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Coccidiostats in unmedicated feedingstuffs for poultry

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Abstract. Coccidiostats are compounds that are widely used as feed additives to prevent and treat coccidiosis, a contagious disease affecting mainly poultry, and which is associated with warm and humid conditions, as can be found on poultry farms. In Serbia and in the EU, specific coccidiostats are authorized as poultry feed additives. A wide range of these products is available for prevention (as additives) and treatment of coccidiosis (as veterinary medicinal products). The aim of this study is to present findings of residues of coccidiostats in unmedicated feed for chickens for fattening and laying hens as possible causes for coccidiostat residues in liver and eggs. The reasons for these compounds occurring in animal tissues and primary products of animal origin could be an inappropriate withdrawal period after the last administration of medicated feed or cross-contamination of unmedicated feed during the production on the same production line as medicated feedingstuffs, because of inadequate cleaning procedures and/or hygiene practices.

1. Introduction

Coccidiosis is a parasitic disease affecting livestock, especially poultry. The disease is caused by protozoan parasites of the genus *Eimeria*. In warm and humid environments, it causes intestinal lesions and it is highly contagious and spreads from one animal to another by contact with infected feces. Even in the presence of high sanitary standards and good farm management, coccidiosis can occur with serious impact on animal health and welfare and possible high mortality rates. The economic damage caused by coccidiosis should also be taken into consideration [1]. Effective coccidiostats are, at the moment, indispensable to protect the health and welfare of poultry and other animals against coccidiosis.

The use of coccidiostats in the EU is regulated by Commission Regulation 2003/1831/EC [2] on additives for use in animal nutrition. This Regulation lays down rules for authorization of feed additives and classifies feed additives into five categories, namely technological, sensory, nutritional, and zootechnical additives and coccidiostats or histomonostats. Currently eleven coccidiostats are authorized as feed additives in accordance with regulation [2] – decoquinate, diclazuril, halofuginone, lasalocid, maduramicin, monensin, narasin, nicarbazin, robenidine, salinomycin and semduramicin. Each coccidiostat can be used at a prescribed concentration and during a certain time interval for broilers and young chickens but not for laying hens. Despite the requirements set for companies that produce feeds (cleaning of equipment and production line, especially between medicated and non-medicated feed) [3], under practical conditions during production, unavoidable carry-over occurs when a certain percentage of a feed batch containing coccidiostats remains in the production circuit and contaminates the subsequent feed batch. This unavoidable carry-over or cross-contamination can result in the exposure of non-target animal species with potential health risks for animals, as well as the potential residues in

foods derived from these species such as eggs. Maximum levels of unavoidable carry-over of certain coccidiostats is regulated by Commission Directive 2002/32/EC on undesirable substances in animal feed [4], Annex I section VII – authorized feed additives in non target feed following unavoidable carry-over, and subsequent Directives and Regulations amending Annex I (Directive 2009/8/EC, Regulations 574/2011 and 744/2012). A carry-over rate of 1% is allowed for feed used during the period before slaughter (withdrawal feed), for other feed to which no coccidiostats are added and for non-target feed for continuous food producing animals (laying hens or dairy cows). Maximum levels for the presence of coccidiostats in food resulting from the unavoidable carry-over of these substances in non-target feed are regulated by the EU [5,6].

In Serbia, the use of these same eleven coccidiostats as feed additives and unavoidable carry-over in non-target feed is regulated by Regulation on quality of feed in article 89 (permitted substances with a coccidiostatic action which can be added to the mixtures and premixtures) and article 99 (maximum permitted harmful substances – authorised feed additives in non target feed following unavoidable carry-over) [7]. Table 1 presents the maximum concentrations of active substances allowed in non-target feed in Serbia given an unavoidable maximum level of carry-over of 1% of the coccidiostats.

Table 1. Maximum levels of unavoidable carry-over (1%) of coccidiostats in non-target feed

Coccidiostat	Maximum levels of unavoidable carry-over (1%) (mg of active substance/kg of feed)
Narasin	0.70
Lasalocid	1.25
Maduramicin	0.05
Semduramicin	0.25
Salinomycin	0.70
Monensin	1.25
Nicarbazin (DNC)	1.25
Diclazuril	0.01
Robenidine	0.70
Decoquinate	0.40
Halofuginone	0.03

Chickens for fattening receive coccidiostats through feed during their entire life, except during the period before slaughter – the withdrawal period (this is 3-5 days for the different coccidiostats). Coccidiostats are added to their feed at different levels: 100-125 mg/kg (monensin), 75-125 mg/kg (lasalocid), 60-70 mg/kg (salinomycin), 5-6 mg/kg (maduramicin), 40-50 mg/kg (narasin), 40-50 mg/kg (nicarbazin), 30-40 mg/kg (robenidine), 1 mg/kg (diclazuril) [7]. A withdrawal period before slaughter is required in order to avoid residues of coccidiostats in edible tissues, although there is no risk to consumer health from ingestion of coccidiostats residues in tissues of animals exposed to feed cross-contaminated up to a level of 10% [8-10]. None of the coccidiostats are licensed for use in egg laying hens and eggs should be free of coccidiostats, but residues of coccidiostats in eggs have been detected in Europe at levels from 0.3 to <40 μg/kg [11-13], and in Serbia within the National Residue Monitoring Programme, at levels from 1 to <20 μg/kg. The reported findings of coccidiostats residues can be attributed to the cross-contamination of unmedicated feed at feed production mills, although illegal use of the drugs cannot be discarded.

2. Materials and Methods

Diclazuril (DICL), robenidine (ROBN), narasin (NAR), nicarbazin (DNC), monensin (MON), salinomycin (SAL), maduramicin (MAD) and lasalocid (LAS) were all purchased from Sigma-Aldrich (St. Louis, USA). Water, methanol, acetonitrile and N,N-dimethylformamide were all HPLC grade and

also purchased from Sigma-Aldrich. Formic acid LC grade was from Merck (Merck KGaA, Darmstadt, Germany). Individual stock solutions at a concentration of 1.00 mg/mL were prepared in methanol for all compounds except for narasin and nicarbazin, which were dissolved in dimethylformamide (DMF); all were stored at -20ºC. Working standard solution (a mixture of analytes) was prepared in acetonitrile by diluting stock solutions into a range that equated to the carryover levels in feed and stored at 4ºC.

Coccidiostats were analyzed with Waters ACQUITY connected to a TQD mass spectrometer (Waters, Miliford, MA, USA). The instrument was controlled by MassLynks software version 4.1.The analytical column used for separation was Kinetex 100 x 2.1 mm, 2.6µ C18 100A with UltraGuard cartridge (Phenomenex, Torrance, CA, USA). The oven temperature was set at 45ºC. The chromatographic separation was achieved in gradient mode using water acidified with 0.1% formic acid (mobile phase A) and acetonitrile acidified with 0.1% formic acid (mobile phase B) at a flow rate of 0.55 mL/min. Electrospray ionization (ESI) was used with both positive and negative mode, with following parameters: capillary voltage 3.5kV, source temperature 130ºC, desolvation temperature 400ºC, desolvation and cone gas 900 and 60 L/h, respectively. Argon was used as collision gas. The precursor and products ions for each analyte, cone voltages and collision energies are presented in Table 2.

Feeds labeled as withdrawal feed or feed for laying hens, from 2014 to May 2017, were used in this study. We also analyzed different batches of rinsing feed, produced when switching from production of medicated feed to unmedicated feed, provided to us by mill owners. Before analysis, feeds were ground using a laboratory mill IKA A11 Basic (IKA Werke, Germany). A 5.0 g portion of each feed was individually weighed into polypropylene jars with caps. Acetonitrile 25 mL was added and the jars were shaken on a horizontal shaker IKA Yellow line (IKA Werke, Germany) for 60 mins. The extracted feeds were filtered individually through nylon 0.22µm syringe filter into HPLC vials.

Quantification was carried out using matrix extracted calibrations curves at four levels. With every batch of feeds examined, blank feed samples were fortified at four different levels with mixed working standard solution and submitted to the full extraction procedure.

Table 2. Mass spectrometry parameters for quantification of coccidiostats in poultry feeds

Compound	Precursor ion (m/z)	Product ions (m/z)	Cone voltage (V)	Collision energies (eV)	Ionization mode
Narasin	787.30	431.30	70	50	ES+
		531.40	70	45	
Lasalocid	613.20	359.30	70	32	ES+
		377.40	70	35	
Maduramicin	934.8	629.50	36	28	ES+
		647.50	36	20	
Salinomycin	773.50	265.10	70	50	ES+
		431.10	70	50	
Monensin	693.00	461.10	70	60	ES+
		479.10	70	60	
Nicarbazin	301.00	107.10	50	40	ES-
		137.00	50	40	
Diclazuril	405.00	334.00	33	21	ES-
	406.90	336.00	33	19	
Robenidine	333.90	138.00	35	30	ES+
		155.00	35	20	
		178.00	54	30	

3. Results and Discussion

Results obtained from analyzed rinsing feed proved that production of medicated and unmedicated feed on the same production line led to unavoidable carry over, i.e. cross-contamination. According to the legislation, SAL can be added to feed for chickens for fattening at concentrations of between 60-70 mg/kg [7]. Based on these concentrations, from up to 4% to less than 1% of the added SAL was left in the production lines after several batches of rinsing feed, intended to clean the lines, were processed. Similar results were obtained for other coccidiostats. Concentrations of residual coccidiostats in the feeds produced after the rinsing feeds were processed are shown in Figure 1. As can be seen, only after several batches of rinsing feed were processed did the concentrations of coccidiostats fall below maximum levels of unavoidable carry-over (1%).

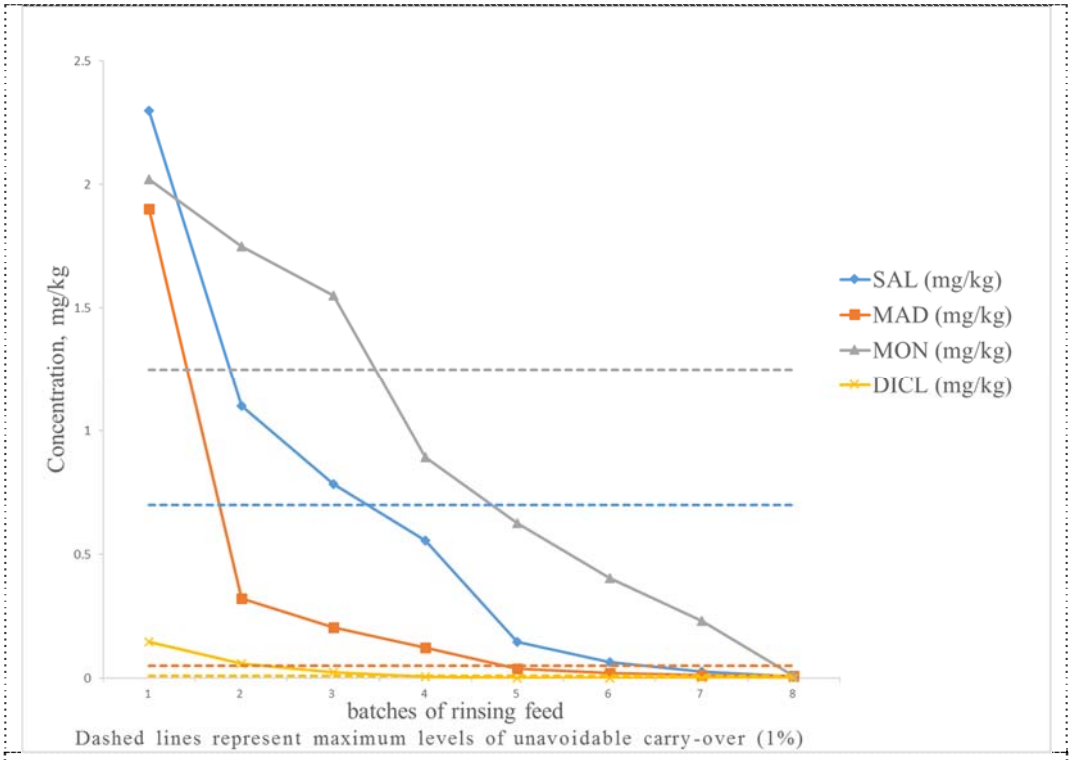

Figure 1. Concentration of different coccidiostats (mg/kg feed) in rinsing feed.

Concentrations of different coccidiostats found in analyzed feed labeled as withdrawal feed or feed for laying hens are presented in Table 3 and Table 4, respectively. For withdrawal feeds, the highest concentration of the coccidiostats SAL, MAD, LAS, DICL and ROBN corresponded to the doses added to starter feed for chickens for fattening [7]. Feeds that had concentrations of coccidiostats above maximum levels of unavoidable carry-over (1%), were declared non-compliant. Among the feeds, 13.7% contained non-compliant concentrations of SAL (Table 3). However, the majority of feeds had concentrations of coccidiostats below maximum levels of unavoidable carry-over (1%) and in 20-25% of feeds, concentrations of coccidiostats were below the limit of detection (LOD). The most disturbing fact was that a large number of feeds (80%) contained concentrations below maximum levels of unavoidable carry-over (1%) of several (2-4) different coccidiostats.

Table 3. Concentration of different coccidiostats (mg/kg feed) in withdrawal feed

Compound	n 1	min – max	n 2	n 3
Salinomycin SAL	124	0.005 – 60	74	17 (13.7%)
Maduramicin MAD	124	0.005 – 6	15	8 (6.5%)
Narasin NAR	124	0.005 – 12	40	4 (3.2%)
Lasalocid LAS	124	0.005 – 116	14	2 (1.6%)
Diclazuril DICL	124	0.005 – 0.920	5	1 (0.8%)
Robenidine ROBN	124	0.005 – 27.6	2	2 (1.6%)
Nicarbazin DNC	124	0.010 – 6.2	18	3 (2.4%)

n 1 – number of feeds; n 2 - number of feeds with coccidiostat level above LOD and below maximum levels of unavoidable carry-over (1%); n 3 - number of feeds with coccidiostat level above maximum levels of unavoidable carry-over (1%)

In feed for laying hens, in which the use of coccidiostats is not licensed, of 133 feeds analyzed, MAD was above maximum levels of unavoidable carry-over (1%) in 13 (9.8%) of feeds (Table 4). Bodi et al. [14] investigated carry-over of MAD from feed into eggs of laying hens. Feeding the hens a diet containing 50µg/kg MAD (the maximum level of unavoidable carry-over 1%), resulted in concentrations of MAD in eggs up to 11 µg/kg. They calculated that the carry-over rate from feed into eggs was 8% for MAD. Kennedy at al. [12] reported the carry-over of LAS from medicated to unmedicated feed during manufacture. LAS was found at levels up to 1mg/kg, level high enough to result in residues in eggs.

Table 4. Concentration of different coccidiostats (mg/kg feed) in feed for laying hens

Compound	n 1	min – max	n 2	n 3
Salinomycin SAL	133	0.005 – 7.3	81	3 (2.3%)
Maduramicin MAD	133	0.005 – 0.832	19	13 (9.8%)
Narasin NAR	133	0.005 – 0.769	13	1 (0.75%)
Lasalocid LAS	133	0.005 – 0.486	17	0
Diclazuril DICL	133	0.005 – 0.660	1	1 (0.75%)
Robenidine ROBN	133	0.005 – 0.125	8	0
Monensin MON	133	0.005 – 3.6	9	1 (0.75%)

n 1 – number of feeds; n 2 - number of feeds with coccidiostat level above LOD and below maximum levels of unavoidable carry-over (1%); n 3 - number (%) of feeds with coccidiostat level above maximum levels of unavoidable carry-over (1%)

4. Conclusion

The obtained results indicate that more attention should be paid to the feed production process in order to avoid cross-contamination from medicated to non-medicated feed. Besides the measures usually taken after any non-compliant findings of coccidiostats in non-medicated feed, adequate education of feed producers is highly recommended. This must be focused on better understanding of rules for production and hygiene practices and implementation of control measures in the use of coccidiostats, including

closer cooperation between producers and competent authorities responsible for the residue surveillance program and official control.

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