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Carryover of Maduramicin from Feed Containing Cross-Contamination Levels into Eggs of Laying Hens

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Supporting Information

ABSTRACT: Maduramicin is a coccidiostat authorized as feed additive in the European Union for chickens and turkeys for fattening but not for laying hens, considering the risk of residues in eggs. The unavoidable cross-contamination of non-target feed with coccidiostats is regulated by Commission Directive 2009/8/EC and resulting carry-over in food by Commission Regulation (EC) No. 124/2009. To verify the compliance of the maximum levels for maduramicin in feed (50 μg/kg) and eggs (2 μg/kg), the carry-over from feed into eggs was investigated. Diets containing 10, 30, and 50 μg of maduramicin/kg of feed were fed to laying hens. Feed, egg white, and yolk were analyzed by LC−MS/MS. Maduramicin residues were only detected in in egg yolk. Feeding the 10 μg/kg maduramicin diet resulted in maduramicin concentrations up to 2.5 μg/kg in whole eggs, already exceeding the maximum level. A carry-over rate of 8% maduramicin from feed into eggs was calculated.

KEYWORDS: *Maduramicin, coccidiostats, feed, eggs, LC−MS/MS, cross-contamination, carryover*

INTRODUCTION

Maduramicin is authorized as coccidiostat for chickens and turkeys for fattening to prevent infection from coccidial parasites.[1] The polyether ionophore affects the parasitic cell by transporting monovalent ions across the cell membrane and thereby disturbing its osmotic balance.

There is no authorization for applying maduramicin to laying hens due to the risk of resulting residues in eggs. Despite maintaining the application of good manufacturing practices in feed production, contamination of non-target feed with traces of maduramicin from a preceding production lot is unavoidable. This so-called "cross-contamination" is regulated by Commission Directive 2009/8/EC, setting a tolerance for the unavoidable cross-contamination to 1% of the maximum authorized content in feed (50 μg/kg for maduramicin) for sensitive non-target species.[2]

For food of animal origin and for deriving products, maximum levels of maduramicin (2 μg/kg in eggs) and other coccidiostats are laid down in Commission Regulation (EC) 124/2009.[3] These maximum levels were set by the European Commission in order to protect public health insofar as no maximum residue levels (MRL) are yet fixed for these substances in specific food from non-target animals. The current maximum cross-contamination levels for coccidiostats were derived from the risk assessment stated in the "Opinion of the Scientific Panel on Contaminants in the Food Chain (CONTAM)" by the European Food Safety Authority (EFSA).[4]

Several ionophoric coccidiostats such as lasalocid,[5] salinomycin,[6] narasin,[7] and monensin[8] have been examined in feeding studies regarding their ability to be transferred from feed into eggs of laying hens. Despite structural similarity, the transferred amounts differed markedly depending on the substance. Therefore, carry-over rates of ionophoric coccidiostats cannot be derived from one substance to another but have to be determined individually.

No data were available on the amount of maduramicin residues that might occur in eggs, milk, or meat and offal from non-target animal species after feeding cross-contaminated feed. The European Commission established maximum values of unavoidable carry-over of coccidiostats in non-target feed of 1% of the highest authorized level. Compliance of the maximum level in feed (Commission Directive (EC) 2009/8)[2] with the specified maximum level in foodstuffs (Commission Regulation 124/2009)[3] was not demonstrated experimentally.

Recent data of Slovenian authorities[9] indicated that feed containing the maximum cross-contamination level of maduramicin may lead to maduramicin levels in eggs that exceed the maximum level in foodstuffs. Consequently, a revision of the maximum levels might be necessary.

This paper presents a comprehensive study of maduramicin transfer from feed into eggs of laying hens, conducted to determine carry-over rates at cross-contamination levels. Homogenization and control of homogeneity and of the concentrations in the contaminated feed during the feeding period formed a major part of the investigation. Experience showed that these factors had a considerable impact on the results. Until now this item was discussed marginally. Mortier et al.[7] were the first who described the method of preparation of the experimental diets in detail and reported measured coccidiostat concentrations in prepared feeds. Focusing on the carry-over subject, no data from previous studies were published concerning evaluation of homogeneity of the artificially contaminated feed or concerning analysis of the diets during the feeding period.[5,7,8,10,11]

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Distribution of maduramicin between egg white and yolk as well as the excretion from eggs were examined. Data were used to evaluate the transfer of maduramicin from contaminated feed into eggs by means of the calculated parameters carry-over rate and transfer factor.[12]

For the determination of maduramicin concentrations in feed and eggs LC−MS/MS detection was used following a quick and simple sample preparation protocol. At present, LC−MS/MS is the method of choice for analysis of coccidiostats in complex matrices as food and feed,[13,14] as in many other fields of food safety.[15]

MATERIALS AND METHODS

Study Design. The feeding trial was permitted by the State Office of Health and Social Affairs Berlin and was performed in compliance with the German Protection of Animals Act. The study consisted of three parts with one individual group of laying hens (Lohmann Brown Classic) for each maduramicin concentration. Animals were adapted to their environment before starting the feeding trial. During a short pre-experimental period (pre-period) maduramicin-free feed was fed in order to reveal a possible background level of maduramicin in the eggs. Maduramicin containing diets were fed in the experimental period (main period) for 21 days. After displacement of the coccidiostat, an 18-day post-experimental period (post-period) with maduramicin-free feed followed. The individual groups of 10 laying hens each were fed with three concentrations of maduramicin (group 1, 10 μg/kg; group 2, 30 μg/kg; group 3, 50 μg/kg) according to 20%, 60%, and 100% of the maximum level in non-target animal feed or 0.2%, 0.6%, and 1.0% of the authorized maximum level in target animal feed, respectively. Animal groups were kept in separate rooms of about 20 m^2 size with free access to water and feed. The daily quantity of diet for each group was calculated assuming an individual average feed intake of 120 g. A sample was taken before feeding to check the maduramicin concentration of the ration. Remaining feed from each daily ration was collected, weighed, and analyzed for maduramicin concentrations. Eggs were collected daily during all periods.

Blending and Homogenization of Maduramicin Diets. Experimental feed was prepared by using the commercial premix Cygro, containing 1% of the coccidiostat. At first an intermediate feed mixture containing 5 mg/kg maduramicin was prepared by mixing the premix and a basic feed for laying hens. The basic feed consisted of corn, wheat, soy extraction meal, calcium carbonate, molasses, corn gluten feed, wheat bran, soy bean oil, sodium phosphate, methionine, and sodium chloride. It was previously tested and proven to be free of maduramicin. A drum hoop mixer was used for agitation. After testing of homogeneity, calculated amounts of the intermediate mixture were weighed for blending with basic feed to achieve the concentration levels of 10 μg/kg (group 1, low level), 30 μg/kg (group 2, mean level), and 50 μg/kg (group 3, high level). Homogenization was accomplished by applying the cross-riffling technique,[16] originally been developed for homogenization of black carbon samples. The entire material was divided into eight portions, which were homogenized by shaking for 1 h. Each portion was divided into eight subunits and recombined following a staggered scheme. The recombined fractions were shaken once again for 1 h, remixed into one main bulk, and finally homogenized for another 2 h. A total of 50 kg feed for each maduramicin diet was prepared consisting of two main batches of 25 kg feed due to the available technical equipment. Homogeneity was verified by analyzing maduramicin concentrations in 10 random samples per batch and evaluating results by analysis of variance (ANOVA). All except one batch proved to be homogeneous. As every batch contained the amount of feed required for 19 of the 21 days, feeding of the inhomogeneous lot of feed for 2 days was accepted. No additional step of homogenization was performed, as no improvement of the distribution of maduramicin in the diet was expected according to our experience. During the main feeding period the total daily diet of each group was shaken overhead for 1 h before feeding to ensure a homogeneous distribution of maduramicin in the feed mixture.

Sampling. Eggs of the entire study were separated into egg white and yolk directly after collection. Both components were weighed, homogenized, and labeled according to their group, day of laying, and individual egg. Samples were stored at 4 °C until analysis. Egg white and yolk were analyzed separately in order to ascertain the distribution of maduramicin in eggs. After substitution of contaminated feed by maduramicin-free feed, eggs were collected for additional 14 days and analyzed to investigate excretion of maduramicin from the eggs. Feed samples of approximately 50 g for testing homogeneity were taken from each feed batch directly after the homogenization procedure by means of an all-layer sampler. During the experimental feeding period samples (∼50 g) of each daily diet were taken using a sample spoon. The point of sampling was after shaking the daily diet, directly before feeding (pre-feeding sample). Remaining feed from each day of the main period was collected from the trough before provision of the new feed ration. No sampling was needed, as the total amount of remaining feed from 1 day was kept each as one sample.

Determination Method. *Reagents and Chemicals.* Acetonitrile from Merck KGaA (Darmstadt, Germany) was of LC−MS quality. All water used was deionized and purified by a TKA water purification system (TKA, Niederelbert, Germany). Ammonium acetate (Fluka, Buchs, Switzerland) and formic acid (Merck) were of analytical grade. The analytical standard maduramicin ammonium salt (91% puritiy) from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and the internal standard nigericin sodium salt (98% purity) were obtained from Fluka. Standard stock solutions were made by dissolving 10 mg of the solid substance in 10 mL of acetonitrile.

Sample Preparation. Feed samples were ground to a particle size of 1 mm using an ultracentrifugal mill (Retsch, Haan, Germany) and homogenized by rotating for 1 h, and aliquots of 5 g were weighed into 50 mL polyethylene screw-cap tubes. Samples were allowed to stand for about 10 min after adding 40 μL of 0.1 ng/mL (egg white/yolk samples) or 25 μL of 10 ng/mL (feed samples) of the internal standard nigericin. If necessary, the calibration curve, volume, and concentration of the added internal standard solution were adapted to the expected concentration range of the samples. Then an amount of 20 mL of acetonitrile containing 1% formic acid (v/v) was added for extraction and tubes were shaken overhead for 1 h. Eggs were separated into egg white and yolk for the analysis. The egg components were homogenized using an Ultra Turrax homogenizer (IKA, Staufen, Germany) before weighing 2 g into 50 mL polyethylene screw-cap tubes. The internal standard was added, and samples were shaken on a vortex mixer. The extraction solvent, 4 mL of acetonitrile with 1% formic acid (v/v), was added while shaking on the vortex mixer. Both feed and egg samples were centrifuged at 3000*g* for 10 min, and 1 mL of the supernatant was evaporated to dryness under a nitrogen stream in a heated (45 °C) metal rack (Barkey, Leopoldshöhe, Germany). Prior to evaporation, egg yolk supernatants were cleaned by an additional nonretentive solid phase extraction (HybridSPE, Supelco, Geisenhofen, Germany). Therefore, an amount of 1.3 mL of the raw egg yolk extract was passed through the cartridge without prior conditioning or equilibration and 1 mL of the cleaned solution was evaporated. The residues were reconstituted in 500 μL (feed samples) or 250 μL (egg samples) of acetonitrile/water 90/10 (v/v) and filtered through centrifuge filters (Nylon, 0.2 μm, VWR) at 10000*g* before analysis.

LC−MS/MS Analysis. An LC−MS/MS system consisting of an Agilent 1200 series HPLC with autosampler, degasser, quaternary pump, and column oven (Agilent Waldbronn, Germany) and an API 4000 QTrap quadrupole tandem mass spectrometer (AB Sciex, Ontario, Canada) was used. The ion source was operated in positive electrospray mode. Acquisition and processing of LC−MS/MS data were performed with the AB Sciex software Analyst 1.5.

Chromatographic separation was carried out on a reversed phase C8 column (Hypersil Gold C8, 100 mm × 2,1 mm, 3 μm; Thermo Electron Corporation, Bellefonte, PA, U.S.) combined with a guard column (Hypersil Gold C8 drop-in guard cartridge 10 mm × 2,1 mm, 3 μm, Thermo) at 50 °C oven temperature. The injected sample volume was 5 μL. Starting conditions of the HPLC run were 10% mobile phase A (buffer, 5 mmol of ammonium acetate) and 90%

mobile phase B (acetonitrile containing 1% (v/v) phase A) at 200 μL/min flow rate. This composition was maintained for 3.5 min. Then phase B was decreased to 50% in 0.1 min and held for 3.5 min to remove potentially interfering compounds of moderate polarity from the column. The eluent was returned to the initial composition in 0.1 min and equilibrated 7.8 min for the next run. Retention times were 2.7 and 3.5 min for maduramicin and nigericin, respectively. A chromatogram of a feed sample fortified at the maximum level (50 μg/kg) and a yolk sample (fortified at 2 μg/kg) is presented in Figure 1.

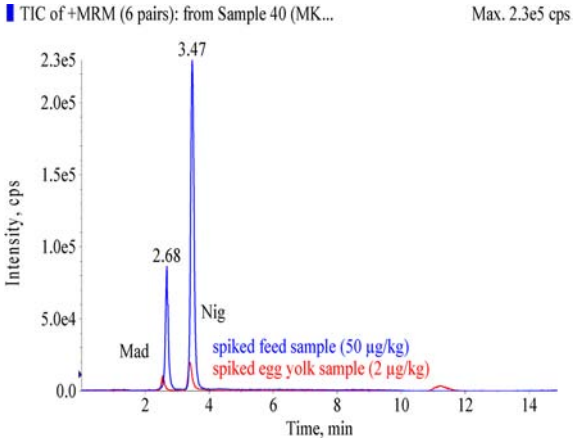

Figure 1. LC−MS/MS chromatogram of blank materials spiked with maduramicin (Mad) and nigericin (Nig). The upper line shows a feed sample spiked at 50 μg/kg. The lower line shows an egg yolk sample spiked at 2 μg/kg.

The source parameters were optimized for maduramicin and nigericin and therefore set to 5000 V ion transfer voltage, 450 °C source temperature, 20 psi of curtain gas, 60 psi of nebulizer gas and 30 psi of heating gas. Both analytes were determined as ammonium adducts $[M + NH_4]^+$. The selected MRM transitions, declustering potentials (DP), and collision energies (CE) were as follows: for maduramicin 1, m/z 934.6 → 629.4 (DP = 91 V, CE = 37 V); for maduramicin 2, m/z 934.6 → 647.4 (DP = 91 V, CE = 29 V); for maduramicin 3, m/z 934.6 → 393.2 (DP = 91 V, CE = 41 V); for nigericin 1, m/z 742.5 → 657.4 (DP = 86 V, CE = 39 V); for nigericin 2, m/z 742.5 → 675.5 (DP = 86 V, CE = 35 V); for nigericin 3, DP = 86 V, CE = 43 V. Dwell times were set to 200 ms for the first transition (quantifier) and 50 ms each for the second and third transitions (qualifiers).

Method Validation and Quantification of Maduramicin. The method described was in-house validated including evaluation of linearity, recovery, limit of detection (LOD), limit of quantification (LOQ), and precision data for the three sample types feed, egg white, and yolk. Blank samples of feed, egg white, and yolk were fortified at three concentration levels (for feed: 25, 50, 100 μg/kg; for egg white/yolk: 0.5, 1, 2 μg/kg) with five replicates to assess the recovery of the method. The spiked samples were analyzed on 3 days to obtain data for intraday and interday repeatability. Detection and quantification limits were assessed according to the calibration curve method of DIN 32645.[17]

Maduramicin concentrations were determined by means of matrix-assisted calibration because of the occurrence of considerable matrix effects in the LC−MS/MS analysis. For graphic charts yolk samples with analyte levels between LOD and LOQ were considered to contain maduramicin concentrations equivalent to the limit of detection.

Data Interpretation. Maduramicin concentrations were converted from yolk to whole egg. For each egg the absolute amount of maduramicin in the yolk was divided by the sum of weights of the respective egg white and yolk. Determined maduramicin concentrations in feed and eggs were used for calculation of the parameters describing the transfer of maduramicin from feed into eggs. The carry-over rate is calculated as the percentage of the maduramicin intake of the laying hens in μg per day which is transferred into eggs per day after reaching a concentration plateau. The transfer factor can be derived from the correlation between the applied contaminant levels in feed and the resulting concentration levels in eggs, where it corresponds to the slope of the correlation function.[8] It is expressed as the concentration unit transferred into eggs (e.g., μg/kg egg) per unit contained in the diet (μg/kg feed).[12]

RESULTS

Method Validation. Different extraction solvents were investigated during method development concerning extraction efficiency and matrix effects (results not shown). Acetonitrile containing 1% formic acid gave the best results for the three matrices. However, signal suppression was observed requiring quantification of maduramicin via matrix calibration. Linearity was proven for matrix calibration curves at 10−100 ng/mL (corresponding to 10−100 μg/kg) for feed and at 1−15 ng/mL (corresponding to 0.25−3.75 μg/kg) in egg white and yolk. Where other calibration ranges were necessary for quantification of the samples, linearity was checked individually. Correlation coefficients of matrix-assisted calibration curves were at least 0.99. LOD and LOQ were found to be 0.3 and 1.0 μg/kg in egg white and 0.3 and 0.9 μg/kg in egg yolk. In feed LOD and LOQ were 2.0 and 5.8 μg/kg. Relative recoveries ranged from 100.6% to 109.8% in fortified feed samples, from 107.5% to 119.0% in egg white, and from 87.0 to 99.0% in yolk samples. Mean intraday repeatability and interday repeatability RSD for the three fortification levels were 5.5% and 7.8% in egg white, 8.5% and 11.7% in egg yolk, 7.8% and 10.1% in feed, respectively. Robustness of the method for a change of personnel and to the state of freshness of egg samples was also proven. No degradation of maduramicin in the samples was expected, as analysis was performed within at least 7 days. However, stability of maduramicin in the sample material was confirmed by repeated analysis of several random samples. Additional in-house validation parameters were determined according to Commission Decision 2002/657/EC and published elsewhere.[18]

General Observations. The daily number of eggs and weights of white and yolk were registered, and possible influences of the experimental diets were examined. In the three groups with durations of 42 days each a total of over 1000 eggs were derived. No correlation of maduramicin levels in eggs and laying performance of hens was observed.

Feed Samples. Mean maduramicin concentrations and standard deviations of analyzed feed samples are shown in Figure 2. In this chart mean levels in the pre-feeding samples of maduramicin diet are compared to those in the remaining feed samples and samples from homogeneity testing of each group. Maduramicin concentrations of the daily pre-feeding samples (group 1, 10.2 μg/kg; group 2, 18.1 μg/kg; group 3, 30.3 μg/kg) were lower than those determined within homogeneity tests (13.6 μg/kg, 26.2 g/kg, 46.3 μg/kg). Highest maduramicin concentrations were determined in the remaining feed samples, with mean values of 13.5, 31.8, and 76.7 μg/kg.

Intake of Maduramicin. Maduramicin levels in pre-feeding samples and remaining feed were determined in order to estimate the mean intake of maduramicin per hen per day. For the calculation of the carry-over rate of maduramicin from feed into eggs, the determined maduramicin intake is related to the amount of maduramicin in eggs. Considering separation of maduramicin in experimental diets, which is demonstrated by

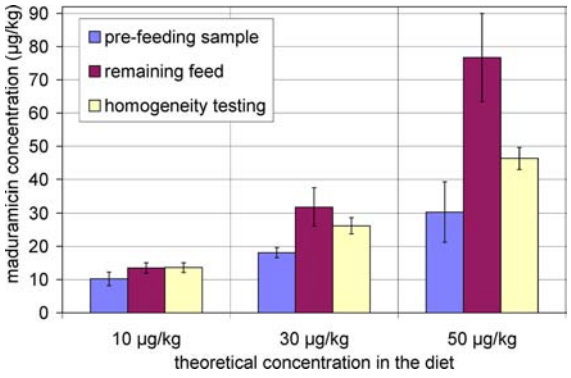

Figure 2. Mean (±SD) maduramicin concentrations determined in pre-feeding samples and remaining feed compared to homogeneity test samples. Standard deviations are shown as error bars.

Figure 2, the intake of maduramicin was calculated using three different approaches. For the first approach it was assumed that the determined concentration in the pre-feeding sample was representative for the whole portion of feed for one specific day. The second and third approach were based on the assumption that the amount of maduramicin in one feed ration corresponded to the initial amount, calculated using the target levels 10, 30, and 50 μg/kg (approach 2) or the levels determined by homogeneity tests (approach 3). In each of the three approaches, maduramicin intake was calculated by subtracting the amount of maduramicin in the remaining feed from the total amount in the daily feed ration.

Egg White Samples. Maduramicin concentrations in egg white samples did not exceed the method's LOD (0.3 μg/kg) during the entire feeding trial.

Yolk Samples. The depletion curve of maduramicin in egg yolks of group 1 and respective graphs of groups 2 and 3 are presented in Figure 3. Detailed graphs for each group also

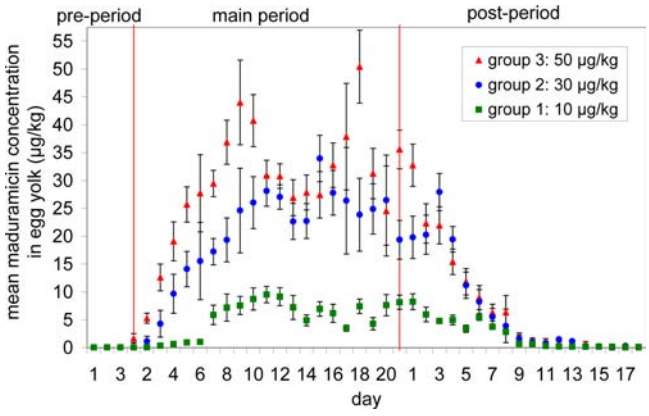

Figure 3. Depletion of maduramicin in egg yolks of the three groups. Mean (±SD) maduramicin concentrations of each day are shown.

containing individual values are available as Supporting Information. Maduramicin levels in yolk samples exceeded the LOD (0.3 μg/kg) on day 3 of the main period when 10 μg/kg maduramicin was fed to the laying hens. A rapid increase to a mean concentration of 9.5 μg/kg on day 12 followed. Maduramicin levels slightly decreased after this first maximum and stabilized toward the end of the main period, indicating a steady state with a mean concentration of approximately 6.8 μg/kg from day 10 to day 21. After a switch to maduramicin-free feed, concentrations of maduramicin in the yolk decreased to the limit of detection within 11 days. The mean concentration in egg yolk at the first maximum deriving from hens fed 30 μg/kg maduramicin was 28 μg/kg (day 11), and the average concentration at the apparent steady state was 26 μg/kg (days 10–21). Respective values in the group 3 fed 50 μg/kg maduramicin were 44 μg/kg (day 9) and 34 μg/kg (days 10–21). The course of maduramicin concentrations in yolk was similar in the three groups.

Maduramicin Concentrations in Whole Eggs. Maduramicin depletion curves in whole eggs for all groups are presented in Figure 4. A detailed graph for group 1 is provided

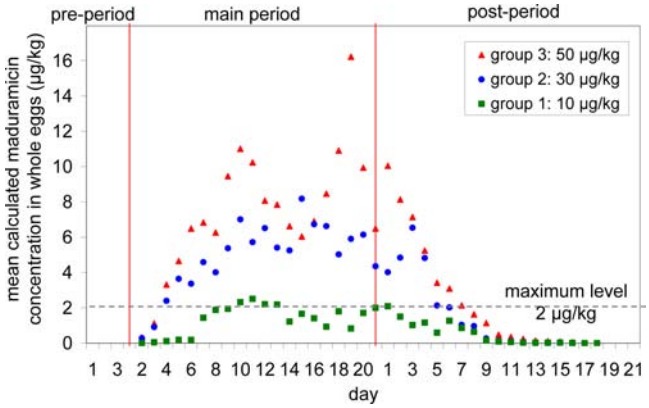

Figure 4. Depletion of maduramicin in whole eggs of the three groups. Mean maduramicin concentrations of each day are shown.

in Supporting Information. Feeding the 10 μg/kg maduramicin diet caused maduramicin concentrations to be up to 2.5 μg/kg (day 10) in whole eggs. The maximum level laid down for food of animal origin by Commission Regulation (EC) 124/2009 was therefore exceeded. The application of the 30 μg/kg feed led to a mean maduramicin concentration at the maximum in eggs of 8.2 μg/kg on day 15 of the main period. Administration of the diet containing the maximum level of unavoidable cross-contamination in non-target feed (group 3, 50 μg/kg) led to mean concentrations up to 16.3 μg/kg in whole eggs on day 19 of the main period. The calculated maduramicin concentrations in eggs of 1 day were spanning the range of up to 15 μg/kg.

To assess the transfer from feed into eggs, the mean maduramicin level in eggs deriving from the period of days 10–21 was considered as an apparent steady state in all groups and was used to calculate the average maduramicin amount transferred into eggs.

Carryover Rate and Transfer Factor. Maduramicin concentrations in whole eggs, intake of maduramicin calculated considering the three approaches, and resulting carry-over rates are shown in Table 1. The carry-over rates calculated from the target concentration (approach 2) and the homogeneity test results (approach 3) were in the same range with a mean value of 8.2% (7.3–8.9%) for the three groups. By use of the concentrations in feed determined in the daily pre-feeding samples (approach 1), the carry-over rates were considerably higher, averaging 11.4% (9.2–12.7%). Figure 5 shows the correlations of the calculated maduramicin levels in whole eggs of the three groups to the target concentrations in feed (approach 2), the concentrations in homogeneity test samples (approach 3), and concentrations in daily pre-feeding samples (approach 1), respectively. The slopes of the correlation

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Table 1. Maduramicin Intake Calculated Using Three Comprehensive Approaches, Maduramicin Plateau Concentrations in Whole Eggs Obtained after Feeding Diets Containing Different Maduramicin Levels,[a] and Calculated Carry-over Rates[b]

	group 1	group 2	group 3	mean
maduramicin intake in μg per laying hen per day				
approach 1	1.11	2.28	3.72	
approach 2	1.03	3.72	5.78	
approach 3	1.25	3.31	5.41	
maduramicin plateau concentrations in whole eggs (μg/kg)				
	1.75	6.10	9.09	
carryover rate (%)				
approach 1	9.2	12.3	12.7	11.4
approach 2	8.9	7.5	8.2	8.2
approach 3	7.3	8.5	8.7	8.2

[a]Group 1: 10 μg/kg. Group 2: 30 μg/kg. Group 3: 50 μg/kg. [b]Approach 1 = intake calculated from concentrations determined in daily pre-feeding samples. Approach 2 = intake calculated from theoretical concentrations in the diets. Approach 3 = intake calculated from concentrations determined in homogeneity tests.

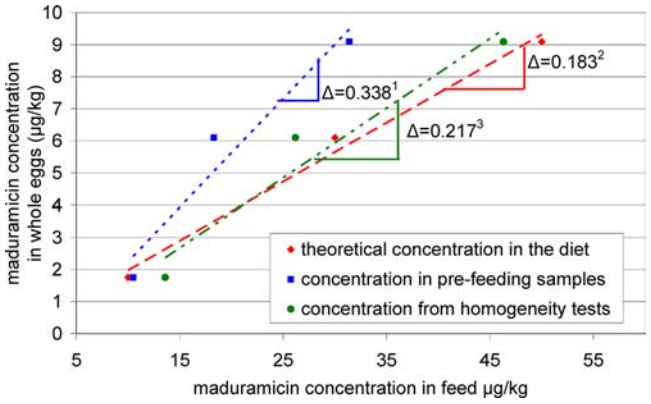

Figure 5. Correlation of maduramicin concentrations in the diet and the resulting maduramicin concentration in whole eggs based on three approaches on maduramicin intake. Superscripts 1, 2, and 3 indicate approaches 1, 2, and 3, respectively. Slopes of the correlation functions correspond to the transfer factor.

functions correspond to the transfer factor of maduramicin from feed into eggs. Depending on the different approaches for the determination of the concentrations in feed, transfer factors ranging from 0.183 to 0.338 μg/kg maduramicin in eggs per μg/kg maduramicin in feed were calculated.

DISCUSSION

Maduramicin in Feed. Analysis of the feed samples demonstrated that maduramicin distribution within the complete feed varied during the feeding trial. Homogeneity was tested and proven directly after the mixing procedure. Despite immediate partitioning and repetitive agitation of the daily feed rations before feeding, daily pre-feeding samples showed lower maduramicin concentrations than those from homogeneity tests. This may be due to segregation during storage of the daily feed rates. The main cause for this segregation obviously is the heterogeneous composition of the basic feed consisting of grains, groats, bran, and a powdery fraction. Small particles of maduramicin are able to sediment to the bottom of the container, causing a reduced concentration in middle and upper layers of feed. Even shaking overhead for 1 h could not prevent or revoke separation. As coccidiostats are known to be strongly electrostatic, a separation of the additive and the basic feed may occur because of this property. Additionally, the inner surface of the plastic container used for agitation might support electrostatic charge. In other feeding trials, different strategies for preparation of experimental diets were used. Feed containing coccidiostats for target animals was milled and added to coccidiostat-free feed meals,[5,7,8] or coccidiostat premixes were mixed into ground pellet feeds.[10,11] However, the studies neither described the mixing procedure and the performance of homogeneity tests nor any analysis in feed during the trial and in remaining feed. Maduramicin levels in remaining feed were remarkably higher than those of pre-feeding samples of the maduramicin diet throughout all groups. Therefore, transport and storage of diet in the container as well as storage in the trough may influence separation of feed material and the coccidiostat. Besides, poultry in general selects feed components. It can be assumed that laying hens preferred grains to powdery components including maduramicin, causing higher maduramicin concentrations in the remaining feed and reduced maduramicin intake by the hens.

Maduramicin in Eggs. Distribution of pharmacologically active substances in egg white and yolk depends on the properties of the respective substance concerning lipid solubility, pK_a, protein binding ability, molecular weight, and structure.[19] However, if a certain threshold concentration in eggs is exceeded, physicochemical properties have decreasing impact on the distribution of a pharmacologically active substance between egg components. This threshold is not necessarily the saturation level in egg white or yolk. Residues of polyether ionophores are known to be more likely deposited in egg yolk than in egg white because of their lipophilic properties.[20] Previous studies revealed that the distribution between egg white and yolk differ in dependence of the investigated coccidiostats.[19] Furthermore, Akhtar et al. demonstrated that distribution of salinomycin between egg white and yolk does not remain constant at changing concentrations in the fed diets. In feeding trials with diets containing four levels of salinomycin fed to laying hens,[21] concentration ratios in egg white and yolk increased from 1:140 to 1:20 with increasing concentrations in feed (30−150 mg/kg). The iononophore concentrations in the diets fed in previous studies were mainly in the range of authorized levels for target animals. In this study, maduramicin levels in eggs were considerably low compared to ionophore levels in other studies. Concentration dependence of maduramicin distribution between egg white and yolk did not occur in the investigated concentration range. Maduramicin concentrations in egg white were below the method's LOD (0.3 μg/kg), which is according to results obtained by Rokka et al. feeding 2.5 mg/kg narasin to laying hens.[20]

Feeding 50 μg of maduramicin per kg of diet led to a maximum average maduramicin level in egg yolk of 50.4 μg/kg according to a concentration of 16.2 μg/kg in whole eggs. The time courses of maduramicin concentrations in egg yolk and calculated concentrations in whole egg did not show a typical dose−response relationship. Following a quasilinear slope of the average maduramicin level, a first maximum was reached on day 10 or 11 of the main period (see group 3 in Figure 3) and no clear plateau was observed. This may be due to inhomogeneous distribution of maduramicin in feed, as well

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Table 2. Transfer Factors of Maduramicin Calculated Using Three Approaches and Transfer Factors of Other Coccidiostats Opposed to Their Maximum Value in Feed for Non-target Animals and Predicted Levels in Eggs When Diets Containing the Maximum Level Are Fed as Well as Predicted Levels in Diets for Compliance with Maximum Levels in Egg

compd[a]	transfer factor (μg/kg egg per μg/kg feed)	max level in feed (μg/kg)[23]	max level in eggs (μg/kg)[24]	predicted level in eggs when feeding max limit in feed[c] (μg/kg)	predicted level in feed for compliance with max limit in eggs[d] (μg/kg)
maduramicin[1]	0.338	50	2	16.9	6
maduramicin[2]	0.183	50	2	9.2	11
maduramicin[3]	0.227	50	2	11.4	9
lasalocid	0.063 [5]	1250	5	78.8	79
salinomycin	0.003 [8]	700	3	2.3	
monensin	0.0001 [8]	1250	2	0.2	
narasin[b]	0.004 [7]	700	2	3	472
halofuginon[b]	0.077[10]	30	6	2.3	
nicarbazin[b]	0.051 [11]	500	100	25.6	

[a]Superscripts 1, 2, and 3 indicate transfer factor of maduramicin calculated by means of approaches 1, 2, and 3, respectively. [b]Transfer factors were calculated from literature information. [c]Predicted level in eggs calculated from the transfer factor and the maximum level in feed. [d]Level in feed that should not be exceeded for compliance with maximum levels in eggs, calculated from the transfer factor and the maximum level in eggs.

as the individual feed intake of the hens regarding the administration ad libidum. Biological variations of maduramicin depletion, excretion, and metabolism may also contribute to the course of maduramicin concentrations in eggs.

From interpreting of the first maximum in the time course (between day 9 and day 12 of the main period) as the beginning of a plateau, maduramicin concentrations approached this apparent steady state later than those of other polyether ionophores. Plateaus were approached after 6 days of applying monensin[8] and lasalocid,[5] after 7 days of applying narasin,[7] and after 9 days of applying salinomycin.[8]

Maduramicin concentrations in the yolk decreased below the limit of detection (0.3 μg/kg) within 10 days after the maduramicin feeding period. Other ionophoric coccidiostats were detected in eggs 9 days (salinomycin, LOD = 10 μg/kg),[6] 10 days (lasalocid, LOD = 50 μg/kg),[5] and 7 days (narasin, CCβ yolk, 0.9 μg/kg)[20] after withdrawal of the artificially contaminated diets. As LODs for the determination of salinomycin and lasalocid were comparably high, it can be assumed that residues were present in eggs for several more days.

Carryover Rate and Transfer Factor. The transfer of contaminants from feed into food products of animal origin can be described by various parameters; however, there is no consistent nomenclature. For the present study, the terms carry-over rate and transfer factor are used to describe the transfer of maduramicin from feed into eggs.

The calculated carry-over rate of maduramicin cannot be compared to other coccidiostats, since reports of respective studies carry-over rates were not published. As the transfer factor can be calculated independent of the absolute feed intake and the weight of eggs, it is appropriate to compare the transfer of different concentrations of various pharmacologically active substances from feed into eggs. Unless transfer factors for maduramicin differ according to the three approaches, transfer of maduramicin is considerably higher than that of other coccidiostats (see Table 2). Reports of other carry-over studies[5,8,10,20] mainly do not include data concerning analysis of concentrations in the prepared diets. It can be assumed that in these studies the transfer of the coccidiostats was evaluated from theoretical concentrations in the diets calculated from the amounts applied for preparation. Transfer factors of other coccidiostats[5,7,8,10,11] are therefore compared to the transfer factor of maduramicin derived by means of approach 2. Despite the structural analogy of maduramicin to salinomycin, narasin, and monensin, carry-over of these compounds into eggs is considerably lower (up to 3 orders of magnitude).

The transfer factor of a substance may be utilized to predict levels in eggs according to an applied level of cross-contamination in feed. Vice versa, a maximum applicable contamination level in feed ensuring compliance with the given, e.g., legislative, maximum level of a coccidiostat in eggs may be calculated from the transfer factor of this substance. These considerations were carried out and are listed in Table 2 for maduramicin and the coccidiostats with reported or calculated transfer factors.

Determination of the exact carry-over rate of a compound from feed into eggs requires the knowledge of the absolute intake by the laying hen. This precondition may be fulfilled if the substance is homogeneously distributed in the diet, if animals are kept in single cages, and if they are fed using a gavage. Therefore, in this study the parameters' carry-over rate and transfer factor are calculated considering a comprehensive model concerning maduramicin concentrations in the feed mixtures. Analysis of the feed samples demonstrated that compounds of mixed feeds may not be homogeneously distributed in the mixture and furthermore, homogeneity of an additive depends on the feed's components size and shape. A once homogeneous mixture can lose this quality during transport and storage. The aim of the study was to give a realistic estimation of the transfer of a feed additive into eggs resulting from unavoidable carry-over; therefore, segregation issues had to be part of the results. Variations of the individual levels in yolk might result from the individual feed intake of the animals. Dispersion of maduramicin levels in eggs of 1 day may result from the sum of biological variations of the individual hen, the described variations of the ingested amounts of maduramicin, and also the uncertainty of measurement. As a sufficient number of samples is necessary to obtain data with a high statistical confidence, yolks of all eggs and selected samples of egg whites were analyzed. Mean levels of maduramicin in eggs and subsequent carry-over rates and transfer factors were thus calculated on a substantial statistical base.

On the other hand, for exposure assessment of the consumer to maduramicin residues in eggs due to cross-contamination of non-target feed, variability of maduramicin levels in eggs seems relevant. Single values within 1 study day ranged widely, causing relative standard deviations of up to 50%. The range of

individual maduramicin levels in eggs within 1 study day was up to 15 μg/kg. The quantity of data can therefore be helpful in the frame of risk assessment, especially for a comprehensive estimation of consumer exposure to residues of maduramicin.

Results of the present work demonstrate that the maximum cross-contamination levels set for maduramicin in feed for non-target animals by Commission Directive 2009/8/EC[2] cannot ensure compliance with maximum levels in eggs set by Commission Regulation (EC) 124/2009.[3] Feeding of diets containing the maximum cross-contamination rate of maduramicin (1%, 50 μg/kg) to laying hens caused mean levels in eggs of 9.1 μg/kg, which is a multiple of the maximum level laid down for foodstuffs (2 μg/kg). On the basis of these results and of the risk assessment stated in the scientific opinions of the EFSA,[4,22] revision of both maximum levels in feed and in eggs required by the European Commission was accomplished. Adaption of the maximum level of maduramicin in eggs to 12 μg/kg is recommended by the Draft Commission Regulation (legislative procedure is ongoing) amending Commission Regulation (EC) 124/2009.

■ ASSOCIATED CONTENT

Supporting Information

Detailed depletion curves of maduramicin in egg yolks and whole eggs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Regulation (EC) No 1831/2003 of the European Parliament and of the Council on additives for use in animal nutrition. *Off. J. Eur. Communities: Legis.* **2003**, *L268*, 29−43.

(2) Commission Directive 2009/8/EC of the European Parliament and of the Council as regards maximum levels of unavoidable carry-over of coccidiostats or histomonostats in non-target feed. *Off. J. Eur. Communities: Legis.* **2009**, *L40*, 19−40.

(3) Commission Regulation (EC) No 124/2009 setting maximum levels for the presence of coccidiostats or histomonostats in food resulting from the unavoidable carry-over of these substances in non-target feed. *Off. J. Eur. Communities: Legis.* **2012**, *L40*, 7−11.

(4) EFSA Panel on Contaminants in the Food Chain. Cross-contamination of non-target feedingstuffs by maduramicin authorised for use as a feed additive: Scientific opinion of the Panel on Contaminants in the Food Chain. *EFSA J.* **2008**, *594*, 1−30.

(5) Kennedy, D. G.; Blanchflower, W. J.; Hughes, P. J.; McCaughey, W. J. The incidence and cause of lasalocid residues in eggs in Northern Ireland. *Food Addit. Contam.* **1996**, *13*, 787−794.

(6) Sinigoj-Gacnik, K.; Rojs, O. Z. Salinomycin concentration in eggs and tissues of laying hens. *Acta Vet. Brno* **2008**, *77*, 423−429.

(7) Mortier, L.; Huet, A. C.; Daeseleire, E.; Huyghebaert, G.; Fodey, T.; Elliott, C.; Delahaut, P.; Van Peteghem, C. Deposition and depletion of five anticoccidials in eggs. *J. Agric. Food Chem.* **2005**, *53*, 7142−7149.

(8) Kennedy, D. G.; Hughes, P. J.; Blanchflower, W. J. Ionophore residues in eggs in Northern Ireland: incidence and cause. *Food Addit. Contam.* **1998**, *15*, 535−541.

(9) Dolenc, J.; Ciglari, R.; Ganik, K. Accumulation of Maduramicin in Eggs. Presented at the 6th International Symposium on Hormone and Veterinary Drug Residue Analysis, Ghent University, Ghent, Belgium, 2010.

(10) Yakkundi, S.; Cannavan, A.; Young, P. B.; Elliott, C. T.; Kennedy, D. G. Halofuginone contamination in feeds as a cause of residues in eggs. *Anal. Chim. Acta* **2002**, *473*, 177−182.

(11) Cannavan, A.; Ball, G.; Kennedy, D. G. Nicarbazin contamination in feeds as a cause of residues in eggs. *Food Addit. Contam.* **2000**, *17*, 829−836.

(12) MacLachlan, D. J. Estimating the transfer of contaminants in animal feedstuffs to livestock tissues, milk and eggs: a review. *Anim. Prod. Sci.* **2011**, *51*, 1067−1078.

(13) Dubreil-Cheneau, E.; Bessiral, M.; Roudaut, B.; Verdon, E.; Sanders, P. Validation of a multi-residue liquid chromatography−tandem mass spectrometry confirmatory method for 10 anticoccidials in eggs according to Commission Decision 2002/657/EC. *J. Chromatogr., A* **2009**, *1216*, 8149−8157.

(14) Delahaut, P.; Pierret, G.; Ralet, N.; Dubois, M.; Gillard, N. Multi-residue method for detecting coccidiostats at carry-over level in feed by HPLC-MS/MS. *Food Addit. Contam., Part A* **2010**, *27*, 801−809.

(15) Malik, A. K.; Blasco, C.; Pico, Y. Liquid chromatography−mass spectrometry in food safety. *J. Chromatogr., A* **2010**, *1217*, 4018−4040.

(16) van der Veen, A. M. H.; Nater, D. A. G. Sample preparation from bulk samples: an overview. *Fuel Process. Technol.* **1993**, *36*, 1−7.

(17) DIN ISO 32645:1994. Chemical Analysis; Decision Limit; Detection Limit and Determination Limi; Estimation in Case of Repeatability; Terms, Methods, Evaluation. Deutsches Institut für Normung, 1994.

(18) Bodi, D.; Fry, H.; Schafft, H.; Lahrssen-Wiederholt, M.; Preiß-Weigert, A. Development, Validation, and Application of LC−MS/MS Methods for the Investigation of Maduramicin Carry-Over from Feed into Eggs. Presented at the EuroResidue VII Conference on Veterinary Drug Residues in Food 2012, Egmond aan Zee, The Netherlands, 2012.

(19) Kan, C. A.; Petz, M. Residues of veterinary drugs in eggs and their distribution between yolk and white. *J. Agric. Food Chem.* **2000**, *48*, 6397−6403.

(20) Rokka, M.; Eerola, S.; Perttila, U.; Rossow, L.; Venalainen, E.; Valkonen, E.; Valaja, J.; Peltonen, K. The residue levels of narasin in eggs of laying hens fed with unmedicated and medicated feed. *Mol. Nutr. Food Res.* **2005**, *49*, 38−42.

(21) Akhtar, M. H.; ElSooud, A.; Shehata, M. A. A. Concentrations of salinomycin in eggs and tissues of laying chickens fed medicated feed for 14 days followed by withdrawal for 3 days. *Food Addit. Contam.* **1996**, *13*, 897−907.

(22) EFSA Panel on Additives and Products or Substances Used in Animal Feed (FEEDAP). Scientific opinion on safety and efficacy of Cygro 10G (maduramicin ammonium α for chickens for fattening. *EFSA J.* **2011**, *9* (1), 1952−1953.

(23) Van De Steene, J. C.; Mortier, K. A.; Lambert, W. E. Tackling matrix effects during development of a liquid chromatographic−electrospray ionisation tandem mass spectrometric analysis of nine basic pharmaceuticals in aqueous environmental samples. *J. Chromatogr., A* **2006**, *1123*, 71−81.

(24) Dubois, M.; Pierret, G.; Delahaut, P. Efficient and sensitive detection of residues of nine coccidiostats in egg and muscle by liquid chromatography−electrospray tandem mass spectrometry. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2004**, *813*, 181−189.