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

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6 1 Doxycycline and sulfadimethoxine transfer from cross-contaminated
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4 15 **Abstract**
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8 16 During feed preparation at feed mills or during feed mixing in bins at farms, the
9
10 17 accidental contamination of feed at trace levels by veterinary drug residues, commonly
11
12 18 known as carry-over, can accidentally but frequently occur. To evaluate the
13
14 19 concentrations of residual antimicrobials in poultry edible tissues, due to contaminated
15
16 20 feed, sulfadimethoxine and doxycycline were administered for 10 days to chickens in
17
18 21 poultry feed incurred at the contamination levels frequently found during national feed
19
20 22 monitoring programs (1-5 mg/kg). Sulfadimethoxine and doxycycline residual
21
22 23 concentrations detected in muscle (<LoD and 31 $\mu\text{g kg}^{-1}$ respectively), liver (13 $\mu\text{g kg}^{-1}$
23
24 24 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
25
26 25 compared with their maximum residue limits (MRLs) fixed by EC 470/2009 and EU
27
28 26 37/2010 Regulations for a preliminary risk evaluation.
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36 28 **Keywords:** sulfonamides, tetracyclines, cross contamination, animal feedstuff, residues
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30 Introduction

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

35
36 In the feed industry, almost all mixed feed formulations are prepared in multi-product
37 plants and cross-contamination cannot be ruled out for carry-over of active ingredients
38 from previous formulations. To prevent cross-contamination several technical steps
39 must be taken by feed operators according to Annex II Reg 183/2005 (European
40 Parliament and Council 2005) further to the separation of medicated feedstuff
41 production lines from the non-medicated (Heberer et al. 2007).

42
43 However, it is well known that contamination of non-medicated feed by residues of
44 active ingredients like antimicrobials or coccidiostats, may occur at several steps during
45 the whole production process. Further to the critical contamination of production lines,
46 contamination can also occur during storage at feed mills, transport to farms and,
47 eventually, at the farm during storage, manipulation and mixing operations.

48
49 The accidental administration of feed contaminated with pharmacologically active
50 residues can cause the marketing of food of animal origin containing drug residues, for
51 which the zero level applies, or at concentrations greater than the authorised MRL.

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

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5 54 opinions on the risk for animal and consumer's health related to the carry-over of
6
7 55 coccidiostats or histomonostats into non-target feed contaminated at the 2%, 5% and
8
9 56 10% of the highest authorised concentrations for target species (EFSA 2007-2008).

10
11 57 For those compounds EFSA generally concluded that adverse effects and consequently
12
13 58 the associated risk would be negligible; however the European Commission lowered the
14
15 59 maximum levels of coccidiostats and histomonostats in non-target feeds, at 1% or 3% of
16
17 60 the maximum adopted concentrations by Commission Directive 2009/8/EC (EU
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19 61 Commission 2009).

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22 62
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25 63 Despite the fact that unavoidable contamination may occur with antimicrobials, no
26
27 64 similar approach has yet been adopted for such veterinary drugs. For these compounds
28
29 65 the carry-over can occur during medicated feed production as reported by McEvoy
30
31 66 (2002) and Kennedy et al. (2000) reviewing the origins of cross-contamination and the
32
33 67 consequences on food safety. The results of the feed monitoring conducted in Northern
34
35 68 Ireland in 1996 confirmed tetracyclines, sulfonamides, penicillins and ionophores as the
36
37 69 most frequently contaminating drugs.

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44 71 The occurrence of residues in eggs after sulfonamide administration via feed to laying
45
46 72 hens was studied by Roudouth and Garnier 2002; Tansakul et al. 2007; the occurrence
47
48 73 of coccidiostats in eggs was studied by Mortier et al. 2005; Rosen J. 2001; Yakkundi et
49
50 74 al., 2002. The distribution of residues between yolk and white in eggs was studied by
51
52 75 Kan and Petz 2000 for veterinary drugs while Mulder et al. 2005 studied the distribution
53
54 76 of halofuginone and toltrazuril. Other papers have presented data on the transfer of
55
56 77 coccidiostats or anthelmintics at cross-contamination levels from bovine feed to milk
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4 78 (Kan and Meijer 2007), of chlortetracycline from feed to pig tissue (Kennedy et al.
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6
7 79 2000) and of diclazuril from poultry feed to meat (Mortier et al. 2005).
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10 80

11 81 The general picture confirmed that further to feed contamination levels the residual
12 82 concentrations in food of animal origin strictly depends on the chemical compound, the
13 83 animal species, and the period of feed administration.
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21 85 As it is currently accepted that carry-over seems to be hardly avoidable under standard
22 86 production, storage and manipulation conditions, the cross-contamination of feedstuffs
23 87 by veterinary drugs is an important issue and still a matter of concern.
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30 89 Doxycycline and sulfadimethoxine are currently used for the preparation of medicated
31 90 feed for swine therapy. For this purpose doxycycline is commercially available in
32 91 premix at 0.05% concentration and medicated feed is prepared by diluting 4000-6000 g
33 92 premix in 1000 kg of feed to get a final maximum concentration of doxycycline at 300
34 93 mg kg⁻¹ in complete medicated feed.
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44 95 Sulfadimethoxine premixes for oral administration to young swine contain 200g of
45 96 active ingredient in 1000g of product. The recommended dose in medicated feed is
46 97 prepared by diluting 1 kg premix in 100 kg feed to get a final maximum concentration
47 98 equal to 2000 mg kg⁻¹ in complete medicated feed.
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56 100 Such high concentrations can give rise to frequent contamination of feed later prepared
57 101 in the same line, for the same or other animal species.
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5 102 This study is therefore intended to generate information on the transfer of
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7 103 sulfadimethoxine and doxycycline to chicken tissues, when feed contaminated at the
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9 104 carry-over concentration magnitude-detected during official monitoring plans- is
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11 105 administered to poultry. The conditions adopted were those of a “worse scenario” in
12
13 106 terms of length of administration and absence of withdrawal time.
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19 108 Eventually the residue concentrations measured were compared with MRLs established
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21 109 by the EU Regulation 470/2009 (EU Parliament and Council 2009) and by EU
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23 110 Regulation 37/2010 (EU Commission 2010) for further consideration.
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28 112 **Materials and methods**

29 30 113 *Feed and feed contamination*

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33 114 Complete feed for fattening chickens was produced by Agricola Tre Valli (Valpanteno,
34
35 115 Verona, Italy). Composition: moisture 12.5%, crude protein 19%, fat 8.8%, crude fiber
36
37 116 3%, crude ash 5%, methionine 0.75%. Integration to 100 g of product: vitamin A 8.0
38
39 117 IU, vitamin D3 2.0 IU, vitamin E 20 mg, Cu(SO₄)5H₂O 10 mg, 6-Phytase 600 FTU.
40
41 118 About 15 kg of feed for fattening chickens (free from any veterinary drug) were spiked
42
43 119 with an aqueous solutions of doxycycline to achieve a theoretical concentration of 4
44
45 120 mg kg⁻¹; while other 15 kg were spiked with an aqueous solution of sulfadimethoxine to
46
47 121 produce a final concentration of about 3 mg kg⁻¹. Briefly: an aqueous solution of
48
49 122 doxycycline (60 mg in about 0.5 l) was sprayed over 15 kg of feedstuff previously
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51 123 spread over a clean surface. Analogously an aqueous solution of sulfadimethoxine (45
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53 124 mg in about 0.5 l) was sprayed over a second lot of 15 kg of feedstuff previously spread
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5 125 over a clean surface. Each spiked material was mixed several times during the drying
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7 126 period, and finally homogenised before sampling preparation.
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11 128 Both contaminated feedstuffs were dried in a semi-dark room to get a final aqueous
12
13 129 content lower than 13%. From each bulk, single samples (150 g each) destined to each
14
15 130 individual animal were prepared in paper bags and stored in the refrigerator until the
16
17 131 day of their use; a total of 8 samples per day per treatment were stored for the study.
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21 132 To assess the average concentration of each compound in the corresponding bulk, one
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23 133 further feed sample per day of administration was prepared, stored in the same
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25 134 conditions and analysed by HPLC: 10 samples from each contaminated bulk were
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27 135 therefore available to determine the actual average concentration and variations which
28
29 136 might occur during storage.
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35 138 Measured doxycycline and sulfadimethoxine concentration in feed were (mean \pm SD):
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37 139 (3.79 ± 0.28) mg kg⁻¹ and (2.28 ± 0.17) mg kg⁻¹, respectively, (see section 3.Results).
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41 141 *Animals and treatments*

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44 142 The project fulfilled the requirements of Italian Law n. 116/92 and further amendments
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46 143 on the protection and welfare of animals used in experiments. Twenty vaccinated
47
48 144 chickens about 20 days old were weighted, allocated in cages (2 birds per cage) and fed
49
50 145 dedicated feed and water *ad libitum*, for acclimatisation. After 18 days the animals
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52 146 were weighted and randomly allocated in three groups: group 1 (control), 4 animals;
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54 147 group 2 (doxycycline) 8 animals; group 3 (sulfadimethoxine) 8 animals.
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5 148 The diet of group 1 remained the same of the acclimatisation period, free of any
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7 149 veterinary drug.

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9 150 The 8 chickens of group 2 were fed the same feed of the acclimatisation period,
10
11 151 contaminated with doxycycline (mean concentration $3.79 \pm 0.28 \text{ mg kg}^{-1}$).

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16 153 The 8 chickens of group 3 were fed the same feed used during the acclimatisation
17
18 154 period, contaminated with sulfadimethoxine (mean concentration $2.28 \pm 0.17 \text{ mg kg}^{-1}$).

19
20 155 Each animal received about 150 g daily of the feedstuff -contaminated or not - over a
21
22 156 period of ten days and was weighted at the beginning and at the end of the “treatment”
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24 157 period.

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30 159 On the eleventh day, animals were sacrificed and muscle (breast), liver and kidney were
31
32 160 collected and stored at -20°C before analyses.

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37 162 ***Analytical methods***

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39 163 Tissues were analysed to detect and quantify doxycycline and sulfadimethoxine by
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41 164 internal methods validated according to the guideline laid down by Decision
42
43 165 657/2002/EC (EU Commission 2002).

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46 166 Briefly the methods are hereby summarised.

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49 167 All solvents (methanol -MeOH, acetonitrile -ACN) were of HPLC grade and all of the
50
51 168 chemicals (succinic acid, oxalic acid, copper sulphate (CuSO_4), citric acid, disodium
52
53 169 hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), hydrochloric acid (HCl) 1N, formic
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55 170 acid, acetic acid, ethylenediaminetetracetic acid (EDTA), sodium chloride (NaCl),
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57 171 aqueous ammonia solution 33% were all of analytical reagent grade. Distilled water
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4 172 was de-ionised by an ultrapure water system (Sartorius Stedim Biotec, France).
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6
7 173 Doxycycline and sulfadimethoxine-d6 (sulfadimethoxine-d6) pure standards were
8
9 174 purchased from Fluka (Milan, Italy), sulfadimethoxine and metacycline pure standards
10
11 175 were purchased from Riedel de Haen (Milan, Italy). A stock solution of 1000 mg l⁻¹ for
12
13 176 each drug was prepared by dissolving the appropriate amount of each substance in
14
15 177 methanol. The intermediate working standard solutions for fortification and calibration
16
17 178 curves (sulfadimethoxine, sulfadimethoxine-d6, doxycycline and metacycline: 1 mg l⁻¹)
18
19 179 were prepared daily by diluting separately the correspondent stock solution with
20
21 180 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
31
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
40
41 188 Agilent (Agilent Tecnologies Inc., DE, USA).
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47 190 Analysis of doxycycline and metacycline (internal standard) was performed on a liquid
48
49 191 chromatographic system (HPLC) Alliance 2695 provided with a quaternary solvent
50
51 192 delivery system, a column heater module and a sample cooling device, coupled to a
52
53 193 diode array detector (DAD, 2996) from Waters (Milford, MA, USA); the
54
55 194 chromatographic column was a Supelco Ascentis Express® C18 column, 2.7 µm, (150
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57 195 x 4.6) mm, purchased from Supelco (Milan, Italy). HPLC analysis of sulfadimethoxine
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4 196 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
10
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,
14
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
26
27 206 with 20 ml of MeOH by mechanical shaking for 30 sec and by sonication in an
28
29 207 ultrasonic bath for 10 min. Liquid phase was separated from solid residue by
30
31 208 centrifugation at 5000 g for 10 min. Extraction was repeated a second time by adding 10
32
33 209 ml of succinic acid 0.1 M pH 4 and 10 ml of MeOH. After centrifugation at 5000 g for
34
35 210 10 min, extracts were re-unified and purified through Metal Chelate Affinity
36
37 211 Chromatography (MCAC) columns activated with 6 ml of distilled water, 3 ml of
38
39 212 CuSO₄ 10 mM and 4 ml of distilled water. After extracts loading, columns were washed
40
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
42
43 214 of distilled water; finally elution was performed by application of 8 ml of McIlvaine
44
45 215 buffer.
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52 216
53
54 217 The eluate was purified by OASIS HLB® SPE columns (60 mg, 3 ml) previously
55
56 218 activated with 3 ml of MeOH, 3 ml of HCl 1 N, 3 ml of distilled water and washed with
57
58 219 3 ml of distilled water. Elution was achieved by 5 ml of MeOH. Solvent was evaporated
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4 220 to dryness under N₂ streaming and the residue was re-dissolved with 0.5 ml of oxalic
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6 221 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
12
13 224 chromatographic and detection conditions reported in table 1.
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16 225 Insert table 1
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19 226 Quantification was performed against external calibration in pure solvent, as matrix
20
21 227 effects were negligible, and quantified concentrations were corrected for recovery.
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24 228 Metacycline was added as internal standard at the beginning of the procedure in
25
26 229 unknown samples, not for strict quantitative purposes, but to prove, by an estimate of its
27
28 230 detection, the good application of the method on each single sample.
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31 231 The relevant method characteristics are reported in table 3.
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35 233 ***Sulfadimethoxine in chicken tissues***
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38 234 To 2 g of homogenised tissue 8 ml of de-ionised water were added and the mixture was
39
40 235 shaken by vortex for 30 sec. Acetic acid (1% in ACN, 10 ml) was added and the tubes
41
42 236 were manually shaken for 30 sec; then an Agilent SampliQ® QuEChERS EN
43
44 237 extraction salt packet was added to each tube. Sample tubes were capped tightly and
45
46 238 shaken vigorously for 1 min.
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51 240 After centrifugation at 5000 g for 5 min, an aliquot of the ACN layer (6 ml) was
52
53 241 transferred into an Agilent SampliQ® QuEChERS EN fatty dispersive-SPE 15 ml tube
54
55 242 containing 150 mg of PSA, 150 mg of C18EC and 900 mg of anhydrous MgSO₄. The
56
57 243 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
6
7 245 streaming. The residue was re-suspended in 0.8 ml of a solution of formic acid 0.05 M
8
9 246 and ACN (85/15, v/v) before LC-MS/MS analysis. Sulfadimethoxine determination was
10
11 247 performed by LC-MS/MS according to the chromatographic and spectrometric
12
13 248 conditions reported in table 2.

14
15
16 249 Insert table 2

17
18 250 Quantitative analysis was achieved by internal standard method where deuterated
19
20 251 sulfadimethoxine (sulfadimethoxine-d₆) was chosen as internal standard introduced in
21
22 252 each sample at the beginning of the analytical process. No correction for recovery was
23
24 253 applied in this case.
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28 254 Relevant method characteristics are reported in table 3.
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4 255 **Results**

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6
7 256 *Feed and dosage*

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9 257 Feed contamination by both doxycycline and sulfadimethoxine was homogeneous. By
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11 258 analysing 10 independent feed samples, stored for the same time and in the same
12
13 259 refrigerating conditions of the feed administered to chickens, no significant differences
14
15 260 in concentrations were observed in any tested samples proving the good homogeneity
16
17 261 and stability of contaminated feed (Grubbs test, $p= 0.95$). Measured mean
18
19 262 concentrations were: (3.79 ± 0.28) mg kg⁻¹ of doxycycline and (2.28 ± 0.17) mg kg⁻¹ of
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21 263 sulfadimethoxine.
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28 265 On the day of their arrival chickens were about 20 days old, with an average weight of
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30 266 1.1 kg (in the range 1.0 – 1.2 kg) and after the acclimatisation period the weights ranged
31
32 267 between 2.0 and 2.5 kg. Each chicken in group 2 received about 0.57 mg day⁻¹ of
33
34 268 doxycycline, while chickens in group 3 received about 0.34 mg day⁻¹ of
35
36 269 sulfadimethoxine. During the treatment period of 10 days, a theoretical daily dose of
37
38 270 0.22 mg kg⁻¹ body weight (b.w.) and of 0.13 mg kg⁻¹ b.w. was estimated for doxycycline
39
40 271 and sulfadimethoxine respectively, considering the average weights of birds.
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47 273 *Analytical methods*

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49 274 Trueness, precision, decision limit, detection capability, limit of detection (LoD) and
50
51 275 limit of quantification (LoQ) were estimated by analyzing 18 replicates of tissue spiked
52
53 276 at 4 concentration levels around the MRL on three different days (6 replicates per level
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55 277 per day) under intra-lab reproducibility conditions. LoD and LoQ were extrapolated
56
57 278 according to ISO 11843 approach based on the same data set.
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4 279 The most relevant performance characteristics of both methods are summarised in table

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9 281 Insert table 3

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11 282 In figure 1 representative HPLC-DAD chromatograms of a standard solution of

12 283 doxycycline (at a concentration corresponding to $50 \mu\text{g kg}^{-1}$ on sample), a negative

13
14 284 muscle sample, a muscle sample fortified with doxycycline at $50 \mu\text{g kg}^{-1}$ and an incurred

15
16 285 muscle sample (doxycycline measured concentration: $48 \mu\text{g kg}^{-1}$).

17
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21 286 Insert figure 1

22
23 287 In figure 2 representative LC-MS/MS chromatograms of a standard solution of

24 288 sulfadimethoxine (at a concentration corresponding to $20 \mu\text{g kg}^{-1}$ on sample), a negative

25
26 289 muscle sample, a muscle sample fortified with sulfadimethoxine at $10 \mu\text{g kg}^{-1}$ and an

27
28 290 incurred muscle sample (estimated concentration: $7 \mu\text{g kg}^{-1}$).

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32 291 Insert figure 2

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34 292

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36
37 293 *Transfer of veterinary drugs in chicken tissues*

38
39 294 Doxycycline and sulfadimethoxine concentrations measured in muscle, liver and kidney

40 295 are reported in table 4. In the case of sulfadimethoxine in chicken muscle, the estimated

41
42 296 concentrations were lower than the extrapolated LoD: when S/N ratio was still adequate

43
44 297 for an approximate quantitative evaluation these figures were considered and reported in

45
46 298 italic in table 4.

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49
50 299 Insert table 4

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55 301 **Discussion**

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5 302 The doxycycline contaminant level adopted in this study (about 3.8 mg kg⁻¹) and that of
6
7 303 sulfadimethoxine (about 2.3 mg kg⁻¹) were based on the cross-over concentrations (1-5
8
9 304 mg kg⁻¹) frequently detected in not-compliant feed samples, during official routine
10
11 305 controls. These levels correspond to about 1.3% and 0.11% respectively of the original
12
13 306 highest dose used in medicated feed for swine. Both the percentage levels adopted are
14
15 307 lower than those chosen by EFSA for the risk assessment of coccidiostats and
16
17 308 histomonostats (3-10%).
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22
23 310 The administration period was chosen on the basis of the worst case scenario:
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25 311 considering that a typical commercial feedlot is produced to satisfy 1 week farming
26
27 312 requirements, a period of 10 days should guarantee residue accumulation comparable to
28
29 313 a real situation. Furthermore, considering that doxycycline and sulfadimethoxine half-
30
31 314 times ($t_{1/2}$) in circulating blood are 15-22 hrs and about 16 hrs, respectively (EMEA
32
33 315 1996; Epstein and Ashworth 1989) the steady state concentrations ($5 \times t_{1/2}$) might be
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35 316 reached also in tissues within the experimental period of 10 days.
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42 318 During treatments the animals did not show symptoms of sufferance and feed intake
43
44 319 was regular. Though feed dose was available in a single pot per cage, and both birds had
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46 320 contemporary access to, the absence of significant different weight gains during the
47
48 321 experimental period minimize the possibility that single animals could be exposed to
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50 322 significantly different drug intake.
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56 324 The method adopted for doxycycline analysis in chicken tissues is actually a multi-
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58 325 residue method further applicable to the detection and quantification of tetracycline,
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4 326 chlortetracycline, oxytetracycline and their epimers (data not shown). It is based on the
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6
7 327 well known MCAC purification approach, followed by a further purification step
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9 328 through an Oasis HLB SPE, which guarantees a much cleaner extract. For quantitative
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11 329 analysis the recovery factor estimated through the validation process was applied.
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16 331 The method adopted for sulfadimethoxine in chicken tissues is also a multi-residue
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18 332 method applicable to the detection and quantification of sulfaquinoxaline,
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21 333 sulfachloropyridazine, suladiazine, sulfadoxine, sulfaisoxazole, sulfamerazine,
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23 334 sulfamethazine, sulfamethoxazole, sulfamethoxipridazine, sulfamonomethoxine,
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26 335 sulfapiridine, sulfathiazole (data not shown) further to sulfadimethoxine. A novel
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28 336 QuEChERS approach, employing two subsequent different dispersive phases, was
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31 337 tested and cleaner purified extracts with higher recoveries were achieved with respect
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33 338 to traditional SCX SPE previously employed. The use of deuterated sulfadimethoxine
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35 339 (sulfadimethoxine-d6), as appropriate internal standard, allowed a quantitative estimate
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37 340 of sulfadimethoxine concentration in all samples without the application of recovery
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40 341 factors. Both methods were validated according to the guidelines established by
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42 342 Decision 657/2002/EC (EU Commission 2002) and were found to be fit-for-purpose.

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44 343 The average residual concentration of doxycycline and sulfadimethoxine were different
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46 344 in all tissues (see table 5). Mean doxycycline concentration was 31 $\mu\text{g}/\text{kg}$ in muscle, 56
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48 345 $\mu\text{g kg}^{-1}$ in liver and 115 $\mu\text{g kg}^{-1}$ in kidney, whereas sulfadimethoxine was found at an
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51 346 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
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53 347 kidney.
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5 349 The different residual concentrations were not only a consequence of the different
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7 350 contamination in administered feed, but likely a result of the different absorption,
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9 351 distribution and metabolic behaviour of the drugs; doxycycline, despite the lower logP
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11 352 than sulfadimethoxine, exhibits a greater persistence in tissues. The low
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13 353 sulfadimethoxine dosages adopted in the experiments could have been eliminated faster
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16 354 than expected; as reported for sulfamethazine and sulfadimethoxine (Tansakul et al.
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18 355 2007; Takahashi 1986) some sulfonamides follow a dose dependent kinetics thus
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21 356 potentially increasing the risk of greater residue concentrations in tissues when present
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23 357 at higher concentration in feed.
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28 359 At the experimental conditions adopted, doxycycline and sulfadimethoxine reached in
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30 360 each tissue average concentrations lower than the correspondent MRL. Only in the case
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32 361 of sulfadimethoxine concentrations close to the MRL were found in kidney.
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363 **Conclusions**

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

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5 372 liver was lower than 20%, and only in kidney it reached a concentration close to the
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7 373 MRL.

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11 375 To ensure good manufacturing practice with pharmacologically active substances and to
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13 376 help official food and feed controls, European levels of tolerance in feed, as acceptable
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15 377 amounts of veterinary drugs in compound feed, could be established based on the
16
17 378 respect of residue tolerance in animal products established by the EU Regulations. Thus
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19 379 the analytical results of this study, associated to consumer exposure data, could
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21 380 represent a starting point for a risk assessment evaluation by competent authorities
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23 381 aimed at the definition of safe maximum feed contamination levels.
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35 385 like to thank Bruna Allegretta and Valentina Mozzo for their technical assistance
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37 386 throughout the study.
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4 518 **Figure captions**
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9 520 **Figure 1.** HPLC-DAD chromatograms. From top to bottom: doxycycline reference
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11 521 standard solution corresponding to 50 $\mu\text{g kg}^{-1}$, a negative muscle sample, a muscle
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13 522 sample spiked at 50 $\mu\text{g kg}^{-1}$ and a muscle sample from a group 2 animal (id. nb.142)
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18 524 **Figure 2.** HPLC-ESI-MS/MS chromatograms. From top to bottom: sulfadimethoxine
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20 525 reference standard solution corresponding to 20 $\mu\text{g kg}^{-1}$, a negative muscle sample, a
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22 526 muscle sample spiked at 10 $\mu\text{g kg}^{-1}$ and a muscle sample from a group 3 animal (id.
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24 527 nb.143) (I.S. sulfadimethoxine-d6 not shown)
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3 **Tables and captions**
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5 Table 1. HPLC-DAD conditions for doxycycline and metacycline.
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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

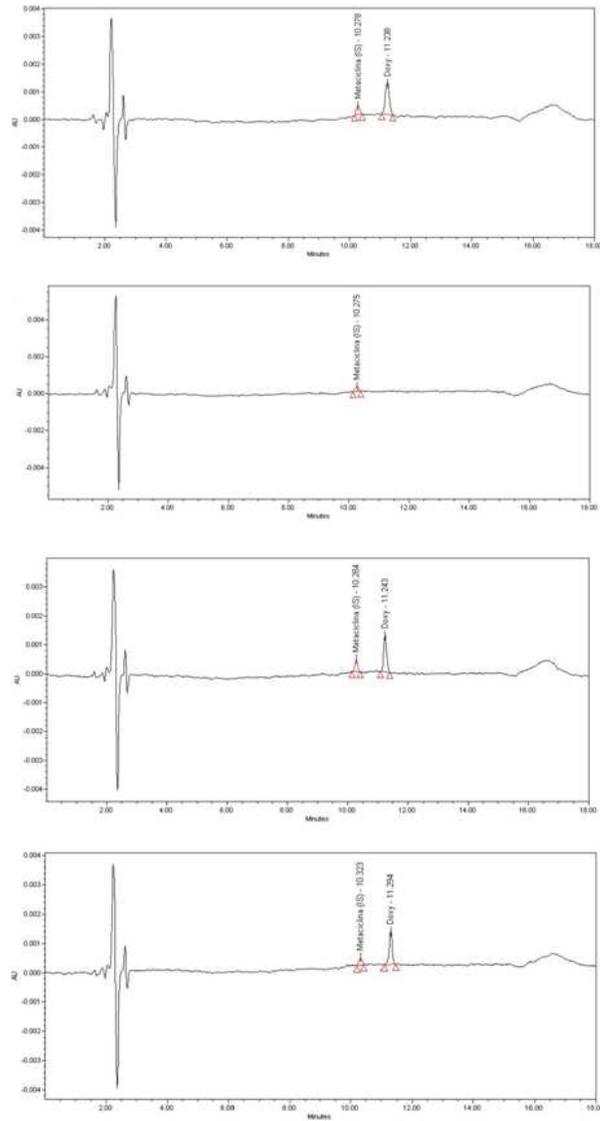


Figure 1. HPLC-DAD chromatograms. From top to bottom: doxycycline reference standard solution corresponding to 50 $\mu\text{g kg}^{-1}$, a negative muscle sample, a muscle sample spiked at 50 $\mu\text{g kg}^{-1}$ and a muscle sample from a group 2 animal (id. nb.142)
138x244mm (96 x 96 DPI)

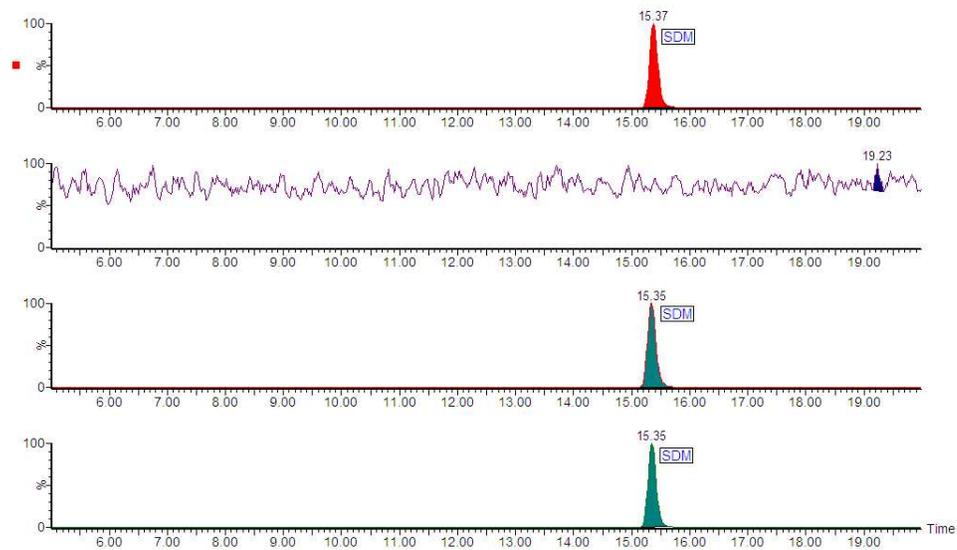


Figure 2. HPLC-ESI-MS/MS chromatograms. From top to bottom: sulfadimethoxine reference standard solution corresponding to $20 \mu\text{g kg}^{-1}$, a negative muscle sample, a muscle sample spiked at $10 \mu\text{g kg}^{-1}$ and a muscle sample from a group 3 animal (id. nb.143) (I.S. sulfadimethoxine-d6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

282x209mm (96 x 96 DPI)