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Doxycycline and sulfadimethoxine transfer from cross-contaminated feed to chicken tissues

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Food Types:	Animal products – meat, Animal feedingstuffs

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Manuscripts

1 Doxycycline and sulfadimethoxine transfer from cross-contaminated
2 feed to chicken tissues

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Abstract

During feed preparation at feed mills or during feed mixing in bins at farms, the accidental contamination of feed at trace levels by veterinary drug residues, commonly known as carry-over, can accidentally but frequently occur. To evaluate the concentrations of residual antimicrobials in poultry edible tissues, due to contaminated feed, sulfadimethoxine and doxycycline were administered for 10 days to chickens in poultry feed incurred at the contamination levels frequently found during national feed monitoring programs (1-5 mg/kg). Sulfadimethoxine and doxycycline residual concentrations detected in muscle ($< \text{LoD}$ and $31 \mu\text{g kg}^{-1}$ respectively), liver ($13 \mu\text{g kg}^{-1}$ and $56 \mu\text{g kg}^{-1}$ respectively) and kidney ($56 \mu\text{g kg}^{-1}$ and $115 \mu\text{g kg}^{-1}$ respectively) were compared with their maximum residue limits (MRLs) fixed by EC 470/2009 and EU 37/2010 Regulations for a preliminary risk evaluation.

Keywords: sulfonamides, tetracyclines, cross contamination, animal feedstuff, residues

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Introduction

Feed containing veterinary drugs and additives are commonly prepared following Good Manufacturing Practice (GMP) guidelines and employed as medicated feed in livestock to treat animal diseases, to guarantee animal welfare and consequently to increase animal weight increase and producer’s economic incomes.

In the feed industry, almost all mixed feed formulations are prepared in multi-product plants and cross-contamination cannot be ruled out for carry-over of active ingredients from previous formulations. To prevent cross-contamination several technical steps must be taken by feed operators according to Annex II Reg 183/2005 (European Parliament and Council 2005) further to the separation of medicated feedstuff production lines from the non-medicated (Heberer et al. 2007).

However, it is well known that contamination of non-medicated feed by residues of active ingredients like antimicrobials or coccidiostats, may occur at several steps during the whole production process. Further to the critical contamination of production lines, contamination can also occur during storage at feed mills, transport to farms and, eventually, at the farm during storage, manipulation and mixing operations.

The accidental administration of feed contaminated with pharmacologically active residues can cause the marketing of food of animal origin containing drug residues, for which the zero level applies, or at concentrations greater than the authorised MRL.

In 2009 a guideline on the GMP and the use of medicated feed (FEFAC 2009) was published, and the European Food Safety Authority (EFSA) has published several

opinions on the risk for animal and consumer's health related to the carry-over of coccidiostats or histomonostats into non-target feed contaminated at the 2%, 5% and 10% of the highest authorised concentrations for target species (EFSA 2007-2008).

For those compounds EFSA generally concluded that adverse effects and consequently the associated risk would be negligible; however the European Commission lowered the maximum levels of coccidiostats and histomonostats in non-target feeds, at 1% or 3% of the maximum adopted concentrations by Commission Directive 2009/8/EC (EU Commission 2009).

Despite the fact that unavoidable contamination may occur with antimicrobials, no similar approach has yet been adopted for such veterinary drugs. For these compounds the carry-over can occur during medicated feed production as reported by McEvoy (2002) and Kennedy et al. (2000) reviewing the origins of cross-contamination and the consequences on food safety. The results of the feed monitoring conducted in Northern Ireland in 1996 confirmed tetracyclines, sulfonamides, penicillins and ionophores as the most frequently contaminating drugs.

The occurrence of residues in eggs after sulfonamide administration via feed to laying hens was studied by Roudouth and Garnier 2002; Tansakul et al. 2007; the occurrence of coccidiostats in eggs was studied by Mortier et al. 2005; Rosen J. 2001; Yakkundi et al., 2002. The distribution of residues between yolk and white in eggs was studied by Kan and Petz 2000 for veterinary drugs while Mulder et al. 2005 studied the distribution of halofuginone and toltrazuril. Other papers have presented data on the transfer of coccidiostats or anthelmintics at cross-contamination levels from bovine feed to milk

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(Kan and Meijer 2007), of chlortetracycline from feed to pig tissue (Kennedy et al. 2000) and of diclazuril from poultry feed to meat (Mortier et al. 2005).

The general picture confirmed that further to feed contamination levels the residual concentrations in food of animal origin strictly depends on the chemical compound, the animal species, and the period of feed administration.

As it is currently accepted that carry-over seems to be hardly avoidable under standard production, storage and manipulation conditions, the cross-contamination of feedstuffs by veterinary drugs is an important issue and still a matter of concern.

Doxycycline and sulfadimethoxine are currently used for the preparation of medicated feed for swine therapy. For this purpose doxycycline is commercially available in premix at 0.05% concentration and medicated feed is prepared by diluting 4000-6000 g premix in 1000 kg of feed to get a final maximum concentration of doxycycline at 300 mg kg⁻¹ in complete medicated feed.

Sulfadimethoxine premixes for oral administration to young swine contain 200g of active ingredient in 1000g of product. The recommended dose in medicated feed is prepared by diluting 1 kg premix in 100 kg feed to get a final maximum concentration equal to 2000 mg kg⁻¹ in complete medicated feed.

Such high concentrations can give rise to frequent contamination of feed later prepared in the same line, for the same or other animal species.

This study is therefore intended to generate information on the transfer of sulfadimethoxine and doxycycline to chicken tissues, when feed contaminated at the carry-over concentration magnitude-detected during official monitoring plans- is administered to poultry. The conditions adopted were those of a “worse scenario” in terms of length of administration and absence of withdrawal time.

Eventually the residue concentrations measured were compared with MRLs established by the EU Regulation 470/2009 (EU Parliament and Council 2009) and by EU Regulation 37/2010 (EU Commission 2010) for further consideration.

Materials and methods

Feed and feed contamination

Complete feed for fattening chickens was produced by Agricola Tre Valli (Valpanteno, Verona, Italy). Composition: moisture 12.5%, crude protein 19%, fat 8.8%, crude fiber 3%, crude ash 5%, methionine 0.75%. Integration to 100 g of product: vitamin A 8.0 IU, vitamin D3 2.0 IU, vitamin E 20 mg, Cu(SO₄)5H₂O 10 mg, 6-Phytase 600 FTU. About 15 kg of feed for fattening chickens (free from any veterinary drug) were spiked with an aqueous solutions of doxycycline to achieve a theoretical concentration of 4 mg kg⁻¹; while other 15 kg were spiked with an aqueous solution of sulfadimethoxine to produce a final concentration of about 3 mg kg⁻¹. Briefly: an aqueous solution of doxycycline (60 mg in about 0.5 l) was sprayed over 15 kg of feedstuff previously spread over a clean surface. Analogously an aqueous solution of sulfadimethoxine (45 mg in about 0.5 l) was sprayed over a second lot of 15 kg of feedstuff previously spread

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125 over a clean surface. Each spiked material was mixed several times during the drying
126 period, and finally homogenised before sampling preparation.

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128 Both contaminated feedstuffs were dried in a semi-dark room to get a final aqueous
129 content lower than 13%. From each bulk, single samples (150 g each) destined to each
130 individual animal were prepared in paper bags and stored in the refrigerator until the
131 day of their use; a total of 8 samples per day per treatment were stored for the study.

132 To assess the average concentration of each compound in the corresponding bulk, one
133 further feed sample per day of administration was prepared, stored in the same
134 conditions and analysed by HPLC: 10 samples from each contaminated bulk were
135 therefore available to determine the actual average concentration and variations which
136 might occur during storage.

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138 Measured doxycycline and sulfadimethoxine concentration in feed were (mean $\pm$ SD):
139 (3.79 $\pm$ 0.28) mg kg⁻¹ and (2.28 $\pm$ 0.17) mg kg⁻¹, respectively, (see section 3.Results).

140

141 ***Animals and treatments***

142 The project fulfilled the requirements of Italian Law n. 116/92 and further amendments
143 on the protection and welfare of animals used in experiments. Twenty vaccinated
144 chickens about 20 days old were weighted, allocated in cages (2 birds per cage) and fed
145 dedicated feed and water *ad libitum*, for acclimatisation. After 18 days the animals
146 were weighted and randomly allocated in three groups: group 1 (control), 4 animals;
147 group 2 (doxycycline) 8 animals; group 3 (sulfadimethoxine) 8 animals.

The diet of group 1 remained the same of the acclimatisation period, free of any veterinary drug.

The 8 chickens of group 2 were fed the same feed of the acclimatisation period, contaminated with doxycycline (mean concentration $3.79 \pm 0.28 \text{ mg kg}^{-1}$).

The 8 chickens of group 3 were fed the same feed used during the acclimatisation period, contaminated with sulfadimethoxine (mean concentration $2.28 \pm 0.17 \text{ mg kg}^{-1}$).

Each animal received about 150 g daily of the feedstuff -contaminated or not - over a period of ten days and was weighted at the beginning and at the end of the “treatment” period.

On the eleventh day, animals were sacrificed and muscle (breast), liver and kidney were collected and stored at -20°C before analyses.

Analytical methods

Tissues were analysed to detect and quantify doxycycline and sulfadimethoxine by internal methods validated according to the guideline laid down by Decision 657/2002/EC (EU Commission 2002).

Briefly the methods are hereby summarised.

All solvents (methanol -MeOH, acetonitrile -ACN) were of HPLC grade and all of the chemicals (succinic acid, oxalic acid, copper sulphate (CuSO_4), citric acid, disodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), hydrochloric acid (HCl) 1N, formic acid, acetic acid, ethylenediaminetetracetic acid (EDTA), sodium chloride (NaCl), aqueous ammonia solution 33% were all of analytical reagent grade. Distilled water

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5 172 was de-ionised by an ultrapure water system (Sartorius Stedim Biotec, France).
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7 173 Doxycycline and sulfadimethoxine-d6 (sulfadimethoxine-d6) pure standards were
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9 174 purchased from Fluka (Milan, Italy), sulfadimethoxine and metacycline pure standards
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11 175 were purchased from Riedel de Haen (Milan, Italy). A stock solution of 1000 mg l⁻¹ for
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14 176 each drug was prepared by dissolving the appropriate amount of each substance in
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16 177 methanol. The intermediate working standard solutions for fortification and calibration
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18 178 curves (sulfadimethoxine, sulfadimethoxine-d6, doxycycline and metacycline: 1 mg l⁻¹)
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21 179 were prepared daily by diluting separately the correspondent stock solution with
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23 180 methanol.
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28 182 Chelating sepharose fast flow was purchased from GE Healthcare (Uppsala, Sweden);
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30 183 OASIS HLB® 60mg/3ml solid phase extraction (SPE) columns were purchase by
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32 184 Waters (Milford, MA, USA).
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37 186 Agilent SampliQ® QuEChERS EN extraction kit 5982-5650 and Agilent SampliQ®
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39 187 QuEChERS EN fatty dispersive-SPE kit for 15 ml 5982-5156 were purchased from
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42 188 Agilent (Agilent Tecnologies Inc., DE, USA).
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47 190 Analysis of doxycycline and metacycline (internal standard) was performed on a liquid
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49 191 chromatographic system (HPLC) Alliance 2695 provided with a quaternary solvent
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52 192 delivery system, a column heater module and a sample cooling device, coupled to a
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54 193 diode array detector (DAD, 2996) from Waters (Milford, MA, USA); the
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56 194 chromatographic column was a Supelco Ascentis Express® C18 column, 2.7 µm, (150
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59 195 x 4.6) mm, purchased from Supelco (Milan, Italy). HPLC analysis of sulfadimethoxine
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5 196 and sulfadimethoxine-d6 was performed by an HPLC Alliance 2695 provided with a
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7 197 quaternary solvent delivery system, a column heater module and a sample cooling
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9 198 device coupled to a QuattroUltima® triple quadrupole system (MSMS) provided with
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11 199 an ESI source by Waters (Milford, MA, USA); the chromatographic column was an
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13 200 XTerra® Phenyl column, 3.5 µm, (100 x 2.1) mm, purchased from Waters (Milford,
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15 201 MA, USA).
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21 *Doxycycline in chicken tissues*

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23 204 The procedure was based on the method proposed by Cristofani et al., 2009.
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25 205 3 g of homogenised tissue were extracted with 20 ml of succinic acid 0.1 M pH 4 and
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27 206 with 20 ml of MeOH by mechanical shaking for 30 sec and by sonication in an
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29 207 ultrasonic bath for 10 min. Liquid phase was separated from solid residue by
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31 208 centrifugation at 5000 g for 10 min. Extraction was repeated a second time by adding 10
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33 209 ml of succinic acid 0.1 M pH 4 and 10 ml of MeOH. After centrifugation at 5000 g for
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35 210 10 min, extracts were re-unified and purified through Metal Chelate Affinity
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37 211 Chromatography (MCAC) columns activated with 6 ml of distilled water, 3 ml of
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39 212 CuSO₄ 10 mM and 4 ml of distilled water. After extracts loading, columns were washed
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41 213 with 2 ml of succinic acid 0.1 M pH 4, 2 ml of distilled water, 2 ml of MeOH and 2 ml
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43 214 of distilled water; finally elution was performed by application of 8 ml of McIlvaine
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54 217 The eluate was purified by OASIS HLB® SPE columns (60 mg, 3 ml) previously
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56 218 activated with 3 ml of MeOH, 3 ml of HCl 1 N, 3 ml of distilled water and washed with
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58 219 3 ml of distilled water. Elution was achieved by 5 ml of MeOH. Solvent was evaporated
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5 220 to dryness under N₂ streaming and the residue was re-dissolved with 0.5 ml of oxalic
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7 221 acid 0.01 M before injection in HPLC-DAD.
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11 223 Doxycycline determination was performed by HPLC-DAD according to the
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13 224 chromatographic and detection conditions reported in table 1.
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16 225 Insert table 1
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18 226 Quantification was performed against external calibration in pure solvent, as matrix
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20 227 effects were negligible, and quantified concentrations were corrected for recovery.
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22 228 Metacycline was added as internal standard at the beginning of the procedure in
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24 229 unknown samples, not for strict quantitative purposes, but to prove, by an estimate of its
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26 230 detection, the good application of the method on each single sample.
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28 231 The relevant method characteristics are reported in table 3.
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33 233 *Sulfadimethoxine in chicken tissues*
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35 234 To 2 g of homogenised tissue 8 ml of de-ionised water were added and the mixture was
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37 235 shaken by vortex for 30 sec. Acetic acid (1% in ACN, 10 ml) was added and the tubes
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39 236 were manually shaken for 30 sec; then an Agilent SampliQ® QuEChERS EN
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41 237 extraction salt packet was added to each tube. Sample tubes were capped tightly and
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43 238 shaken vigorously for 1 min.
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47 240 After centrifugation at 5000 g for 5 min, an aliquot of the ACN layer (6 ml) was
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49 241 transferred into an Agilent SampliQ® QuEChERS EN fatty dispersive-SPE 15 ml tube
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51 242 containing 150 mg of PSA, 150 mg of C18EC and 900 mg of anhydrous MgSO₄. The
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53 243 samples were vortexed for 2 min and centrifuged at 5000 g for 5 min. 2 ml of each
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5 244 purified extract were transferred into an empty tube and evaporated to dryness under N₂
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7 245 streaming. The residue was re-suspended in 0.8 ml of a solution of formic acid 0.05 M
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9 246 and ACN (85/15, v/v) before LC-MS/MS analysis. Sulfadimethoxine determination was
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11 247 performed by LC-MS/MS according to the chromatographic and spectrometric
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13 248 conditions reported in table 2.

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16 249 Insert table 2
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18 250 Quantitative analysis was achieved by internal standard method where deuterated
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20 251 sulfadimethoxine (sulfadimethoxine-d₆) was chosen as internal standard introduced in
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22 252 each sample at the beginning of the analytical process. No correction for recovery was
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24 253 applied in this case.
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26 254 Relevant method characteristics are reported in table 3.
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Results

Feed and dosage

Feed contamination by both doxycycline and sulfadimethoxine was homogeneous. By analysing 10 independent feed samples, stored for the same time and in the same refrigerating conditions of the feed administered to chickens, no significant differences in concentrations were observed in any tested samples proving the good homogeneity and stability of contaminated feed (Grubbs test, $p= 0.95$). Measured mean concentrations were: (3.79 ± 0.28) mg kg⁻¹ of doxycycline and (2.28 ± 0.17) mg kg⁻¹ of sulfadimethoxine.

On the day of their arrival chickens were about 20 days old, with an average weight of 1.1 kg (in the range 1.0 – 1.2 kg) and after the acclimatisation period the weights ranged between 2.0 and 2.5 kg. Each chicken in group 2 received about 0.57 mg day⁻¹ of doxycycline, while chickens in group 3 received about 0.34 mg day⁻¹ of sulfadimethoxine. During the treatment period of 10 days, a theoretical daily dose of 0.22 mg kg⁻¹ body weight (b.w.) and of 0.13 mg kg⁻¹ b.w. was estimated for doxycycline and sulfadimethoxine respectively, considering the average weights of birds.

Analytical methods

Trueness, precision, decision limit, detection capability, limit of detection (LoD) and limit of quantification (LoQ) were estimated by analyzing 18 replicates of tissue spiked at 4 concentration levels around the MRL on three different days (6 replicates per level per day) under intra-lab reproducibility conditions. LoD and LoQ were extrapolated according to ISO 11843 approach based on the same data set.

279 The most relevant performance characteristics of both methods are summarised in table

280 3.

281 Insert table 3

282 In figure 1 representative HPLC-DAD chromatograms of a standard solution of
283 doxycycline (at a concentration corresponding to $50 \mu\text{g kg}^{-1}$ on sample), a negative
284 muscle sample, a muscle sample fortified with doxycycline at $50 \mu\text{g kg}^{-1}$ and an incurred
285 muscle sample (doxycycline measured concentration: $48 \mu\text{g kg}^{-1}$).

286 Insert figure 1

287 In figure 2 representative LC-MS/MS chromatograms of a standard solution of
288 sulfadimethoxine (at a concentration corresponding to $20 \mu\text{g kg}^{-1}$ on sample), a negative
289 muscle sample, a muscle sample fortified with sulfadimethoxine at $10 \mu\text{g kg}^{-1}$ and an
290 incurred muscle sample (estimated concentration: $7 \mu\text{g kg}^{-1}$).

291 Insert figure 2

293 *Transfer of veterinary drugs in chicken tissues*

294 Doxycycline and sulfadimethoxine concentrations measured in muscle, liver and kidney
295 are reported in table 4. In the case of sulfadimethoxine in chicken muscle, the estimated
296 concentrations were lower than the extrapolated LoD: when S/N ratio was still adequate
297 for an approximate quantitative evaluation these figures were considered and reported in
298 italic in table 4.

299 Insert table 4

301 Discussion

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302 The doxycycline contaminant level adopted in this study (about 3.8 mg kg⁻¹) and that of
303 sulfadimethoxine (about 2.3 mg kg⁻¹) were based on the cross-over concentrations (1-5
304 mg kg⁻¹) frequently detected in not-compliant feed samples, during official routine
305 controls. These levels correspond to about 1.3% and 0.11% respectively of the original
306 highest dose used in medicated feed for swine. Both the percentage levels adopted are
307 lower than those chosen by EFSA for the risk assessment of coccidiostats and
308 histomonostats (3-10%).

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310 The administration period was chosen on the basis of the worst case scenario:
311 considering that a typical commercial feedlot is produced to satisfy 1 week farming
312 requirements, a period of 10 days should guarantee residue accumulation comparable to
313 a real situation. Furthermore, considering that doxycycline and sulfadimethoxine half-
314 times (t_{1/2}) in circulating blood are 15-22 hrs and about 16 hrs, respectively (EMEA
315 1996; Epstein and Ashworth 1989) the steady state concentrations (5 x t_{1/2}) might be
316 reached also in tissues within the experimental period of 10 days.

317
318 During treatments the animals did not show symptoms of sufferance and feed intake
319 was regular. Though feed dose was available in a single pot per cage, and both birds had
320 contemporary access to, the absence of significant different weight gains during the
321 experimental period minimize the possibility that single animals could be exposed to
322 significantly different drug intake.

323
324 The method adopted for doxycycline analysis in chicken tissues is actually a multi-
325 residue method further applicable to the detection and quantification of tetracycline,

chlortetracycline, oxytetracycline and their epimers (data not shown). It is based on the well known MCAC purification approach, followed by a further purification step through an Oasis HLB SPE, which guarantees a much cleaner extract. For quantitative analysis the recovery factor estimated through the validation process was applied.

330

The method adopted for sulfadimethoxine in chicken tissues is also a multi-residue method applicable to the detection and quantification of sulfaquinoxaline, sulfachloropiridazine, suladiazine, sulfadoxine, sulfaisoxazole, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfamethoxipiridazine, sulfamonomethoxine, sulfapiridine, sulfathiazole (data not shown) further to sulfadimethoxine. A novel QuEChERS approach, employing two subsequent different dispersive phases, was tested and cleaner purified extracts with higher recoveries were achieved with respect to traditional SCX SPE previously employed. The use of deuterated sulfadimethoxine (sulfadimethoxine-d6), as appropriate internal standard, allowed a quantitative estimate of sulfadimethoxine concentration in all samples without the application of recovery factors. Both methods were validated according to the guidelines established by Decision 657/2002/EC (EU Commission 2002) and were found to be fit-for-purpose.

The average residual concentration of doxycycline and sulfadimethoxine were different in all tissues (see table 5). Mean doxycycline concentration was 31 $\mu\text{g/kg}$ in muscle, 56 $\mu\text{g kg}^{-1}$ in liver and 115 $\mu\text{g kg}^{-1}$ in kidney, whereas sulfadimethoxine was found at an average concentration of 5 $\mu\text{g kg}^{-1}$ in muscle, 13 $\mu\text{g kg}^{-1}$ in liver and 56 $\mu\text{g kg}^{-1}$ in kidney.

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The different residual concentrations were not only a consequence of the different contamination in administered feed, but likely a result of the different absorption, distribution and metabolic behaviour of the drugs; doxycycline, despite the lower logP than sulfadimethoxine, exhibits a greater persistence in tissues. The low sulfadimethoxine dosages adopted in the experiments could have been eliminated faster than expected; as reported for sulfamethazine and sulfadimethoxine (Tansakul et al. 2007; Takahashi 1986) some sulfonamides follow a dose dependent kinetics thus potentially increasing the risk of greater residue concentrations in tissues when present at higher concentration in feed.

At the experimental conditions adopted, doxycycline and sulfadimethoxine reached in each tissue average concentrations lower than the correspondent MRL. Only in the case of sulfadimethoxine concentrations close to the MRL were found in kidney.

Conclusions

This work verified the role played by feed, cross-contaminated with doxycycline or sulfadimethoxine, in residue accumulation in chicken tissues. At the feed concentrations chosen for this study and in absence of any withdrawal period, in none of the analysed tissue were violative concentrations of doxycycline or sulfadimethoxine found, notwithstanding the long administration period. In the case of doxycycline the highest concentration found in muscle was lower than 50% of the MRL, whereas in liver and kidney they were lower than 30%. In the case of sulfadimethoxine, the highest residual concentration in muscle was lower than 10% of the corresponding MRL, the highest in

liver was lower than 20%, and only in kidney it reached a concentration close to the MRL.

To ensure good manufacturing practice with pharmacologically active substances and to help official food and feed controls, European levels of tolerance in feed, as acceptable amounts of veterinary drugs in compound feed, could be established based on the respect of residue tolerance in animal products established by the EU Regulations. Thus the analytical results of this study, associated to consumer exposure data, could represent a starting point for a risk assessment evaluation by competent authorities aimed at the definition of safe maximum feed contamination levels.

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518 **Figure captions**

519

520 **Figure 1.** HPLC-DAD chromatograms. From top to bottom: doxycycline reference
521 standard solution corresponding to 50 µg kg⁻¹, a negative muscle sample, a muscle
522 sample spiked at 50 µg kg⁻¹ and a muscle sample from a group 2 animal (id. nb.142)

523

524 **Figure 2.** HPLC-ESI-MS/MS chromatograms. From top to bottom: sulfadimethoxine
525 reference standard solution corresponding to 20 µg kg⁻¹, a negative muscle sample, a
526 muscle sample spiked at 10 µg kg⁻¹ and a muscle sample from a group 3 animal (id.
527 nb.143) (I.S. sulfadimethoxine-d6 not shown)

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Tables and captions

Table 1. HPLC-DAD conditions for doxycycline and metacycline.

Time [minutes]	% Acetonitrile	% Oxalic acid 0.01M	% Methanol
0.0 - 1.0	9	70	21
1.0 - 6.0	22	70	8
6.0 - 12.0	22	70	8
12.0 – 13.0	9	70	21
Flow rate		0.6 ml min ⁻¹	
HPLC Column		C18, 2.7µm, (150 x 4.6)mm, Ascentis Express (Supelco)	
Injection Volume		20 µl	
Autosampler temperature:		5° C	
Column temperature:		30° C	
Detection system: UV-DAD. Operative conditions			
Monitoring wavelenght		355 nm	
UV spectrum range		210 – 450 nm	

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Table 2. HPLC-MSMS conditions for Sulfadimethoxine and Sulfadimethoxine-d6

Time [minutes]	% Formic acid 0.1%	% Acetonitrile
0.0 - 1.0	95	5
1.0 - 15.0	70	30
15.0 – 16.5	70	30
16.5 – 17.0	10	90
17.0 – 19.0	9	70
19.0 – 20.0	95	5
Flow rate 0.25 ml min ⁻¹		
HPLC column	Phenyl, 3.5µm, (100 x 2.1)mm, XTerra (Waters)	
Injection volume	10 µl	
Autosampler temperature:	5° C	
Column temperature:	30° C	
Detection system: MS/MS analyzer. Operative conditions		
Sulfadimethoxine MSMS transitions (Collision Energy)	m/z: 311 > 156 (20 eV)	m/z: 311 > 108 (28 eV)
Sulfadimethoxine-d6 MSMS transition (Collision Energy)	m/z: 317 > 156 (20 eV)	
Ionization mode	ESI +	
Capillary voltage	3.20 kV	
Cone voltage	40 V	
Source temperature	125° C	
Desolvation temperature	350° C	
Desolvation gas (nitrogen) flow	900 l hr ⁻¹	
Cone gas flow	50 l hr ⁻¹	

Table 3. Recovery, precision, decision limit ($CC\alpha$) and detection capability ($CC\beta$), limit of detection (LoD) and limit of quantification (LoQ) of the analytical methods for doxycycline and sulfadimethoxine determination in chicken muscle

	Fortification levels [$\mu\text{g kg}^{-1}$]	Mean found ^a [$\mu\text{g kg}^{-1}$]	Recovery (%)	Precision R.S.D. (%)	LoD [$\mu\text{g kg}^{-1}$]	LoQ [$\mu\text{g kg}^{-1}$]	$CC\alpha$ [$\mu\text{g kg}^{-1}$]	$CC\beta$ [$\mu\text{g kg}^{-1}$]
Doxycycline	25	21.5	85.9	11.0	14.6	24.8	109	118
	50	41.0	82.1	8.9				
	100	80.7	80.7	6.7				
	150	119.7	79.8	7.0				
Sulfadimethoxine	20	22.2	110.8	7.2	8.6	14.6	106	112
	50	47.9	95.8	3.1				
	100	93.9	93.9	4.0				
	150	141.5	94.3	4.0				

Note: ^aMean value of 6 repetitions for each fortification level on each day, three series on three different days

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Table 4. Measured concentrations of doxycycline and sulfadimethoxine in muscle, liver and kidney after 10 days administration of contaminated feed.

Group	Animal number	Muscle [$\mu\text{g kg}^{-1}$]	Liver [$\mu\text{g kg}^{-1}$]	Kidney [$\mu\text{g kg}^{-1}$]
CONTROL	131	N.D. ^a	N.D.	N.D.
	132	N.D.	N.D.	N.D.
	133	N.D.	N.D.	N.D.
	134	N.D.	N.D.	N.D.
Doxycycline	135	34.2	52.9	107.4
	136	20.2	38.1	77.1
	137	20.2	43.3	77.7
	138	22.5	40.1	89.2
	139	22.6	35.4	91.7
	140	49.7	95.5	185.2
	141	29.3	64.6	123.3
	142	47.5	78.6	165.7
Average		30.8	56.1	114.7
Standard dev.		12.0	21.8	40.8
MRL		100	300	600
Sulfa-dimethoxine	143	6.9	18.9	80.1
	144	2.2	5.8	34.7
	145	6.2	15.5	87.4
	146	6.8	17.6	82.7
	147	4.4	9.5	40.2
	148	3.8	12.7	50.6
	149	5.9	16.3	48.6
	150	2.1	5.7	27.0
Average		4.8	12.8	56.4
Standard dev.		2	5.2	23.6
MRL		100	100	100

Note: ^aN.D. = not detected

Figure 1. HPLC-DAD chromatograms. From top to bottom: doxycycline reference standard solution corresponding to 50 µg kg⁻¹, a negative muscle sample, a muscle sample spiked at 50 µg kg⁻¹ and a muscle sample from a group 2 animal (id. nb.142)
138x244mm (96 x 96 DPI)

Figure 2. HPLC-ESI-MS/MS chromatograms. From top to bottom: sulfadimethoxine reference standard solution corresponding to 20 $\mu\text{g kg}^{-1}$, a negative muscle sample, a muscle sample spiked at 10 $\mu\text{g kg}^{-1}$ and a muscle sample from a group 3 animal (id. nb.143) (I.S. sulfadimethoxine-d6 not shown)

282x209mm (96 x 96 DPI)