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Depletion of tylosin residues in feathers, muscle and liver from broiler chickens after completion of antimicrobial therapy

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ABSTRACT
Tylosin is one of the most commonly used antimicrobial drugs from the macrolide family and in broiler chickens it is used specially for the treatment of infectious pathologies. The poultry industry produces several by-products, among which feathers account for up to 7% of a chicken’s live weight, thus they amount to a substantial mass across the whole industry. Feathers have been repurposed as an animal feed ingredient by making them feather meal. Therefore, the presence of high concentrations of residues from antimicrobial drugs in feathers might pose a risk to global public health, due to re-entry of these residues into the food chain. This work aimed to characterise the depletion behaviour of tylosin in feather samples, while considering its depletion in muscle and liver tissue samples as a reference point. To achieve this goal, we have implemented and validated an analytical methodology suitable for detecting and quantifying tylosin in these matrices. Sixty broiler chickens, raised under controlled conditions, received an oral dose of 32 mg kg\(^{-1}\) of tylosin for 5 days. Tylosin was quantified in muscle, liver and feathers by liquid chromatography coupled with a photodiode array detector (HPLC-DAD). High concentrations of tylosin were detected in feather samples over the whole experimental period after completing both the therapy and the recommended withdrawal time (WDT). On the other hand, tylosin concentrations in muscle and liver tissue samples fell below the limit of detection of this method on the first sampling day. Our results indicate that the WDT for feather samples is 27 days, hence using feather meal for the formulation of animal diets or for other agricultural purposes could contaminate with antimicrobial residues either other livestock species or the environment. In consequence, we recommend monitoring this matrix when birds have been treated with tylosin, within the context of poultry farming.

Introduction
Tylosin is one of the most commonly used antimicrobials from the macrolide family when treating infectious diseases of poultry. Such a widespread usage stems from it being restricted exclusively to veterinary medicine, making it a valuable tool in the prevention of cross-resistance problems (Ji et al. 2014; Soliman and Sedeik 2016). Additionally, though this antimicrobial is usually regarded to have bacteriostatic activity at therapeutical concentration levels, it can also have mild bactericidal activity against streptococci (Landoni and Albarellos 2015; Soliman and Sedeik 2016).

However, administering antimicrobials to animals destined for human consumption poses two major risks to public health. The first one relates to drug residues that may persist in edible tissues and are potentially capable of causing adverse effects such as direct toxicity, allergic reactions, mutagenesis, carcinogenicity and teratogenicity (Anadón et al. 2012; Beyene 2016). In particular, the presence of tylosin residues in edible tissues has been associated with toxicological effects in the short-term or hypersensitivity reactions in allergic individuals, as well as long-term inhibition of Gram positive bacteria present in human gastrointestinal gut flora (Prats et al. 2002; Jeong et al. 2010; Nasr et al. 2014). The second equally important threat to public health arises from the constant selection pressure that these residues impose on bacteria, eventually leading to the development of resistant strains (Beyene 2016).

Little data have been published about depletion of tylosin residues in edible tissues of poultry, especially about therapeutic dose regimes. In one study,
Vandenberge et al. (2012) analysed several poultry matrices to determine tylosin’s transfer from feed at concentration levels representative of cross-contamination events. They found no residue concentration levels above the limit of quantification in muscle matrix, therefore this molecule did not exceed maximum residue limits established at 100 µg kg⁻¹ by EU legislation (EMA 2002). Another two studies concluded that the administration route determines the concentration of tylosin in different organs. Specifically, orally administered tylosin resulted in lower concentration levels than those from tylosin injections, and it could be a consequence of low oral bioavailability (Kowalski et al. 2001; Lewicki 2006).

As for the egg matrix, Muñoz et al. (2014) reported meeting maximum residue limits in egg yolks after 3 days of withdrawing tylosin in laying hens. They also found that tylosin’s oral bioavailability, distribution, molecular weight and solubility had strongly influenced their results. Tylosin residues in eggs were also studied by Kan and Petz (2000), who characterised their distribution between yolk and white. These authors found that tylosin residues in egg white reflected its concentration level in blood plasma and would reach a constant level after 2–3 days. They also concluded that tylosin depletion from egg yolks generally takes about 10 days.

Meanwhile, for non-edible tissues such as feathers there is no information regarding behaviour of tylosin residues. However, current evidence for other antimicrobials shows that they accumulate in feathers of broiler chickens at higher concentration levels and for longer periods than in edible tissues. Reports from San Martin et al. (2007), Cornejo et al. (2011) and Cornejo-Kelly et al. (2012) showed that when poultry had been treated with therapeutical doses of fluoroquinolones, that drug could persist in higher concentration levels in their feathers than in their edible tissues (muscle and liver). Such a high affinity and bioaccumulation of fluoroquinolones in feathers has recently been confirmed in works by Mestorino et al. (2016) and Jansen et al. (2016).

Cornejo, Pokrant, Krogh, et al. (2017) also determined concentration levels of oxytetracycline (OTC) and 4-epi-oxytetracycline residues in feathers and edible tissues of broiler chickens. After administering an oral treatment based on a commercial formulation of 10% OTC to their birds, these authors found higher concentration levels of analytes in feathers than in edible tissues. Such results are consistent with previous work of Berendsen et al. (2013), who showed that OTC could bioaccumulate in different feather matrix after exposing chickens to different OTC treatments and then assessing drug disposition in several feather segments.

A final example of antimicrobials bioaccumulating in feathers is florfenicol (FF). This drug and its metabolite, florfenicol amine (FFA), were recently studied by Cornejo, Pokrant, Riquelme, et al. (2017) to determine residue concentration and depletion. They concluded that these analytes depleted slowly and might persist for a long time in broiler chicken feathers.

The above information becomes highly relevant when we consider that the poultry industry produces at least 1 kilogram of carcass by-products (such as heads, bones, offals and feathers) for every 3 kilograms of chicken meat. From these by-products, 2,000 metric tons of feathers are generated in the United States of America alone (Love et al. 2012). These feathers can then be industrially processed to transform them into feather meal (Meeker and Hamilton 2006) following a sequence of steps: collection of feathers from poultry carcasses, rinsing and chopping, physical hydrolysis by autoclaving (at 115–140°C for 45 minutes), pressing the cake to remove fats from it, drying, and grinding. This feather meal can then be used for several purposes, such as an organic fertiliser, raw material for biodiesel production or even as an ingredient for bioplastics (Arunlertaree and Moolthongnoi 2008; Love et al. 2012; Nachman et al. 2012), and as a low cost source of aminoacids for animal diets. The latter makes it an interesting ingredient for feeding poultry, swine, ruminants and fishes (Divakala et al. 2009) and therefore, a potential vehicle for re-entry of antimicrobial drug residues into the food chain (Meeker and Hamilton 2006; Divakala et al. 2009). Consequently, the presence of antimicrobial residues in feather meal poses a serious risk to public health.

To this date, the presence of tylosin residues has not proved to be a serious risk in the edible tissues of poultry, but in light of the aforementioned potential re-entry, it becomes necessary to study tylosin’s transfer, bioaccumulation and depletion. This work aimed to evaluate the depletion phase of tylosin in broiler chicken feathers for a tylosin tartrate pharmaceutical formula.
(80% w/w), as well as to implement and validate an analytical methodology for detection and quantification of tylosin in feathers.

**Materials and methods**

**Reagents and solutions**

Tylosin of certified purity (Sigma Aldrich Chemie Gmbh, Munich, Germany) was used to prepare stock and working solutions that were suitable for analysis and quantification. Stock solution was prepared by diluting tylosin in water of HPLC grade at a concentration of 2500 ng mL\(^{-1}\). A working solution to spike blank samples was also prepared by diluting tylosin in water of HPLC grade at a concentration of 1,000 ng mL\(^{-1}\).

Besides tylosin, the following reagents and solvents were also used: sodium phosphate monobasic monohydrate (\(\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}\)) (N° CAS: 10049-21-5), HPLC-grade water, orthophosphoric acid 85% (EMSURE\(^{®}\)), sodium phosphate dibasic (\(\text{Na}_2\text{HPO}_4\)) (N° CAS: 7558-79-4), methanol, acetonitrile and dibutylamine. All of these products were sourced from Merck (Kenilworth, New Jersey) or a similar manufacturer.

Sample analyses required phosphate buffer solutions at different pH values (2.5, 4.0 and 9.0). To prepare pH 2.5 buffer solution, 3.5 g of \(\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}\) were dissolved in 200 mL of water and then its pH was adjusted to 2.5 ± 0.1 by using orthophosphoric acid (85% w/w). For pH 4.0 phosphate buffer solution, 3.5 g of \(\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}\) were dissolved in 200 mL of water and its pH was adjusted to 4.0 ± 0.1 by using orthophosphoric acid (85% w/w). The pH 9.0 phosphate buffer solution was prepared by dissolving 1.4 g of \(\text{Na}_2\text{HPO}_4\) in 80 mL of water and its pH was adjusted to 9.0 ± 0.1 by using orthophosphoric acid (85% w/w). Both pH 2.5 and 4.0 buffer solutions were transferred to a 250 mL flask and filled up to the mark with water. As for the pH 9.0 buffer solution, this was transferred to a 100 mL flask and then filled up to the mark with water.

**Experimental animals and controlled treatment study**

Animal welfare guidelines were drawn up on the basis of Directive 2010/63/UE21 recommendations for the protection of animals used for scientific purposes (EPCEU 2010). Ethics approval was sought and granted by the Bioethics committee from the Veterinary Sciences Faculty of the University of Chile, before commencement of the experimental phase.

The experimental population were 60 male 1-day-old broiler chicks (Ross 308 genetic), who were individually caged under controlled environmental conditions (25 ± 5°C and 50–60% relative humidity). These cages were provided with a raised wire-mesh floor to avoid faecal contamination on feathers.

Throughout the entire experiment, all birds had *ad libitum* access to water and non-medicated feed that was formulated with ingredients certified tylosin-free by HPLC-DAD analyses. Chicks were reared for 22 days, when they reached an average weight of 736 ± 74.9 g. These birds were then randomly assigned to either group A (48 birds) or B (12 birds).

A commercial tylosin premix formulation (tylosin tartrate 80% w/w) registered for veterinary use in poultry in Chile was chosen for this experiment. The product label recommends a WDT of 7 days to reach the MRL of 100 mg kg\(^{-1}\) as established by EU (EC 2010). An exact therapeutic dose of 32 mg kg\(^{-1}\) of tylosin was sourced from this premix and administered through an orogastric catheter, once a day for five consecutive days, to birds from group A. Meanwhile, Group B birds (control group) were subjected to the same treatment regime as group A regarding frequency, volume and period, but instead of tylosin they received saline solution.

**Sample collection and preparation**

After completing the treatment period, eight birds from group A and two from group B were randomly sampled and euthanised via cervical dislocation on days 4, 7, 9, 11, 13 and 15 post-treatment. The experimental group size was defined following directive VICH GL48 from the European Medicines Agency (EMA 2009). According to these directives, the minimum size for any given sampling point is 6 birds; therefore 8 birds were deemed a convenient safety margin. As for the control group, 2 birds were slaughtered on each sampling point to provide enough control matrices for analytical method testing (EMA 2009).
These samples were then collected in properly labelled plastic bags and individually stored at \(-20^\circ\text{C}\), waiting to be processed for analyte extraction and chromatographic analysis. Once in the laboratory, feather samples were washed out, subjected to cryogenic treatment with liquid nitrogen and then all samples were ground in a Robot Coupe R4® food processor (Robot-coupe, France).

**Extraction from feather samples**

De Liguoro et al. (1998), Civitareale et al. (2004) and Muñoz et al. (2014) published analytical methods that, with minor modifications, became the basis for implementing a technique to determine tylosin concentration levels in feather samples.

Analyte extraction began by weighing 5 ± 0.05 g of feather samples in 50 mL centrifuge tubes and adding 10 mL of pH 2.5 phosphate buffer solutions, 15 mL of methanol and another 15 mL of HPLC-grade water. This mixture was then subject to vortex agitation for 20 minutes, sonication and centrifugation at 1780 g. The resulting supernatant was sieved through a 50 mL syringe body filled with glass fibre, collected in another set of 50 mL centrifuge tubes and then centrifuged at 1780 g for 5 minutes.

The solid phase extraction columns (SPE) for strong cation exchange (SCX) with BAKERBOND spe™ aromatic sulfonic acid (sorbent size 500 mg, column size 6 mL, packing volume 400 units) manufactured by J.T. Baker, were conditioned with 5 mL of methanol and 5 mL of pH 4.0 phosphate buffer before passing samples through them (flow rate 1 mL min\(^{-1}\)). Afterwards, columns were washed out with 1 mL of water and 1 mL of pH 9.0 phosphate buffer solution. Column elution was performed then using 7 mL of methanol and evaporated under mild nitrogen flow at 40–50°C.

Analytes were reconstituted with 350 µL of a 50:50 solution of water and acetonitrile, vortex agitated for 5 minutes and sonicated for another 5 minutes. The resulting solution was transferred to Eppendorf tubes, centrifuged at 15,890 g for 5 minutes and then transferred its supernatant to HPLC injection vials.

**HPLC-DAD analysis**

An Hitachi LaChrom Elite® liquid chromatograph (Hitachi America, Ltd. Tarrytown, New York), coupled to L-2455 photodiode array detector was used for the samples instrumental analysis.

For the chromatographic separation, we used a Chromolith® HighResolution RP 18e 100 × 4.6 mm (reference number 1.52022.0001) column from Merck Millipore (Massachusetts, USA) with a Chromolith Guard Cartridge RP 18e 10 × 4.6 mm (reference number 1.51452.0001) from Merck Millipore (Massachusetts, USA) pre-column and a mobile phase composed of a 1 M dibutylamine buffer solution, a 300 mL acetonitrile solution (solution A), 300 mL of water (solution B) and 0.02 M dibutylamine (solution C).

The mobile phase was kept at a constant flow rate of 1 mL min\(^{-1}\), an oven temperature of 50°C and a wavelength of 287 nm to ensure the best chromatographic conditions during the analysis.

**In-house validation of the analytical method**

The internal validation procedure was designed following European Union recommendations from the Commission Decision 2002/657/EC (EC 2002) for linearity, recovery and precision of analytical methods. We calculated the limit of detection (LOD) as the mean of the assay results for 20 fortified control samples at 50 µg kg\(^{-1}\), with a 3:1 signal-to-noise ratio. The limit of quantification (LOQ) was set as the mean of those same results plus 1.64 times the standard deviation (SD) of the mean.

**Withdrawal time (WDT) determination**

To determine WDT for tylosin in feather samples we followed the recommendations from the Guidance Towards Harmonization of Withdrawal Periods document published by The European
Agency for the Evaluation of Medicinal Products (EMA 2016). According to these recommendations, WDT were estimated by performing a regression analysis that included the concentration levels observed for each sampling period, as well as by plotting a depletion curve on a semi-logarithmic scale based on the K Stange equation for a 95% confidence level.

Considering that there is no established maximum residue limits (MRL) for the feather matrix, we decided to use the calculated LOD as the cut-off mark for determination of its WDT.

Results

In-house validation

While for the muscle matrix we chose an analytical method that had previously been validated, on the feather matrix it was necessary to validate our selected method before relying on its results for calculating WDTs.

Method specificity was proved by HPLC-DAD analyses on blank liver and feather samples from broiler chickens. Tylosin LOD was set at 50 µg kg⁻¹ for feathers and liver and its LOQ was set for feather of 64.1 µg kg⁻¹ and for liver 66.03 µg kg⁻¹. The LOD for muscle was set at 25 µg kg⁻¹ and the LOQ at 25.9 µg kg⁻¹. Blank samples were then analysed and showed no interferences at the average retention time (RT) for tylosin, 32.99 minutes with a CV of 3.5%. (Figure 1).

On matrix-matched calibration curves (spiked to 50, 100, 150, 200 and 250 µg kg⁻¹) our results showed linearity, which was evidenced by a coefficient of determination (R²) >0.97 for both feather, muscle and liver samples, as well as coefficients of variation (CV) of 1.03%, 6.85% and 0.34% for feather, muscle and liver samples, respectively. Tylosin recovery in feather, muscle and liver samples varied between 61% and 105%.

To assess precision of this analytical method for tylosin recovery we calculated its repeatability and within-laboratory reproducibility parameters. Table 1 shows analytical method validation results for detection and quantification of tylosin in feathers.

Quantification of tylosin in feathers, muscle and liver

In light of the absence of an established MRL for feathers, as this tissue is not destined for human consumption, we decided to compare concentration levels in feathers with liver and muscle MRLs. Such comparison provided a reference frame to assess the degree of persistence of tylosin residues in this matrix. Furthermore, for the quantification of the samples, each batch were analysed with fortified samples at increasing and equidistant concentration, in order to avoid extrapolation. High concentration levels of tylosin were detected in feather samples starting on day 4 after treatment, averaging 9246.5 µg kg⁻¹. By day 7 after treatment, concentration levels of this analyte decreased by approximately 67% in this matrix, reaching an average of 3001.9 µg kg⁻¹. Day 7 is the established WDT for muscle tissue corresponding to the pharmaceutical formulation of tylosin tartrate (80% w/w) that we used in this work. However, in spite of the declining concentration we detected and quantified in feather samples over the span of this work, these values exceeded the MRL as set by both the Codex Alimentarius and the EU for muscle tissue samples (100 µg kg⁻¹).

Table 2 lists tylosin concentration levels as they were quantified in each sampling point in feathers matrix. In the case of both muscle and liver samples, tylosin concentration levels after day 4 were not detected (ND) as they fell below the LOD of this analytical method.

Depletion time in feathers

The European Medicines Agency Guide: Approach Towards Harmonization of Withdrawal Periods EMA/CVMP/SWP/036/2012 (EMA 2016) provides a statistical method suitable to calculate the persistence of tylosin in feathers. Therefore, we determined the depletion time for feather samples by plotting a semi-logarithmic scale of concentration levels versus time and then performed a linear regression analysis at a 95% confidence level. Based on this curve, we determined the day when
concentration levels were equal or less than the established LOD for this analytical technique.

Figure 2 presents depletion-time for tylosin in feathers samples only. Depletion time could not be calculated for liver and muscle samples as their concentration levels fell below the established LOD for this technique. Considering 50 µg kg\(^{-1}\) as the cut-off point for this analytical

Figure 1. Chromatograms of: (a) tylosin standard; (b) feather blank sample without tylosin signal present at expected retention time for tylosin; (c) feather positive control sample fortified to LOD of 50 µg kg\(^{-1}\); (d) feather positive control sample fortified to 250 µg kg\(^{-1}\).
method, withdrawal time for tylosin was set at 26.4 (rounded up to 27 days).

**Discussion**

The internal validation protocol for the analytical method was designed on the basis of Commission Decision 2002/657/EC with the aim of proving that the method was suitable for its use with feather samples.

The tests conducted on the fortified matrix allowed us to establish an LOD at 50 μg kg⁻¹ with a signal-to-noise ratio greater than 3:1. As subsequent repetitions for the LOD yielded a CV value of less than 25% (which indicated statistical homogeneity), we accepted both the LOD and the calculated LOQ.

The method was proved to be linear, reproducible and repetitive according to Commission Decision 2002/657/EC guideline as the results for all parameters met its acceptance criteria (EC 2002). Therefore we deemed this method to be precise and quantitative for the analysis of tylosin in feather matrix.

This work joins several studies that have investigating the deposition into poultry feathers of drug residues from several families of antimicrobials such as fluoroquinolones, tetracyclines and phenicols (San Martin et al. 2007; Cornejo et al. 2011; Cornejo-Kelly et al. 2012; Berendsen et al. 2013; Jansen et al. 2016; Cornejo, Pokrant, Krogh, et al. 2017; Cornejo, Pokrant, Riquelme, et al. 2017). In line with those

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| Table 1. Linearity, recovery, repeatability, reproducibility, LOD and LOQ of Tylosin in biological matrices: feathers, muscle and liver. |
|-----------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Matrix/Analyte  | Linearity     | Recovery (%) to LOD | Repeatability (%) to LOD | Reproducibility (%) to LOD | LOD (μg kg⁻¹) | LOQ (μg kg⁻¹) |
| Feathers/Tylosin | $R^2 > 0.97$  | 101.6%         | 7.99%         | 11.07%         | 50            | 64.1          |
| Muscle/Tylosin  | $R^2 > 0.99$  | CV = 1.03%     | 61.1%         | 1.7%           | 20            | 25.9          |
| Liver/Tylosin   | $R^2 > 0.98$  | CV = 6.85%     | 105.3%        | 7.77%          | 13.76%        | 50            | 66.03 |

| Table 2. Tylosin concentration in feathers after oral administration of a dose of 32 mg kg⁻¹ of tylosin tartrate (80% w/w), for 5 days. |
|-----------|---------------|---------------|---------------|---------------|
| Matrix    | Post-treatment day | Lifetime (in days) | Tylosin average concentration (µg kg⁻¹) |
| Feathers  | 1 4 30          | 9246.53       |
|           | 2 7 33          | 3001.93       |
|           | 3 9 35          | 1710.59       |
|           | 4 11 37         | 664.77        |
|           | 5 13 39         | 552.23        |
|           | 6 15 41         | 356.59        |

Figure 2. Depletion-time for tylosin concentrations. Calculated depletion time in broiler chicken feathers was 27 days when plotted against a cut-off value equivalent to the LOD of the analytical methodology (50 μg kg⁻¹).
studies, our results indicate that these drugs bioaccumulate at high concentration levels in feathers from birds that have been treated with them, and even though the established WDT for a given pharmaceutical formulation has been fulfilled.

The importance of studying antimicrobial concentration levels present in feathers is due to the inclusion of feather meal as an ingredient in the diet of other animals that are destined for human consumption. In fact, it has been calculated that approximately 37% of the live weight of poultry is not directly consumed by human beings but instead becomes a source of raw material for animal food (Meeker and Hamilton 2006; Divakala et al. 2009).

Tylosin depletion from feather samples was slow throughout this study and concentration levels were high. Such high levels cannot be attributed to foreign contamination as our birds were reared in cages with an elevated mesh floor, precisely to avoid faecal contamination, and their only sources for food and water were not medicated. Unlike feather samples, edible tissues exhibited fast depletion for tylosin that brought concentration levels down to <50 µg kg⁻¹ (LOD for the technique) as soon as day 4 after treatment. These results are consistent with those reported for edible tissues by other authors (Kowalski et al. 2001; Lewicki 2006; Vandenberghe et al. 2012).

Our work shows that tylosin concentration levels in feather samples remained elevated throughout the whole experiment, even though in muscle samples these fell below the established MRL (100 µg kg⁻¹). Such high concentration levels in feather samples could be explained by tylosin’s pharmacokinetic characteristics, such as being readily absorbed at the gastrointestinal level, having low plasma protein binding affinity (hence it is widely distributed in body fluids and tissues) and is highly soluble in lipids (Lewicki 2006; Vandenberghe et al. 2012), which means it could possibly reach high concentration levels in peripheral tissues such as skin and feathers. It could also be due to contamination by secretions from the uropygial gland (mainly composed of fatty acids and esters), that birds widely distribute over their plumage when grooming themselves (Sandilands et al. 2004). Furthermore, Cornejo et al. (2011) point to growth and moulting as stages that would determine drug kinetics within feathers themselves. Additionally, the highly vascular pulp within the calamus is reabsorbed into the skin as feathers mature and could release the drug back after therapy has been discontinued. In turn, this could help explain the slow elimination of a drug in birds.

Feathers are, therefore, an important re-entry route for antimicrobial drugs into the food chain; an event that presents a significant risk to public health, especially in regards to development of antimicrobial resistance. Hence, it becomes imperative monitoring and controlling that animal diets do not include in their formulation feathers that have been sourced from animals who were treated with antimicrobial drugs.

Finally, our results also allow us to reinforce a proposal stated not long ago by other authors, that feathers are a suitable analytical matrix for detecting antimicrobial drugs, such as tylosin, in the context of poultry farming (Jansen et al. 2016; Wegh et al. 2016).

Disclosure statement
No potential conflict of interest was reported by the authors.

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