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To cite this version:

Giulia Segato, Cristiana Benetti, Roberto Angeletti, Clara Montesissa, Giancarlo Biancotto. Doxycycline and sulfadimethoxine transfer from cross-contaminated feed to chicken tissues. Food Additives and Contaminants, 2011, 28 (07), pp.860-868. <10.1080/19440049.2011.569574>. <hal-00704671>

HAL Id: hal-00704671
https://hal.archives-ouvertes.fr/hal-00704671
Submitted on 6 Jun 2012

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Doxycycline and sulfadimethoxine transfer from cross-contaminated feed to chicken tissues
Doxycycline and sulfadimethoxine transfer from cross-contaminated 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 (<LoD and 31 µg kg\(^{-1}\) respectively), liver (13 µg kg\(^{-1}\) and 56 µg kg\(^{-1}\) respectively) and kidney (56 µg kg\(^{-1}\) and 115 µg 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
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
(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(SO\textsubscript{4})\textsubscript{5}H\textsubscript{2}O 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\textsuperscript{-1}; while other 15 kg were spiked with an aqueous solution of sulfadimethoxine to produce a final concentration of about 3 mg kg\textsuperscript{-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.
over a clean surface. Each spiked material was mixed several times during the drying period, and finally homogenised before sampling preparation.

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

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

Measured doxycycline and sulfadimethoxine concentration in feed were (mean ± SD):

(3.79 ± 0.28) mg kg\(^{-1}\) and (2.28 ± 0.17) mg kg\(^{-1}\), respectively, (see section 3. Results).

**Animals and treatments**

The project fulfilled the requirements of Italian Law n. 116/92 and further amendments on the protection and welfare of animals used in experiments. Twenty vaccinated chickens about 20 days old were weighted, allocated in cages (2 birds per cage) and fed dedicated feed and water *ad libitum*, for acclimatisation. After 18 days the animals were weighted and randomly allocated in three groups: group 1 (control), 4 animals; 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 $3.79 \pm 0.28$ mg kg$^{-1}$).

The 8 chickens of group 3 were fed the same feed used during the acclimisation period, contaminated with sulfadimethoxine (mean concentration $2.28 \pm 0.17$ 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 (Na$_2$HPO$_4$.2H$_2$O), hydrochloric acid (HCl) 1N, formic acid, acetic acid, ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl), aqueous ammonia solution 33% were all of analytical reagent grade. Distilled water
was de-ionised by an ultrapure water system (Sartorius Stedim Biotec, France).
Doxycycline and sulfadimethoxine-d6 (sulfadimethoxine-d6) pure standards were purchased from Fluka (Milan, Italy), sulfadimethoxine and metacycline pure standards were purchased from Riedel de Haen (Milan, Italy). A stock solution of 1000 mg l\(^{-1}\) for each drug was prepared by dissolving the appropriate amount of each substance in methanol. The intermediate working standard solutions for fortification and calibration curves (sulfadimethoxine, sulfadimethoxine-d6, doxycycline and metacycline: 1 mg l\(^{-1}\)) were prepared daily by diluting separately the correspondent stock solution with methanol.

Chelating sepharose fast flow was purchased from GE Healthcare (Uppsala, Sweden); OASIS HLB® 60mg/3ml solid phase extraction (SPE) columns were purchase by Waters (Milford, MA, USA).

Agilent SampliQ® QuEChERS EN extraction kit 5982-5650 and Agilent SampliQ® QuEChERS EN fatty dispersive-SPE kit for 15 ml 5982-5156 were purchased from Agilent (Agilent Tecnologies Inc., DE, USA).

Analysis of doxycycline and metacycline (internal standard) was performed on a liquid chromatographic system (HPLC) Alliance 2695 provided with a quaternary solvent delivery system, a column heater module and a sample cooling device, coupled to a diode array detector (DAD, 2996) from Waters (Milford, MA, USA); the chromatographic column was a Supelco Ascentis Express® C18 column, 2.7 µm, (150 x 4.6) mm, purchased from Supelco (Milan, Italy). HPLC analysis of sulfadimethoxine
and sulfadimethoxine-d6 was performed by an HPLC Alliance 2695 provided with a quaternary solvent delivery system, a column heater module and a sample cooling device coupled to a QuattroUltima® triple quadrupole system (MSMS) provided with an ESI source by Waters (Milford, MA, USA); the chromatographic column was an XTerra® Phenyl column, 3.5 µm, (100 x 2.1) mm, purchased from Waters (Milford, MA, USA).

**Doxycycline in chicken tissues**

The procedure was based on the method proposed by Cristofani et al., 2009. 3 g of homogenised tissue were extracted with 20 ml of succinic acid 0.1 M pH 4 and with 20 ml of MeOH by mechanical shaking for 30 sec and by sonication in an ultrasonic bath for 10 min. Liquid phase was separated from solid residue by centrifugation at 5000 g for 10 min. Extraction was repeated a second time by adding 10 ml of succinic acid 0.1 M pH 4 and 10 ml of MeOH. After centrifugation at 5000 g for 10 min, extracts were re-unified and purified through Metal Chelate Affinity Chromatography (MCAC) columns activated with 6 ml of distilled water, 3 ml of CuSO$_4$ 10 mM and 4 ml of distilled water. After extracts loading, columns were washed with 2 ml of succinic acid 0.1 M pH 4, 2 ml of distilled water, 2 ml of MeOH and 2 ml of distilled water; finally elution was performed by application of 8 ml of McIlvaine buffer.

The eluate was purified by OASIS HLB® SPE columns (60 mg, 3 ml) previously activated with 3 ml of MeOH, 3 ml of HCl 1 N, 3 ml of distilled water and washed with 3 ml of distilled water. Elution was achieved by 5 ml of MeOH. Solvent was evaporated.
to dryness under N$_2$ streaming and the residue was re-dissolved with 0.5 ml of oxalic acid 0.01 M before injection in HPLC-DAD.

Doxycycline determination was performed by HPLC-DAD according to the chromatographic and detection conditions reported in table 1.

Insert table 1

Quantification was performed against external calibration in pure solvent, as matrix effects were negligible, and quantified concentrations were corrected for recovery.

Metacycline was added as internal standard at the beginning of the procedure in unknown samples, not for strict quantitative purposes, but to prove, by an estimate of its detection, the good application of the method on each single sample.

The relevant method characteristics are reported in table 3.

**Sulfadimethoxine in chicken tissues**

To 2 g of homogenised tissue 8 ml of de-ionised water were added and the mixture was shaken by vortex for 30 sec. Acetic acid (1% in ACN, 10 ml) was added and the tubes were manually shaken for 30 sec; then an Agilent SampliQ® QuEChERS EN extraction salt packet was added to each tube. Sample tubes were capped tightly and shaken vigorously for 1 min.

After centrifugation at 5000 g for 5 min, an aliquot of the ACN layer (6 ml) was transferred into an Agilent SampliQ® QuEChERS EN fatty dispersive-SPE 15 ml tube containing 150 mg of PSA, 150 mg of C18EC and 900 mg of anhydrous MgSO$_4$. The samples were vortexed for 2 min and centrifuged at 5000 g for 5 min. 2 ml of each
purified extract were transferred into an empty tube and evaporated to dryness under N\textsubscript{2} streaming. The residue was re-suspended in 0.8 ml of a solution of formic acid 0.05 M and ACN (85/15, v/v) before LC-MS/MS analysis. Sulfadimethoxine determination was performed by LC-MS/MS according to the chromatographic and spectrometric conditions reported in table 2.

Quantitative analysis was achieved by internal standard method where deuterated sulfadimethoxine (sulfadimethoxine-d\textsubscript{6}) was chosen as internal standard introduced in each sample at the beginning of the analytical process. No correction for recovery was applied in this case.

Relevant method characteristics are reported in table 3.
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: $(3.79 \pm 0.28)$ mg kg$^{-1}$ of doxycycline and $(2.28 \pm 0.17)$ 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.
The most relevant performance characteristics of both methods are summarised in table 3.

In figure 1 representative HPLC-DAD chromatograms of a standard solution of doxycycline (at a concentration corresponding to 50 µg kg⁻¹ on sample), a negative muscle sample, a muscle sample fortified with doxycycline at 50 µg kg⁻¹ and an incurred muscle sample (doxycycline measured concentration: 48 µg kg⁻¹).

In figure 2 representative LC-MS/MS chromatograms of a standard solution of sulfadimethoxine (at a concentration corresponding to 20 µg kg⁻¹ on sample), a negative muscle sample, a muscle sample fortified with sulfadimethoxine at 10 µg kg⁻¹ and an incurred muscle sample (estimated concentration: 7 µg kg⁻¹).

Transfer of veterinary drugs in chicken tissues

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

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

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

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

The method adopted for doxycycline analysis in chicken tissues is actually a multi-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.

The method adopted for sulfadimethoxine in chicken tissues is also a multi-residue method applicable to the detection and quantification of sulfaquinoxaline, sulfachloropiridazine, suladiazone, 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 µg/kg in muscle, 56 µg kg\(^{-1}\) in liver and 115 µg kg\(^{-1}\) in kidney, whereas sulfadimethoxine was found at an average concentration of 5 µg kg\(^{-1}\) in muscle, 13 µg kg\(^{-1}\) in liver and 56 µg kg\(^{-1}\) in kidney.
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.

Acknowledgements

The project was financially supported by Italian Ministry of Health. The authors would like to thank Bruna Allegretta and Valentina Mozzo for their technical assistance throughout the study.
References


EMEA-Committee for Veterinary Medicinal Products. 1996. Summary report on Doxycycline hyclate EMEA/MRL/101/96-Final.


Figure captions

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)

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

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

<table>
<thead>
<tr>
<th>Time [minutes]</th>
<th>% Acetonitrile</th>
<th>% Oxalic acid 0.01M</th>
<th>% Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 - 1.0</td>
<td>9</td>
<td>70</td>
<td>21</td>
</tr>
<tr>
<td>1.0 - 6.0</td>
<td>22</td>
<td>70</td>
<td>8</td>
</tr>
<tr>
<td>6.0 - 12.0</td>
<td>22</td>
<td>70</td>
<td>8</td>
</tr>
<tr>
<td>12.0 – 13.0</td>
<td>9</td>
<td>70</td>
<td>21</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Time [minutes]</th>
<th>% Formic Acid 0.1%</th>
<th>% Acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 - 1.0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>1.0 - 15.0</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>15.0 – 16.5</td>
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<td>30</td>
</tr>
<tr>
<td>16.5 – 17.0</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>17.0 – 19.0</td>
<td>9</td>
<td>70</td>
</tr>
<tr>
<td>19.0 – 20.0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.25 ml min⁻¹</td>
<td></td>
</tr>
<tr>
<td>HPLC column</td>
<td>Phenyl, 3.5µm, (100 x 2.1)mm, XTerra (Waters)</td>
<td></td>
</tr>
<tr>
<td>Injection volume</td>
<td>10 µl</td>
<td></td>
</tr>
<tr>
<td>Autosampler temperature:</td>
<td>5° C</td>
<td></td>
</tr>
<tr>
<td>Column temperature:</td>
<td>30° C</td>
<td></td>
</tr>
</tbody>
</table>

Detection system: MS/MS analyzer. Operative conditions

| Sulfadimethoxine MSMS transitions | m/z: 311 > 156 (20 eV) | m/z: 311 > 108 (28 eV) |
| Sulfadimethoxine-d6 MSMS transition | 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              |

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Table 3. Recovery, precision, decision limit (CCα) and detection capability (CCβ), limit of detection (LoD) and limit of quantification (LoD) of the analytical methods for doxycycline and sulfadimethoxine determination in chicken muscle

<table>
<thead>
<tr>
<th>Fortification levels [µg kg⁻¹]</th>
<th>Mean found [µg kg⁻¹]</th>
<th>Recovery (%)</th>
<th>Precision (R.S.D. (%))</th>
<th>LoD [µg kg⁻¹]</th>
<th>LoQ [µg kg⁻¹]</th>
<th>CCα [µg kg⁻¹]</th>
<th>CCβ [µg kg⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Doxycycline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>21.5</td>
<td>85.9</td>
<td>11.0</td>
<td>14.6</td>
<td>24.8</td>
<td>109</td>
<td>118</td>
</tr>
<tr>
<td>50</td>
<td>41.0</td>
<td>82.1</td>
<td>8.9</td>
<td></td>
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Note: a Mean value of 6 repetitions for each fortification level on each day, three series on three different days
Table 4. Measured concentrations of doxycycline and sulfadimethoxine in muscle, liver and kidney after 10 days administration of contaminated feed.

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<th>Group</th>
<th>Animal number</th>
<th>Muscle [µg kg(^{-1})]</th>
<th>Liver [µg kg(^{-1})]</th>
<th>Kidney [µg kg(^{-1})]</th>
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Note: *N.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 µg kg⁻¹, a negative muscle sample, a muscle sample spiked at 10 µg 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)