{-1}). Next, each sample was evaporated under a mild nitrogen flux at a temperature of 40-50 °C and reconstituted with 250 µL of mobile phase solution.

The resulting solutions were agitated in a vortex for 5 min, sonicated for 5 min, and centrifuged for 2 min at 1,800 g. The supernatant of each centrifuged sample was then transferred to a micro tube (1.5 mL) for a final centrifugation at 1,700 g for 10 min. Finally, this fully processed samples were transferred to a glass vial and ready to be analyzed chromatographically.

Validation procedure

An internal protocol was established for in-house validation of these analytical methods. In the one hand, the assessed parameters like retention time, linearity, recovery and precision (through repeatability and intralaboratory reproducibility) was based on the recommendations from the European Community Commission Decision EC No. 657/2002.[23] On the other hand, the parameters of limits of detection (LOD) and quantification (LOQ) were based on the VICH GL49 Guidance for Industry document regarding "Validation of analytical methods used in residue depletion studies" from the FDA.[22]

In absence of certified reference material (CRM) for OTC, CTC, 4-epi-OTC and 4-epi-CTC in feathers, muscle and liver, the recovery performance for this method was evaluated on the basis of in-house standard materials. Samples of chicken feather, muscle and liver tissue were analyzed for OTC, CTC, 4-epi-OTC and 4-epi-CTC content to rule out contamination and certified as blank. Then they were fortified with these analytes at five different concentration levels (20, 40, 60, 80 and 100 µg kg^{-1}).

To assess precision for this method, its repeatability was determined by analyzing six sample sets that were fortified at three different concentration levels (20, 60 and 100 µg kg^{-1}) on the same day. To determine intralaboratory reproducibility, six sample sets that were fortified at three different concentration levels (20, 60 and 100 µg kg^{-1}), were analyzed on different days and by different analysts.

To determine linearity of these methods, the matrix calibration curves were analyzed for five different concentration levels (20, 40, 60, 80 and 100 µg kg^{-1}).

Blank feathers, muscle and liver samples from different sources were analyzed to assess both selectivity and specificity of these methods. LOD and LOQ were determined based on 20 spiked blank samples of feather, muscle and liver tissue. The criteria for selecting the LOD was to achieve a signal-to-noise ratio greater than 3:1. The LOQ was calculated as being equal to the LOD plus 1.64 times the standard deviation (SD) of the quantified concentration from 20 blank samples fortified at the LOD.

Results

Implementation and optimization of analytical methodologies

To determine if the analytical method effectively extracted OTC, CTC, 4-epi-OTC and 4-epi-CTC from the chosen biological matrices, all analytes were chromatographically detected by their masses and specific retention times (t_R). At the same time, linearity ($R^2 > 0.95$) was determined by fortified calibration curves at five different and equidistant concentrations (20, 40, 60, 80 and 200 µg kg^{-1}).

Validation of the analytical methodologies

The analytical methods developed in this work for feathers, muscle and liver samples were validated against an internal protocol. Results for the assessed were the following:

Selectivity and specificity

Retention time for the standards were kept constant in all six analysis and exhibited a coefficient of variation (CV) lower than five percent for all four analytes. Average retention times for OTC and its metabolite were 12.7 and 8.2 min, respectively, while they averaged 14.6 min for CTC and 10.1 min for its metabolite. On the other hand, the TC-d_6 IS averaged 11.7 min. To rule out the existence of interferences on the specific retention time of each analyte, 20 samples (certified to be free of residues from these antimicrobials) were analyzed and sourced separately for every matrix (feather, muscle and liver). The results showed that within the analyzed sample group there were no interfering signals on the specific retention time for these analytes, for all three biological matrices.

Detection range

The LOD was defined at 20 μg kg^{-1} as this concentration level provided a signal-to-noise ratio greater than 3:1. To validate this parameter, 20 repetitions were performed at the chosen concentration level in a fortified matrix and then their average value, standard deviation and CV (%) were calculated. The observed variation of all repetitions for all analytes was less than 25% (Table 1).

To define the LOQ, the data for LOD calculated above was used as a starting point. To this, to the LOD value was added 1.64 times the standard deviation of all repetitions (20) in the fortified matrix at the LOD. This allowed reaching a signal-to-noise ratio greater than 10:1 for each analyte (Table 1) and, therefore, to accept these LOQ values. Figures 1 and 2 show chromatograms from standards injection and feather samples spiked with OTC, CTC, 4-epi-OTC and 4-epi-CTC standards to LOD concentration.

Linearity: calibration curves

For this study we plotted three calibration curves for five increasing and equidistant concentration levels: 20, 40, 60, 80 and 100 μg kg^{-1}.

The R^2 obtained from the calibration curves was analyzed to evaluate the linearity of the method and the individual variations of these coefficients (Table 2). The statistical values obtained for the CV% are found in values below 1.1, 0.9, and 0.7% for feathers, muscle, and liver samples, respectively. The values determined that the linearity does not suffer significant variations that can affect the robustness of the analytical result.

All these calibration curves had a R^2 higher than 0.95 and a CV (%) lower than 25%, as presented on Table 2, which shows the average R^2 of each analyte according to matrix.

Precision and recovery

The recovery rates were calculated for every analyte based on target samples that were fortified at three concentration levels (1, 2 and 5 times the LOD). For the feather matrix all analytes exhibited recovery rates that ranged from 92 to 107%, while for the muscle samples these fluctuated from 94 to 108% and in the case of liver samples they ranged from 93 to 108% (Table 3).

Table 1. Limit of detection, average concentration level, coefficient of variation of 20 repetitions on fortified samples to LOD and limit of quantification for OTC, 4-epi-OTC, CTC and 4-epi-CTC analytes, by biological matrix

Biological matrix	Analyte	LOD[a] / (μg kg^{-1})	Average concentration / (μg kg^{-1})	CV[b] / %	LOQ[c] / (μg kg^{-1})
Feather	OTC	20	20.551	7.6	22.5
	4-epi-OTC		14.833	17.40	24.2
	CTC		18.965	4.91	21.5
	4-epi-CTC		22.926	8.03	22.9
Muscle	OTC	20	21.028	3.62	21.2
	4-epi-OTC		19.030	1.45	21.3
	CTC		20.090	3.96	21.2
	4-epi-CTC		20.077	4.81	21.6
Liver	OTC	20	24.173	12.65	25.0
	4-epi-OTC		21.064	20.82	27.2
	CTC		21.122	15.95	25.5
	4-epi-CTC		20.075	23.45	27.7

[a]Limit of detection of the analytical methodology; [b]coefficient of variation; [c]limit of quantification.

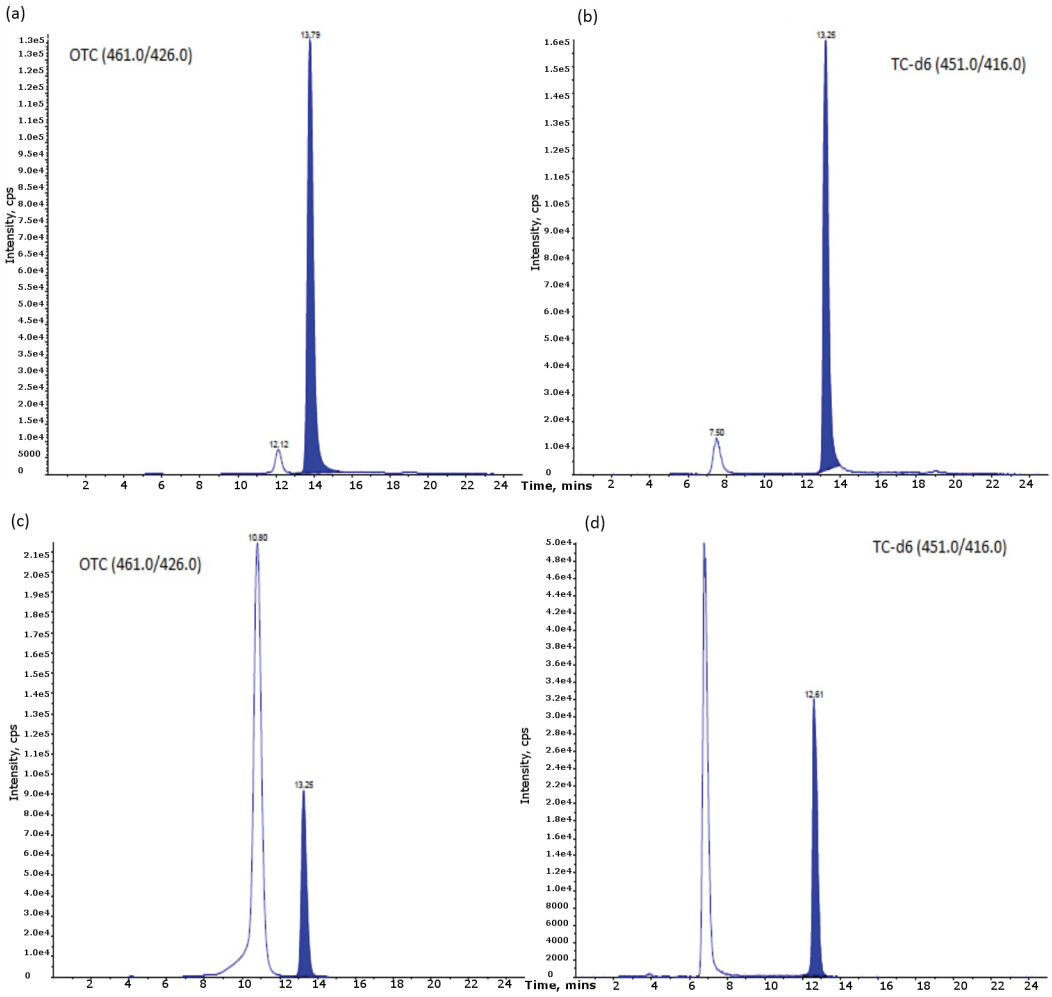

Figure 1. Chromatograms representative of (a) 6 standard injections of OTC analyte (461.0/426.0); (b) EI TC-d_6 (451.0/416.0); (c) fortified feathers samples with OTC to LOD; (d) EI TC-d_6.

The precision for the methodologies was assessed through repeatability and intralaboratory reproducibility analyses. All four analytes exhibited a similar behavior in this regard. For the fortified concentration levels of 20 and 60 μg kg^{-1} the intralaboratory reproducibility CV were less than 35%. For the 100 μg kg^{-1} concentration level, intralaboratory reproducibility CV was 23%. Meanwhile, CV values for repeatability were lower than those observed for intralaboratory reproducibility (Table 4).

Discussion

In 2012, Love *et al.*,[29] designed a study to build up on previous findings reported in 2007 and 2011 by San Martin *et al.*[9] and Cornejo *et al.*,[10] respectively, regarding the presence of antimicrobials in chicken feathers. Love *et al.*[29] decided to sample feather meals sourced from several states in the USA. These researchers not only detected that antimicrobial drugs were present in every analyzed sample but also found multiple (from 2 to 10) antimicrobial drugs present in each sample. Among them, the most frequently found were sulfonamides, macrolides, fluoroquinolones, tetracyclines, folic acid antagonists and streptogramins. Thereby, they concluded that feathers do represent a possible source for re-entry of residues of antimicrobial drugs into the food chain and therefore, they pose a risk to public health by favoring the development of antimicrobial resistance. The latter is especially relevant as it has been shown that selection of resistant bacterial populations and the genes that confer them such resistance, is a consequence of using antimicrobial drugs at therapeutic doses as well as bacteria being exposed to much lower concentration levels. Even levels below the minimum inhibitory concentration (MIC) have been found to play an important role in this selection process.[30]

In this regard, Gullberg *et al.*[31] developed highly sensitive competition experiments to determine if exposing

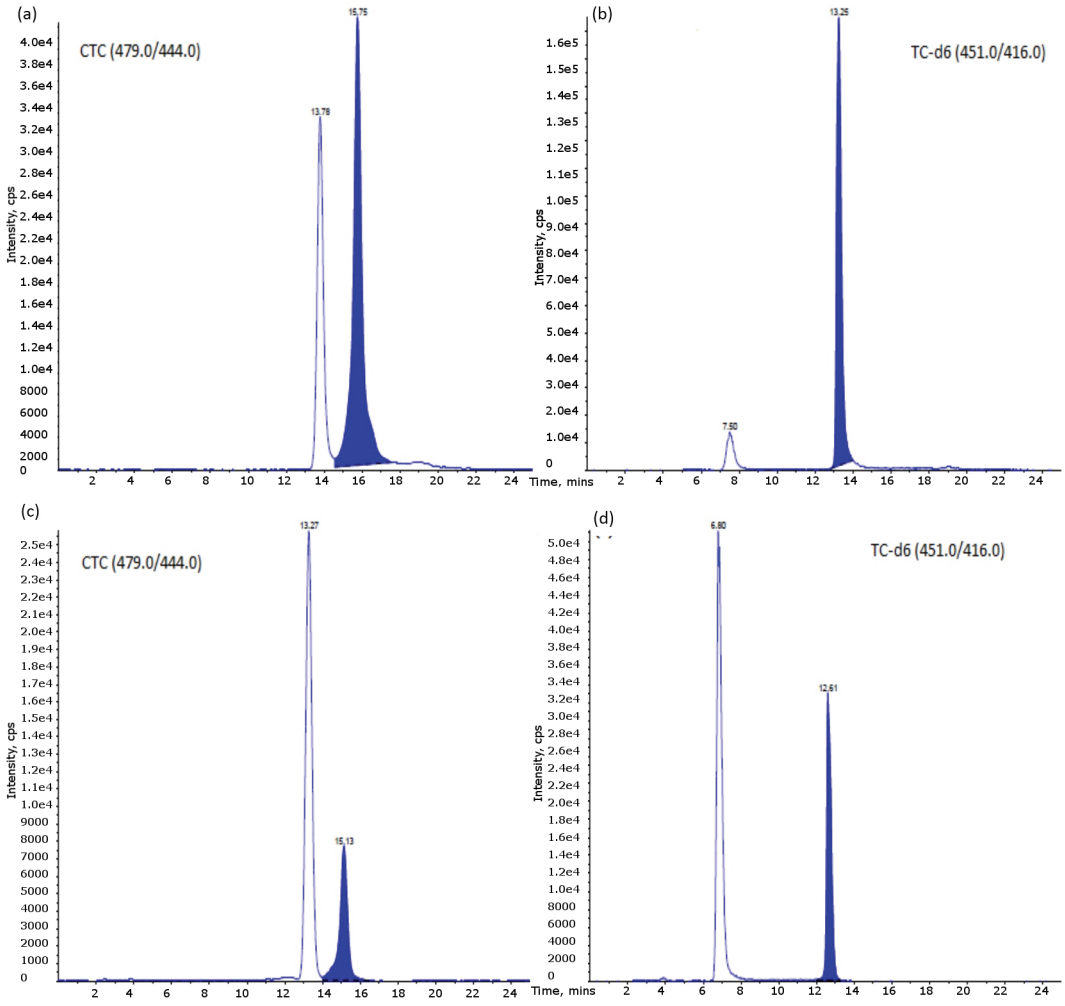

Figure 2. Chromatograms representative of (a) 6 standard injections of CTC analyte (479.0/444.0); (b) EI TC-d_6 (451.0/416.0); (c) fortified feathers samples with CTC to LOD (d) EI TC-d_6.

Table 2. Method linearity parameters for three calibration curves. R^2 average and coefficient of variation for OTC, 4-epi-OTC, CTC and 4-epi-CTC analytes, by biological matrix

Biological matrix	Analyte	R^2 average[a]	SD[b]	CV[c] / %
Feather	OTC	0.986	0.005	0.60
	4-epi-OTC	0.985	0.011	0.97
	CTC	0.981	0.009	1.14
	4-epi-CTC	0.985	0.005	0.56
Muscle	OTC	0.995	0.003	0.32
	4-epi-OTC	0.997	0.001	0.19
	CTC	0.991	0.007	0.72
	4-epi-CTC	0.997	0.003	0.40
Liver	OTC	0.966	0.005	0.60
	4-epi-OTC	0.975	0.008	0.97
	CTC	0.960	0.009	0.90
	4-epi-CTC	0.964	0.006	0.67

[a]Coefficient of determination, average from three calibration curves according analyte and matrix; [b]standard deviation; [c]coefficient of variation.

bacteria to very low concentration levels of antimicrobial drugs (below MIC) could create enrichment conditions for resistant mutant bacteria. These researchers chose several well defined mutant strains of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium LT2 bacteria along with three different antimicrobial classes that are

Table 3. Average recovery rate and coefficient of variation (CV) for 20, 60 and 100 μg kg^{-1} concentration levels by analyte, and by biological matrix

Analyte	Concentration[a] / (μg kg^{-1})	Feather		Muscle		Liver	
		Average recovery / %	CV[b] / %	Average recovery / %	CV / %	Average recovery / %	CV / %
OTC	20	96	19	104	11	93	13
	60	103	12	97	7	105	8
	100	99	4	100	2	99	2
4-epi-OTC	20	107	14	106	8	89	5
	60	95	11	95	6	107	3
	100	101	3	101	2	98	1
CTC	20	92	15	108	8	89	16
	60	105	9	94	10	108	9
	100	98	3	101	3	98	3
4-epi-CTC	20	103	13	104	8	96	8
	60	98	9	97	6	103	5
	100	101	3	100	2	99	2

[a]Fortification concentration (levels correspond to 20, 60 and 100 μg kg^{-1}); [b]coefficient of variation.

Table 4. Precision parameters: repeatability and intralaboratory reproducibility in feather, muscle and liver samples for 20, 60 and 100 μg kg^{-1} concentration levels by analyte

Analyte	Concentration[a] / (μg kg^{-1})	CV[b] of repeatability / %			CV of intralaboratory reproducibility / %		
		Feather	Muscle	Liver	Feather	Muscle	Liver
OTC	20	16.0	9.7	13.1	19.0	10.2	16.3
	60	9.1	6.9	7.8	11.8	7.1	11.8
	100	2.9	2.0	2.5	3.7	3.3	3.4
4-epi-OTC	20	13.0	7.9	15.5	15.6	10.6	13.7
	60	9.0	5.8	8.5	9.1	7.0	9.9
	100	2.8	1.7	2.8	4.5	2.1	2.8
CTC	20	8.1	12.9	5.1	14.0	12.2	15.1
	60	6.4	9.8	3.1	10.5	9.3	10.4
	100	1.8	2.7	0.9	3.0	2.6	3.0
4-epi-CTC	20	8.6	6.6	8.4	13.0	8.9	8.6
	60	6.6	4.6	5.2	9.1	6.5	7.0
	100	1.9	1.4	1.6	2.7	1.8	1.9

[a]Fortification concentration (levels correspond to 20, 60 and 100 μg kg^{-1}); [b]coefficient of variation.

greatly important in veterinary medicine (tetracyclines, fluoroquinolones and aminoglycosides). Their results show that selection of both pre-existent and newly mutant resistant bacteria occurs in extremely low concentration levels of antimicrobial drugs, even several folds below MIC for susceptible bacteria.

Additionally, Fairchild *et al.*[32] researched the effects of tetracyclines administration over *Enterococcus* spp., *Escherichia coli* and *Campylobacter* spp. bacteria that had been sourced from commercial birds. They observed on the sensitivity tests that both *Enterococcus* spp. and *Escherichia coli* were resistant to tetracyclines, in samples sourced from chicken that had been exposed to these drugs as well as from chickens that have not been exposed to them. They also noted that these bacteria possessed different resistance genes.

Consequently, we deemed important to study the behavior of tetracyclines in feathers in order to avoid transferring residues back to the food chain or even off to the environment. In the case of edible tissues of chickens for fattening, there is scientific evidence derived from studies by Anadón *et al.*[4] in CTC and by Ziółkowski *et al.*[5] in OTC showing that antibiotics are widely distibuted in muscle, liver and kidney of these animals.

For the HPLC-MS/MS method, the Sunfire C18 (Waters) column allowed the chromatographic separation of all analytes, including the epimers 4-epi-OTC and 4-epi-CTC, obtaining resolutions higher than 1 in each analyte. Thus, a highly selective method, fulfilling the requirements of specificity, was obtained. These same results were not performed using Symmetry C18 chromatographic columns. The use of oxalic acid modified the medium pH in the mobile

phase, and also allowed to improve the chromatographic separation of the analytes, in a total reading time of 25 min. In this analysis time all analytes were detected decisively against chromatographic interferences.

The first step in our study was to implement procedure charts for our methodologies. These procedure charts detailed all required steps to ensure chemical extraction for all analytes from the different biological matrices we worked with. We established two different SOP based on modifications from methods previously reported by Reveurs and Díaz,[26] Khong *et al*.,[27] Castellari *et al*.[28] and Berendsen *et al*.[13] These two SOP had a few differences due to the divergent structure and composition of the biological matrices, as well as to the presence of interfering elements such as fat and protein in them.

Next, the analytical conditions were established for the three matrices. To this end, we established an internal validation protocol, based on guidelines from both the European Community and the FDA, that allowed to prove that the method was suitable for the purpose of detecting and quantifying OTC, CTC and their active metabolites in either feather, muscle or liver samples.

Conclusions

Oxytetracycline, chlortetracycline and their metabolites can be confidently and precisely detected in feather, muscle and liver samples through the implementation and validation of analytical methodologies. Validation is a fundamental step to perform in depletion studies on these three matrices, thus the present study allows to carry out further residues studies about this family of antimicrobial drugs in the above mentioned tissues.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br.

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