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Suitability of feathers as control matrix for antimicrobial treatments detection compared to muscle and liver of broilers

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ABSTRACT

Widespread antibiotic use and the antimicrobial resistance phenomenon demand new analytical methods and the use of non-conventional matrices increasingly necessary for safe food control. We present a method developed to detect six common antibiotics used in poultry breeding, in the unconventional matrix, feathers, compared to muscle and liver. The analysis for the presence of two β-lactams (penicillin V, amoxicillin), two fluoroquinolones (enrofloxacin, ciprofloxacin), one phenicol (thiamphenicol) and one macrolide (tylosin) was validated and achieved by HPLC–HRMS, with the ultimate aim to identify untargeted metabolites in broilers subjected to different therapeutic protocols. All the validated method parameters met the regulatory requirements. Muscle and liver were not effective matrices when the withdrawal periods were largely respected. Conversely, feathers proved a promising matrix for the detection of all the studied antibiotics, in the range of 8.72–1885.32 ng g^{-1}, except penicillin V. Like other nonconventional matrices, such as teeth, the antibiotics detected in feathers existed in their unmetabolised form.

1. Introduction

Antibiotics play an important role in ensuring the health and welfare of poultry and are commonly administered to treat and prevent respiratory diseases and other microbial infections, but are often illicitly used in poultry breeding, via the drinking water or feed. Antibiotics are overused in poultry farms because animals are intensively reared in crowded conditions that can encourage the spread of diseases. Moreover, the European Union (EU) allows poultry producers to routinely mass-medicate flocks of birds with antibiotics, for prophylactic purposes, even when no illnesses have been noticed (Wasley & Parsons, 2016).

Fluoroquinolones are the most adopted molecules used in poultry productions for the treatment and prevention of serious infections. like septicaemia, gastroenteritis, respiratory diseases and for mycoplasma infections, including the highly infectious Mycoplasma galliseptum. One ubiquitous and potentially devastating disease in poultry is coccidiosis that destroys the normal gut environment of the animal, causing malabsorption of essential nutrients and potentially result in suffering or even death (Jeremy Coller Foundation, 2016). Fluoroquinolones were first banned in the USA poultry productions in 2005 (FDA, 2005) and since then, have been prohibited in Australia, Finland and Denmark because resistant forms of Campylobacter were found in seriously ill human patients, thereby increasing the risk of death or infections (EMEA/CVMP/SAGAM/184651/2005). UK poultry farmers continue to use fluoroquinolones in adult birds, although significant reductions have been implemented (Jeremy Coller Foundation, 2016). Few antibiotics, currently approved by the US Food and Drug Administration (FDA) for use in livestock and poultry have also been prescribed in humans. Their primary function is to prevent necrotic clostridial enteritis, an intestinal infection in the birds that causes dehydration, loss of appetite, diarrhoea and rapid death.

Penicillin V, also known as phenoxymethylpenicillin, is usually administered via drinking water for the treatment and control of this disease in Europe (European Medicines Agency, 2012). Routine preventative administration of antibiotics to food-producing animals has already been banned or phased out in several European countries. In 2006, the EU banned the use of antimicrobials for growth promotion purposes, in response to evidence suggesting growth-promoting antibiotics as a major cause of the spread of resistance (European Union, 1996, 2003). Consequently, to minimise the exposure of humans to antibiotics, maximum residue

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limits (MRLs) of antibiotics in different matrices have been established by the European Commission (European Union, 2010). Nonetheless, treating infections is becoming a challenge due to antimicrobial resistance, with drugs commonly used no longer effective and a lack of new, alternative antibiotics. Thus, the widespread antibiotic use and abuse, and the antimicrobial resistance phenomenon demand new analytical methods and the use of non-conventional matrices increasingly necessary for safe food control.

Due to its ready availability and low price, feather flour is currently added to animal feed as a protein (Love, Halden, Davis, & Nachman, 2012; Pastorelli et al., 2005). In analogy to hair (Dunnett & Lees, 2004), teeth (Chiesa et al., 2017b), bones (Kühne, Wegmann, Kobe, & Fries, 2000) and claws (Cornejo et al., 2017b), feathers have been proposed as an alternative sample material for detection of chemical residues in a longer time window. If teeth, bones and claws proved to be directly or indirectly a source of antimicrobial residue entry into the food chain, they are invasive towards the animals. As for hair, feather samples can be collected easily in a non-invasive way and offer certain advantages compared to other biological samples, including easily shipping and storing. In the few literature studies of drug residues in feathers from treated animals, most focus on the analysis of a single molecule (Berendsen, Bor, Gerritsen, Jansen, & Zuidema, 2013; Cornejo, Lapierre, Iragüen, Pizarro, Hidalgo, & San Martin, 2011; San Martin, Cornejo, Iragüen, Hidalgo, & Anadón, 2007) or a single class of antibiotics (Jansen, Bolck, & Berendsen, 2016). The slow excretion of the drugs from this matrix was also hypothesised, by suggesting reabsorption during the maturation process of the vascularized pulp that fills the calamus, the part of the shaft held in the feather follicle (Cornejo et al., 2011).

In this paper, some treatment protocols were conducted in various broiler groups, to study the differences in accumulation of some of the most used antibiotics (enrofloxacin and its metabolite ciprofloxacin, amoxicillin, penicillin V, thiamphenicol, tylosin A) in the muscle, liver and feathers of poultry, by using high-performance liquid chromatography–high-resolution mass spectrometry (HPLC–HRMS) multiclass analysis. This approach is also useful for untargeted analysis of eventual metabolites present in the matrices studied. The versatility and effectiveness of the same validated protocol used on the three different matrices facilitated the analysis, demonstrating the applicability of the method. The aim of this study was to assess the suitability of feathers as an effective matrix for retrospective detection of several antimicrobial treatments in poultry compared to muscle and liver.

2. Materials and methods

2.1. Chemicals and reagents

All HPLC solvents were of analytical grade and purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Formic (98–100%) and hydrochloric acid (37%) were from Riedel-de Haën (Sigma-Aldrich). Purified water was obtained through a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). Enrofloxacin, ciprofloxacin, amoxicillin, Penicillin V, thiamphenicol, tylosin A and enrofloxacin d5 (which was used as the internal standard [IS]), were purchased from Fluka. All reagents used to prepare the EDTA-McIlvaine buffer solution (pH 4.0), described in a previous work (Chiesa, Nobile, Panseri, & Arioli, 2017a), and trichloroacetic acid 20% (w/v) aqueous solution were purchased from Fluka. The extraction cartridges (Oasis HLB 3 mL, 60 mg) were provided by Waters (Milford, MA, USA).

2.2. Standard solutions

Stock solutions of the studied molecules were prepared at 1 mg mL^{-1} in methanol and kept at −20 °C. Working solutions at 10 and 100 ng mL^{-1} were made daily for the validation sessions or to construct the calibration curves. Each working solution was maintained at 4 °C, during the validation process.

2.3. Sample collection

Eighty Ross®308 (Aviagen group, Huntsville, AL, USA) broiler chickens from the food chain, of 2.25 ± 0.62 kg average weight, reared in sheds, were studied. Six groups of 10 animals were treated with different therapeutic agents commonly used in poultry breeding; the seventh group (control) consisted of 20 non-treated animals (Table 1). The withdrawal period were largely respected. After slaughter, the muscle, liver and feathers of each broiler were collected. The samples were immediately frozen, transported to the laboratory and stored at −20 °C, until analysis.

2.4. Sample extraction

2.4.1. Muscle and liver

The extraction protocol for muscle, also carried out for liver in this work, is described in our previous study (Chiesa, Nobile, Panseri, & Arioli, 2017a,b). Briefly, 1 g of muscle/liver sample was spiked with the IS to a final concentration of 2 ng g^{-1}. The analytes were then extracted, by adding 5 mL of McIlvaine buffer (pH 4.0). Trichloroacetic acid (100 μL, 20% w/v) was added for protein precipitation, and the sample was vortexed and sonicated for 10 min. After centrifugation (2500 × g, 4 °C, 10 min), the supernatant was transferred to an empty polytetrafluoroethylene centrifuge tube and defatted with 2 × 3 mL of *n*-hexane. After each centrifugation (2500 × g, 5 min), the hydrophobic supernatant was removed. The sample was then purified using Oasis HLB cartridges preconditioned with 3 mL of methanol and 3 mL of Milli-Q water; the flow through SPE column was facilitated by aspiration under vacuum. After loading the sample, the cartridge was washed with 2 × 3 mL methanol:water (5:95 v/v). Finally, the compounds were eluted with 5 mL of methanol and were collected in a 15-mL polypropylene tube. The eluate was evaporated using a Hei-VAP rotary vacuum evaporator (Heidolph, Germany). The dried extract was reconstituted in 200 μL of methanol:water (10:90 v/v) and then transferred to a vial.

2.4.2. Feathers

The procedure described in section 2.4.1 was used, with the following modifications: three washing steps, a drying and a grinding stage preceded the extraction. In particular, a sufficient amount of intact feathers (1 g) was successively washed with 3 × 50 mL of water, chosen as the preferred solvent following preliminary trials, because it did not favour the extraction of the molecules during the washing phases. After oven-drying at 50 °C for 30 min, the feathers were shredded with scissors and then ground by a ball mill (30 Freq s^{-1}, 60 s). Next, 0.5 g of ground feathers was spiked with the IS to a final concentration of 50 ng g^{-1} and extracted with 10 mL of McIlvaine buffer (pH 4.0) instead of 5 mL. The defatting step with *n*-hexane was unnecessary and substituted by filtration through Whatman No. 1 filter paper, before purification by solid-phase extraction.

2.5. HPLC-HRMS analyses

The HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) was equipped with a Surveyor MS quaternary pump and degasser, a