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Residues of sulfadiazine and doxycycline in broiler liver and muscle tissues due to cross-contamination in the feed

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Abstract:	Veterinary drugs, such as antimicrobial compounds are widely used in poultry and may lead to the presence of residues in matrices of animal origin such as muscle and liver tissue. In this study, broilers received experimental feed, containing sulfadiazine or doxycycline at cross contamination levels of 2.5%, 5% and 10 % of the therapeutic dose in feed. Breast- and thigh muscle and liver samples were collected during treatment and depletion period and analyzed via liquid chromatography-tandem mass spectrometry (LC-MS/MS). Concentrations reached a plateau phase 3 to 5 days after starting the experimental feed. A rapid depletion of residues was noted after withdrawal of the experimental feed. No significant differences in measured concentrations were observed between the different muscle types. The residue concentrations for some experimental groups, the 10 % group of sulfadiazine and the 5% and 10 %

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Residues of sulfadiazine and doxycycline in broiler liver and muscle tissues due to cross-contamination of the feed

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Abstract

Veterinary drugs, such as antimicrobial compounds are widely used in poultry and may lead to the presence of residues in matrices of animal origin such as muscle and liver tissue. In this study, broilers received experimental feed, containing sulfadiazine or doxycycline at cross-contamination levels of 2.5%, 5% and 10 % of the therapeutic dose in feed. Breast- and thigh muscle and liver samples were collected during treatment and depletion period and analyzed via liquid chromatography-tandem mass spectrometry (LC-MS/MS). Concentrations reached a plateau phase 3 to 5 days after starting the experimental feed. A rapid depletion of residues was noted after withdrawal of the experimental feed. No significant differences in measured concentrations were observed between the different muscle types. The residue concentrations for some experimental groups, the 10% group of sulfadiazine and the 5% and 10% group of doxycycline, however exceeded the corresponding Maximum Residue Limits (MRLs).

Keywords: Residue; sulfadiazine; doxycycline; muscle; liver; feed; cross-contamination; depletion

Introduction

The veterinary drugs used therapeutically in poultry industry are generally given via mass application in feed or drinking water (Kan and Petz 2000). Antibiotics are used to raise production efficiency , as they enhance growth and feed efficiency and reduce disease (Donoghue 2003). Antibiotics have been used as growth promoters in the member states of the EU but concerns about development of antibacterial resistance led to the withdrawal of these agents as growth promoters in the EU since January 1, 2006 (Castanon 2007). Some antimicrobial drugs, such as sulfonamides are used to treat coccidiosis (Roudaut and Garnier 2002; Sentepe and Eraslan 2010). In veterinary medicine the sulfonamides are effective chemotherapeutics for bacterial or protozoal diseases (EMA 1995; BCFI 2010). Doxycycline belongs to the group of tetracycline antibiotics, which have a broad spectrum activity (EMA 1996). These drugs are widely used as mass medication in both pig and poultry industry. Concerning feed medication, in Belgium, sulfadiazine in combination with trimethoprim is available as pre-mix for pigs and non-laying hens and as oral powder to be mixed in the feed of pigs. Doxycycline is registered as a premix only for pigs and also an oral powder to be mixed in the feed of pigs is available (BCFI 2010). These drugs work systemically meaning that they must cross the intestinal wall and distribute in the body to exert their function (Kan and Petz 2000). After absorption from the gastrointestinal tract, the drug reaches the blood stream and is distributed throughout the whole body. This distribution can be quantified by some pharmacokinetic characteristics such as volume of distribution, which is largely determined by the physicochemical parameters of the compounds (Kan and Petz 2000), plasma and tissue protein binding and animal physiological status (Toutain and Lees 2004).

Residues exceeding established safety tolerances may be the result of misuse of antibiotics, due to misreading of the product label or not respecting the withdrawal time or the accidental administration of feed contaminated with pharmacologically active residues (Reyes-Herrera et al. 2005; Segato et al. 2011). According to Regulation 178/2002, a feed or feeding stuff is any substance or product, including additives, whether processed, partially processed or unprocessed, intended to be used for oral feeding to animals. In Directive 2001/82/EC, a medicated feeding stuff is described as any mixture of a veterinary medicinal product or products and feed or feeds that is ready prepared for marketing and intended to be fed to animals without further processing, because of its curative, preventive or other

properties. Medicated feeding stuffs are prepared in multi-product plants following good manufacturing practice guidelines (Segato et al. 2011). Carry-over, transfer from one production batch to the following batch may result in cross-contamination and may occur during feed processing for many reasons (Borras et al. 2011). In the EFSA reports, cross-contamination is defined as: “contamination of feeds that are produced after the production of a mixed feed, containing additives with residual amounts of the previous feed batch”. Cross-contamination may be product or establishment related. Some feed additives and premixes properties; adhesive strength-adhesion to walls, particle size and density (carrier, substance) and electrostatic properties influence cross-contamination behavior and affect how cross-contamination occur. The technological equipment in the feed mill; the design of dosage and grinding and mixing equipment can influence the level of cross-contamination (EFSA 2007; Borras et al. 2011). Under practical conditions, cross-contamination of residues is unavoidable, even when preventive measures are taken (EFSA 2007). A totally risk/residue free food production system does not exist. Consequently, active surveillance and compliance programs to ensure the proper use of antibiotics and the safety of the food supply are needed (Sasanya et al. 2005). Many governments have established antibiotic residue tolerances in edible animal tissues and have determined the target tissues for residue monitoring (Reyes-Herrera et al. 2005). In the European Union, these Maximum Residue Limits (MRLs) for doxycycline for pigs and poultry are set at 100 $\mu\text{g kg}^{-1}$ (muscle), 300 $\mu\text{g kg}^{-1}$ (skin and fat, liver) and 600 $\mu\text{g kg}^{-1}$ (kidneys) (EMEA 1996; BCFI 2010). For all sulphonamides, the MRL is 100 $\mu\text{g kg}^{-1}$ in muscle, fat, liver and kidneys for all food-producing animals (AFSCA 2004; BCFI 2010). Taking into account the unavoidable cross-contamination in practical field conditions, the aims of this study were to investigate the transfer ratios of two frequently used drugs from the feed to poultry edible tissues when provided at cross-contamination levels and to evaluate this transfer in relation to current MRL's.

Material and methods

Premix and oral powders, reagents and standards

The premix containing sulfadiazine and thrimetoprim was Tucoprim® powder, kindly provided by Pfizer (Brussels, Belgium). Doxycycline oral powder to be mixed in the feed was Doxycycline 75 % Kela®, kindly provided by Kela Veterinaria (Sint-Niklaas, Belgium). Analytical standards of sulfadiazine and doxycycline hyclate were from Sigma (Bornem,

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93 Belgium) and Acros (Geel, Belgium), respectively. The internal standards, namely
94 sulfachloropyridazine and demethylchlortetracycline were purchased from Sigma.
95 Acetonitrile, distilled water and methanol came from Biosolve (Valkenswaard, The
96 Netherlands). Water was HPLC grade and generated by a Q Gard 2 system (Millipore,
97 Billerica, MA, USA). The anhydrous sodium sulphate came from Merck (Darmstadt,
98 Germany). Filters for filtration of the sulfadiazine extract, i.e. 0.22 µm Millex® GV were from
99 Millipore (Billerica, MA, USA). The solid-phase extraction (SPE) columns used for the clean-
100 up of doxycycline containing samples were Oasis® columns (HLB SPE column 60 mg/3 ml,
101 Waters, Milford, MA, USA). The Robot Coupe 2, used to mince muscle and liver samples,
102 came from C-Tech Systems (Lille, Belgium).

103 ***Preparation of the experimental feed and animal experiments***

104 The feed preparation and the animal experiments were carried out at ILVO's Animals
105 Sciences Unit with approval from the ILVO ethical committee (EC no. 2008/89, 2009/108).

106 The experimental feed was mixed with sulfadiazine or doxycycline at cross-
107 contamination levels of 10 %, 5 % and 2.5 % of the maximum allowed dose of 250 mg kg⁻¹
108 feed for both drugs. The premix resp. oral powder was first mixed with a small amount of
109 blank feed in the Varimixer Bear (Dehaeck construct, Ghent, Belgium), which was then
110 added to the remaining blank feed in the feed mixer constructed by Silobouw (Zulte,
111 Belgium). The final experimental feed was mixed at 35 rpm during 30 minutes with a switch
112 of direction every five minutes. Before and between the preparations of each experimental
113 feed, rinsing batches were carried out. Ten samples were taken from the moving stream of
114 experimental feed and from the top (n=3), the middle (n=4) and the bottom (n=3) of each
115 experimental feed to determine the concentration achieved and two samples of each rinsing
116 batch were analyzed to exclude cross-contamination. Because of the large amount needed,
117 the preparation of each experimental feed was performed in two phases.

118 A growth trial was set-up with 575 one-day-old broilers, male Ross 308, which were
119 housed per concentration in a concrete floor pen under conventional conditions of
120 ventilation, temperature and lighting (22 hours light/day). The animals were randomly
121 assigned to three treatments, distributed among three pens and separated from each other
122 by a narrow walkway. Each study group counted 188 chickens, of which 102 were

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3 123 euthanized. A small group of 11 broilers was used as control group. Each study consisted of a
4 124 standard 2-phase feeding including a starter and grower diet. After an adaptation period of
5 125 12 days, experimental feed was provided during 14 days, followed by a depletion period of
6 126 17 days, during which the animals received blank feed. The animals were given free access to
7 127 water and feed; they were vaccinated for Infectious Bronchitis (IB), New Castle Disease
8 128 (NCD) and were treated for coccidiosis. The diets were supplemented with an anticoccidial
9 129 (salinomycin at 60 ppm for 1-35 days) and a NSP enzyme (Ronozyme WX at 50 FXU/kg feed)
10 130 but without any growth promoting antibiotic. The animals and housing facilities were
11 131 inspected daily for general and individual health, sufficient feed and water supply,
12 132 temperature and ventilation as well as for dead animals and unexpected events. The
13 133 zootechnical parameter, chicken weight, was noted at day 10 of pre-treatment period, days
14 134 1, 3, 5, 7, 9, 11, 13 of treatment period and days 1, 3, 5, 7, 9, 11, 13, 15, 17 of post treatment
15 135 period. From the age of 10 days onwards (day 10 of pre-treatment period), six animals per
16 136 concentration group were slaughtered by cervical dislocation every 2 days, chicken weight
17 137 was noted, breast feathers were removed and the complete breast muscles, the upper thigh
18 138 muscles and the liver (without the gall-bladder) were sampled. All samples were minced and
19 139 grounded with a Robot Coupe 2 and stored at -18 °C until analysis. A pooled sample was
20 140 prepared by mixing 5 to 10 g of every specific tissue from each of the 6 chickens.

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141 Analysis of the breast muscle was performed every 2-days starting from day 10 of the
142 pre-treatment period. Samples from the upper thigh muscles and the liver were only
143 analyzed for the 10% carry-over concentration and only during steady state.

144 ***Extraction and clean-up of feed samples***

145 For the analysis of sulfadiazine, 5 g of feed was weighed, 100 µL of the internal standard
146 sulfachloropyridazine (1 mg mL⁻¹) was added and the sample was allowed to stand for 10
147 minutes. After addition of 25 mL of methanol, the tube was vortex mixed and afterwards
148 placed on the horizontal shaker for 30 minutes. After centrifugation during 10 minutes at
149 3000 rpm, 5 mL of the supernatant was transferred into a tube and evaporated under
150 nitrogen to dryness in a water bath at 45 ° C. After redissolving the residues in 10 ml
151 acetonitrile:water (ACN: H₂O, 50:50), a dilution of 1:15 was performed in ACN:H₂O followed
152 by filtration using a 0.22 µm Millex®Gv. For the analysis of doxycycline, 5 g of feed was

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weighed and 1 ml of the internal standard demethylchlortetracycline ($20\text{ }\mu\text{g mL}^{-1}$) was added. After the addition of 25 mL of methanol, the samples were placed on a rotary shaker for 20 minutes and centrifuged for 10 min at 4000 rpm. Two hundred μL of the supernatant was transferred in a vial, 800 μL of HPLC water was added and the vial was vortex mixed.

Extraction and clean-up of broiler samples

For sulfadiazine analysis in muscle and liver, we used the method described by Mortier et al. (2005). Two grams of minced muscle or liver were weighed and the internal standard ($20\text{ }\mu\text{L}$ of $10\text{ }\mu\text{g mL}^{-1}$) was added. The samples were vortex mixed, were allowed to stand for 10 min and 6 g (muscle) or 8 g (liver) of anhydrous sodium sulphate was added. The tissue was carefully mixed with a spatula until a powdery mixture was obtained and 10 mL (muscle) or 20 mL (liver) of acetonitrile was added. After vortex mixing, the tubes were placed on a horizontal shaker for 30 min. The tubes were centrifuged (15 min, 4000 rpm), 5 mL (muscle) or 10 mL (liver) of the supernatant was transferred into a tube and was evaporated to dryness under nitrogen in a water bath of 45°C . Then, the sample was redissolved in 1 mL of an acetonitrile/water mixture (50:50,v:v) containing 0.1 % formic acid, vortex mixed, sonicated for 5 min and filtered through a $0.22\mu\text{m}$ filter into a HPLC vial.

For doxycycline analysis in tissues, 2 g of minced tissue were weighed and extracted as described by Cherlet et al (2003). After the addition of the internal standard demethylchlortetracycline ($50\text{ }\mu\text{L}$ of $10\text{ }\mu\text{g mL}^{-1}$), the samples were vortex mixed and were allowed to stand for 5 min. Ten mL of a 0.1 M sodium succinate buffer was added, the samples were placed on a rotary shaker for 20 minutes and centrifuged at 4000 rpm for 10 min. The supernatant was added to a centrifuge tube containing 1 mL of a 20 % trichloroacetic acid, vortex mixed and centrifuged at 4000 rpm for 10 min. The supernatant was filtered through a Whatman® filter paper and was ready for a further solid-phase clean-up step. In the solid-phase clean-up, the Oasis® HLB (hydrophilic lipophilic balance) column was preconditioned consecutively with 3 mL methanol, 3 mL of a 1N HCl solution and 3 mL of water and the extract was allowed to pass slowly through the HLB column. The HLB column was washed with 1 mL of water and dried. The analytes were eluted with 3 mL of methanol and the eluate was evaporated to dryness at 40°C under nitrogen. After redissolution with 250 μL of a 0.5 % formic acid solution and vortex mixing, the sample was transferred to an autosampler vial.

184 ***LC-MS/MS analysis***

185 The sulfadiazine analysis was performed on a Waters Alliance 2695 Separation Module
186 combined with a Waters Micromass Quattro MS instrument. An X-terra® column (C₁₈ 5 µm,
187 150 x 2.1 mm) was used and the analysis was performed with a gradient of 0.1 % formic acid
188 (FA) in H₂O (solvent A) and 0.1 % FA in ACN (solvent B). The analysis of doxycycline was
189 performed on a Waters Alliance 2695 Separation Module combined with a Waters
190 Micromass Quattro Ultima MS instrument. Separation was performed on a PLRP-S polymer
191 column (5 µm, 150 x 2.1 mm). An isocratic mobile phase consisting of 70 % solvent A (HPLC
192 H₂O+0.5% formic acid and 0.001 M oxalic acid; 0.5 % tetrahydrofurane) and 30 % of solvent
193 B (ACN) was used. The analysis of sulfadiazine and doxycycline was performed in MRM mode
194 and electrospray positive ion mode (sulfadiazine: m/z 250.94 → 107.95, 250.94 → 92.18,
195 250.94 → 156.06; doxycycline: m/z 462.45 → 428.1). In this study, the limit of quantification
196 (LOQ) of the method was 5 mg kg⁻¹ and 2.5 mg kg⁻¹ for sulfadiazine and doxycycline
197 respectively for feed. For muscle and liver, the method LOQ was 2 and 10 µg kg⁻¹ for
198 sulfadiazine and doxycycline.

199 ***Pharmacokinetic and statistical analysis***

200 These studies were not repeated, therefore, no statistical analysis of the effect of the
201 experimental feed on the zootechnical parameters was performed. Due to the limited
202 dataset of the experimental feed, no statistical analysis to determine the homogeneity of the
203 experimental feeds could be carried out. The terminal elimination half-life and elimination
204 rate constant in tissues were calculated using pharmacokinetic (PK) functions for Excel and
205 were based on the terminal slope of the tissue concentration-time curve after withdrawal of
206 the experimental feed (Usansky et al, Allergan, Irvine, USA). Statistical analysis was
207 performed with Statistica 9.0 (StatSoft. Inc., Tulsa, OK, USA). Significance level α was set at
208 0.05. Statistical analysis was performed on the mean of the residue concentrations of the
209 individual chickens and the pooled sample for each of the different matrices. The
210 comparison between measured residue concentrations in thigh muscle and in breast muscle
211 was performed with a t-test for dependent samples. The pooled sample on day 13 of
212 treatment period was analysed six times. The mean of these results was compared with the
213 mean of the individual chickens by an independent t-test.

Results

Zootechnical parameters and experimental feed

The effects of experimental feed on the performance parameter, broiler weight, were studied. The mean broiler weights are presented for sulfadiazine and doxycycline in Figure 1.

For both sulfadiazine and doxycycline the maximum authorized dose is 250 mg kg⁻¹ (instruction leaflets). Therefore a value of 25, 12.5 and 6.25 mg kg⁻¹ was calculated for the 10 %, 5 % and 2.5 % carry-over groups, respectively. Mean concentrations ± standard deviation and recovery for the 10 %, 5 % and 2.5 % groups were 21.2 ± 1.8 (79 %), 12.7 ± 0.9 (93 %) and 5.5 ± 0.4 (88 %)mg kg⁻¹ for sulfadiazine and 24.7 ± 4.6 (99 %), 13.2 ± 4.1 (106 %), 5.5 ± 1.2 (88 %) mg kg⁻¹ for doxycycline.

Residue concentrations in breast muscle, thigh muscle and liver

The residue concentrations for respectively sulfadiazine and doxycycline for all groups in breast muscle are shown in Figure 2. The plateau phase was reached at day 3 for all dietary treatments of the doxycycline and the 2.5 and 5 % carry over group of sulfadiazine. A rapid depletion phase was noted after withdrawal of the experimental feed. The breast muscle tissue elimination half-life was calculated for both molecules but due to the rather fast decline below the LOQ, elimination half-life could only be calculated for the 5 and 10 % group of doxycycline, namely 3.1 and 3.5 days, respectively. The concentrations in the three tissue matrices for the 10 % group during plateau phase are presented in Figure 3. Small differences in concentrations were noted between the different types of muscles for sulfadiazine as well as for doxycycline but these were not statistically significant. Measured residue concentrations in liver were similar from the concentrations in muscle for sulfadiazine although higher concentrations were measured in liver compared to muscle for doxycycline. The ratio liver/breast muscle was 1.2 and 1.8; the ratio liver/thigh muscle was 1.3 and 1.9 for sulfadiazine and doxycycline respectively. Transfer factors,

$$\frac{\text{concentration in the matrix on day 13 of treatment period for the 10 \% group}}{\text{measured concentration in the feed}} \times 100$$

are presented for liver, breast muscle and thigh muscle for sulfadiazine and doxycycline. Transfer factors of breast muscle, thigh muscle and liver were 1.04, 0.95 and 1.37 % for sulfadiazine and 1.27, 1.62 and 2.90 % for doxycycline.

The measured residue concentrations of the tissues from the 6 individual chickens and the six repetitive analyses' of the pooled sample were compared. The calculated means and standard deviations are presented in Table 1. For both molecules no significant difference between the 2 means was noted for all matrices ($P>0.05$).

Discussion

For the experimental feed of sulfadiazine and doxycycline, the target concentrations of 2.5, 5 and 10 % of the maximum allowed dose were reached (79 – 106 %) but no statements can be made about the homogeneity of the experimental feeds. The achievement of adequate mixing of an active ingredient in final feed depends on the composition of the final feed, the precision and the size of samples taken for analysis and other factors such as particle size, electrostatic properties, types of mixing machinery and mixing in stages or trituration (EMEA 1996). The ideal way to mix a drug is to add it at the beginning of the pre-mix process just prior to pelleting (Daniel 2009). Good sampling requires sufficient samples of adequate size and sampling from a moving stream is better than static sampling (Borras et al. 2011). Since very small volumes of pre-mixes were added to large amounts blank finished feed and because the experimental feed differs in composition from the feed used for target species for which the medicated pre-mix is intended, it is not easy to guarantee a homogeneous feed. For each experimental feed, ten dynamic samples were taken after 30 min of mixing at the top ($n = 3$), the middle ($n= 4$) and the bottom ($n=3$) of the final feed in order to provide as good as possible sampling procedure.

For both compounds studied, no significant differences were found between the concentrations in breast and thigh muscle. Most of the sampling occasions, the residue concentrations in breast muscle were slightly higher compared to the concentrations in thigh muscle. For doxycycline, this was confirmed by Atef et al. (2002) while Reyes-Herrera et al. (2005, 2008) found significantly higher concentrations of enrofloxacin in breast muscle compared to thigh muscle but no difference in concentrations between different breast sections (Reyes-Herrera et al, 2005; Reyes-Herrera et al, 2008). Other authors reported differences between residual concentrations in thigh and breast muscle, which may be explained by a different amount of intramuscular fat content. According to Reyes-Herrera, different antibiotics may have different sites of preferential deposition and therefore it may

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be interesting to determine the edible tissue with the highest concentration if the target tissue is muscle (Reyes-Herrera et al. 2005). Elimination half-lives were calculated for breast muscle. Atef et al. (2002) found an elimination half-life of 1.8 days for breast muscle in contrast to our calculated value of 3.1 to 3.5 days. This difference may be due to the different ways of oral administration and the duration of the administration, a dose of 15 mg/kg was provided orally twice daily during 5 successive days. Doxycycline had higher residue concentrations in the liver compared to muscle as reflected by the feed/tissue transfer ratio's, which has also been described by other researchers (Atef et al. 2002; Ismail and El-Kattan 2004). Doxycycline is known to be well absorbed from the gastro-intestinal tract (Yoshimura et al. 1991), to have a high volume of distribution with the highest detected levels in liver and kidney (EMEA 1996) and to have a high tissue binding (Santos et al. 1997). Doxycycline has an absolute oral bioavailability of 73.4 % and may be metabolised up to 40 % by the liver and is largely excreted in faeces, via bile (EMEA 1996; Laczay et al. 2001). After oral dosing, a good bioavailability (approximately 80 %) of sulfadiazine is obtained in non-fasted chickens (Baert et al. 2003). Sulphonamides may be metabolized in various tissues, mainly the liver at varying degrees and through varying mechanisms such as acetylation and oxidation and are mainly excreted via the kidneys (Sentepe and Eraslan 2010).

In this study, besides individual tissue analysis we also analyzed a pooled sample. We may conclude that pooled tissue samples are a good reference for the mean of the 6 individual chickens for the studied matrices. An important remark is the animal dependent variability, noted for each of the different matrices. This needs to be kept in mind while sampling and interpreting the results in case of a cross contamination problem occurs on a farm.

For muscle, MRL's have been set at 100 $\mu\text{g kg}^{-1}$ for sulfadiazine as well as for doxycycline (BCFI 2010). The 10 % group of sulfadiazine and the 5 and 10 % group of doxycycline generated concentrations above the MRL, whereas intake of 5 % carry-over of sulfadiazine and the 2.5 % of doxycycline resulted in concentrations around the MRL. Only the 2.5 % group of sulfadiazine generated concentrations that were below the MRL. For liver, MRL's have been set at 100 $\mu\text{g kg}^{-1}$ and 300 $\mu\text{g kg}^{-1}$ for sulphonamides and doxycycline resp. (BCFI 2010). Only liver tissue from the animals receiving the 10% concentration in feed was analyzed and resulted in concentrations exceeding the MRLs. It may be concluded that

cross-contamination may lead to the presence of residual concentrations above MRL. However, once the “cross-contaminated feed” was no longer administered, we observed a fast decline in measured concentrations.

The use of cross-contaminated feed may have several consequences for animal as well as human health. Besides the possible adverse effect on the health of non-target species and the indirect toxic effect by the promotion of resistant strains of bacteria, the MRL may also be exceeded in food matrices of animal origin, and consequently food safety may not be guaranteed (Donoghue et al. 1997; Segato et al. 2011; Vincent et al. 2011). These experiments indicate that important residual levels of both molecules occur in both muscle and liver after feeding cross-contamination levels. An unaware farmer, who provides feed accidentally contaminated with pharmacologically active residues to his animals, may also face legal repercussions because of exceeding the MRL in matrices of animal origin. However, the problem of residual concentrations due to the use of cross-contaminated feed can be resolved within days after switching to blank feed since both molecules showed quick residue depletion once the experimental feed was no longer administered.

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420

421 Tables

422 Table 1: Mean and standard deviation ($\mu\text{g kg}^{-1}$) of the pooled sample and the six individual
423 chickens on day 13 of the treatment period.

424

425 Figures

426 Figure 1: Mean broiler weight (n= 6/conc. group) of the 3 concentration groups of the
427 sulfadiazine and doxycycline study during pre-treatment period (day 1 -> day 12 included),
428 treatment period (day 13 -> day 26 included) and depletion period (day 27 -> day 43).

429 Figure 2: Residue concentrations of the 3 sulfadiazine and doxycycline concentration groups
430 (2.5; 5 and 10 %) in the breast muscle during treatment period (day 1 -> day 14 included)
431 and depletion period (day 15 -> day 31).

432 Figure 3: Residue concentration in breast muscle, thigh muscle and liver for sulfadiazine 10%
433 and doxycycline 10 % during treatment period (day 5 -> day 14 included) and depletion
434 period (day 15).

Molecule	Sample type	Matrix	Mean and standard deviation ($\mu\text{g kg}^{-1}$)
Sulfadiazine	Six analyses of the pooled sample	Breast muscle	201.6 ± 15.7
		Thigh muscle	172.2 ± 7.7
		Liver	358.0 ± 20.4
	Analysis of six individual chickens	Breast muscle	202.6 ± 49.8
		Thigh muscle	173.1 ± 30.3
		Liver	355.8 ± 109.0
Doxycycline	Six analyses of the pooled sample	Breast muscle	485.7 ± 88.8
		Thigh muscle	344.4 ± 13.3
		Liver	728.4 ± 74.4
	Analysis of six individual chickens	Breast muscle	474.8 ± 107.5
		Thigh muscle	327.7 ± 32.7
		Liver	726.2 ± 74.7