Presence and distribution of residues of the anticoccidials diclazuril, dimetridazole, halofuginone, robenidine, nicarbazin and narasin in eggs after administration of medicated feed and influence of the presence of these anticoccidials in feed on zootechnical parameters, yolk colour and shell quality

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Summary

Anticoccidials are compounds that are widely used as feed additives to prevent and treat coccidiosis, a contagious amoebic disease affecting poultry. In the European Union these compounds are registered as feed additives according to Regulation 1831/2003/EC (European Commission, 2003). In Belgium and in other countries a lot of these compounds are licensed for use as feed additive in a prescribed concentration and during a certain time interval for broilers and pullets but not for laying hens. It was shown in the past that accidental cross contamination of feed could lead to residues of the compounds in eggs. Especially carry-over at the feed mill is found to be a main reason for the presence of residues in eggs.

An animal experiment was set up in which 10 groups of 12 laying hens were treated, after an adjustment period, with anticoccidial-containing feed during 14 days. For the compounds diclazuril, robenidine, halofuginone and nicarbazin in combination with narasin, two concentrations levels were tested, namely the maximum level that can be present in feed for broilers or pullets and a lower concentration level, corresponding to 5% carry-over at the feed mill. Also dimetridazole was included in the experiment. But since it is a forbidden compound and hence, carry-over is not likely to occur, for this compound only one concentration level was tested. Eggs were sampled during treatment and 30 days after withdrawal of the anticoccidial-containing feed. Residues were determined and deposition and depletion curves could be generated. For all compounds, substantial residues could be found in the 5% groups, which points out the risk of carry-over at the feeding mill. The distribution of the residues between egg yolk and white was determined by analysing both fractions. Major differences in distribution between yolk and white could be observed for the compounds studied. Also the influence on the zootechnical parameters, yolk colour and eggshell quality was evaluated.

Introduction

Anticoccidials are compounds that are widely used as feed additives to prevent and treat coccidiosis, a contagious amoebic disease affecting poultry. The disease is carried by unicellular organisms belonging to the genus *Eimeria* in the class Sporozoa and is the most important parasitic disease in poultry. In the European Union anticoccidials are registered as feed additives according to Regulation 1831/2003/EC (European Commission, 2003). In Belgium and in other countries a lot of these compounds are licensed for use as feed additive in a prescribed concentration and during a certain time interval for broilers and pullets but not for laying hens.

It was shown in the past that accidental cross contamination of feed could lead to residues of the compounds in eggs. Especially carry-over at the feed mill is found to be a main reason for the presence of residues in eggs. A wide range of anticoccidial drugs is available to treat and prevent coccidiosis. Besides the ionophoric anticoccidials, such as narasin, monensin, lasalocid and salinomycin, there is also a class of chemical anticoccidial drugs. The most common chemical anticoccidials are nicarbazin, halofuginone, diclazuril and robenidine.

An extensive animal experiment was set up to investigate the effect of carry-over at the feed mill on the presence of residues in eggs. In this experiment 10 groups of 12 laying hens were treated, after an adjustment period, with anticoccidial-containing feed during 14 days. Eggs were sampled during treatment and 30 days after withdrawal of the anticoccidial-containing feed. For the compounds diclazuril, robenidine, halofuginone and nicarbazin, two concentrations were tested, namely the maximum level that can be present in feed intended for broilers or pullets and a lower level, corresponding to 5% carry-over at the feed mill. Since 2001, nicarbazin can no longer be administered as such but only in combination with narasin as Maxiban® and hence, narasin was also included in the experiment. Dimetridazole, which belongs to the group of nitroimidazoles, formerly was used as anticoccidial but is now listed in Annex 4 of Council Directive 2377/90 (European Commission, 1990) and is, as a consequence, a forbidden compound. As a result, carry-over is not likely to occur for dimetridazole. Therefore, only one concentration level was included for this compound. Nicarbazin is the generic name of the equimolar complex of 4,4'-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP). When chickens are given nicarbazin in the feed, the HDP fraction is absorbed and excreted more rapidly than the DNC fraction and consequently most residue analyses for nicarbazin are based on methods for the DNC molecule (Porter et al., 1955). Thus, in this experiment, we focussed only on the DNC compound. As mentioned above, dimetridazole or 1,2-dimethyl-5-nitroimidazole belongs to a group of compounds called the nitroimidazoles. The major pathway of elimination of dimetridazole is hydroxylation of the 2-methyl group to 2-hydroxymethyl-1-methyl-5-nitroimidazole (Matusik et al., 1992). The fact that dimetridazole is metabolized rapidly and that the main metabolite, 2-hydroxydimetridazole, is present in higher concentrations in tissues and eggs emphasizes the need to monitor for both of these compounds when one is performing residue analysis.

Preparation of diets

Experimental diets were prepared at the mill of CLO-DVV (Agricultural Research Centre, Department of animal nutrition and husbandry).

For the preparation of the diets containing diclazuril, halofuginone and robenidine, Clinacox® (Janssen Animal Health, Beerse, Belgium), Stenerol® (Intervet, Mechelen, Belgium) and Cycostat® (Alpharma, Antwerpen, Belgium) were used as premix, respectively. Maxiban® (Elanco, Brussel, Belgium) is a mixture of nicarbazin and narasin in a 1/1 ratio. It was used to prepare the nicarbazin and narasin containing feed. For dimetridazole, we were not able to obtain an applicable premix. Therefore, we used an analytical standard bought at Sigma (Bornem, Belgium) to prepare the dimetridazole containing feed. The concentrations corresponding to the maximum allowed concentration for broilers or pullets, further referred to as the 100% groups, were 1 mg/kg for diclazuril, 3 mg/kg for halofuginone, 36 mg/kg for robenidine, 40 and 40 mg/kg for narasin and nicarbazin and 100 mg/kg for dimetridazole. For each compound, except dimetridazole, a second concentration corresponding to 5% carry-over also was prepared.

The appropriate amount of premix was weighed and added to the blank feed. The experimental diets were least-cost formulated according to the requirements of the laying hens during the first half of their production cycle. All feedstuffs were coarsely milled with a hammer mill and carefully mixed in the feed unit. After mixing the diets were, however, not pelleted.

Animal experiment

Animal experiments were conducted at the poultry experimental facility of CLO-DVV. A flock of medium weight laying hens (ISA-brown) was used for the trial during the 1st half of their production cycle (31-39 weeks of age).

The hens were randomly divided into 10 groups of 12 animals each. These laying hens were housed in three tier battery pens of four laying hens each, under conventional conditions of ventilation, temperature (18-22 °C) and lighting (16 h light/day). During the study, they were given free access to water and feed. Each group was previously controlled for their laying persistency in order to improve

the homogeneity of the entire flock. During the entire experiment, the hens were monitored daily for general health by qualified personnel supervised by a veterinarian. Eggs were collected daily during the entire experimental period. After the animals were placed in their pens, they were allowed to adjust to their environment for 4 weeks. During this adjustment period, all animals were kept on an anticoccidial-free feed. The eggs collected during this period were used as blank control material. After the adjustment period, group 1 continued to receive blank feed while the other nine groups received the feed containing an anticoccidial during 14 days (day 1 – 14). From day 15 on, all 10 groups were fed again the anticoccidial-free feed. The collection of the eggs was finished at day 44 i.e. 30 days after cessation of administration of the anticoccidial containing feed.

Of each experimental group, 10 eggs were homogenized daily and stored at –18°C until analysis. On Mondays, also the eggs collected during the weekend were homogenized and frozen. As each group consisted of 12 laying hens, usually 10 eggs per day were available. Moreover, for most groups and at most days, 11 or 12 eggs were available. The remaining eggs were stored at 4°C. At the end of the experiment, in those cases when more than 10 eggs were available for a certain group on a certain day, one egg was used to split the egg yolk and albumen. Both fractions were stored separately at –18°C.

The following conventional zootechnical data were recorded: average feed intake (g/day), laying rate (%=eggs/100 hens), egg weight (g), daily egg mass (g/hen) and feed efficiency (feed intake/egg mass: g/g). To determine these parameters, the experimental period was subdivided into 3 sub-periods: (1) 7 days on the blank reference diet, (2) 14 days on the respective 'anticoccidial' diets, and (3) another 30 days on the blank reference diet.

Materials and methods

Analysis of the feed and egg samples was performed with a liquid chromatographic tandem mass spectrometric method (LC-MS/MS). The methods were validated according to the most recent European legislation concerning residue analysis, i.e. Commission Decision 2002/657/EC (European Commission, 2002). The decision limit or CCα was 0.5 µg/kg for diclazuril, 1 µg/kg for dimetridazole, halofuginone, robenidine and dinitrocarbanilide, and 2 µg/kg for 2-hydroxydimetridazole. For each series of samples analysed, all criteria set by Commission Decision 2002/657/EC were checked. Analyses performed by LC-MS/MS were quantitative analyses. A matrix calibration curve was made using the multiple reaction monitoring (MRM)-data of the transition of the precursor ion into the most abundant product ion. Quantification was conducted by internal calibration using a weighing factor of 1/x. For each series of samples, a calibration curve was made in a specific concentration range to make sure that the concentrations in the samples of that particular series were covered. Also in each series of samples, 2 unknown samples were included as a control.

Results and discussion

ANALYSIS OF THE FEED SAMPLES

The results of the analyses of the feed samples are presented in table 1.

As can be seen in this table, satisfying results were obtained for diclazuril, robenidine, nicarbazin and narasin. For halofuginone and dimetridazole, results were less satisfying. Remarkably, only for the group with the highest concentration of halofuginone, only about 50% of the intended concentration was achieved while good results were obtained for the 5% group. This indicates that most likely a human mistake during the feed preparation is the cause of the lower concentration achieved. A possible explanation for the result for dimetridazole is that no premix but an analytical standard was used for feed preparation. This analytical standard is less suitable for preparing medicated feed. But since dimetridazole is a forbidden compound, the concentration achieved was less important.

Table 1 results of the analyses of the feed samples.

group	compound	premix used	theoretical concentration	measured concentration	% of theoretical concentration
2	diclazuril	Clinacox®	1000 µg/kg	926 µg/kg	93
3			50 µg/kg	47 µg/kg	93
4	halofuginone	Stenerol®	3000 µg/kg	1475 µg/kg	49
5			150 µg/kg	162 µg/kg	108
6	robenidine	Cycostat®	36 mg/kg	39 mg/kg	108
7			1800 µg/kg	1597 µg/kg	89
8	narasin	Maxiban®	40 mg/kg	41 mg/kg	102
	nicarbazin			41 mg/kg	102
9	narasin		2000 µg/kg	2114 µg/kg	106
	nicarbazin			2144 µg/kg	107
10	dimetridazole	analytical standard Sigma	200 mg/kg	101 mg/kg	50

ANALYSIS OF THE WHOLE EGG SAMPLES

Analysis of the whole egg samples showed that substantial amounts of residues could be found. This was not only the case with the 100% groups but also with the 5% groups, which points out the risk of carry-over at the feeding mill. A summary of the main results is presented in table 2.

Table 2 Summary of the main results of the residue analyses of the whole egg samples.

compound	group	% of maximum allowed concentration for broilers	first positive sample (concentration (µg/kg))	plateau concentration (µg/kg)	# days withdrawal needed to obtain negative sample*
diclazuril	2	92.6	day 2 (0.6)	100	22
	3	4.7	day 3 (0.9)	5	11
halofuginone	4	49.2	day 2 (3)	450	19
	5	5.4	day 3 (18)	30	8
robenidine	6	107.8	day 3 (90)	1300	26
	7	4.4	day 3 (6)	no plateau max conc. = 70	13
narasin	8	102.3	day 3 (40)	90	18
nicarbazin		101.5	day 2 (3)	6500	23
narasin	9	5.4	day 3 (1)	6	8
nicarbazin		5.3	day 3 (11)	300	15
dimetridazole	10	50.4	day 2 (676)	650	10
2-hydroxy dimetridazole			day 2 (1513)	1700	10

* : negative sample = concentration below CCα

For each compound, deposition and depletion curves could be generated. Due to a lack of space, only one curve is shown as an example (figure 1). For most compounds, after a few days, a plateau was reached.

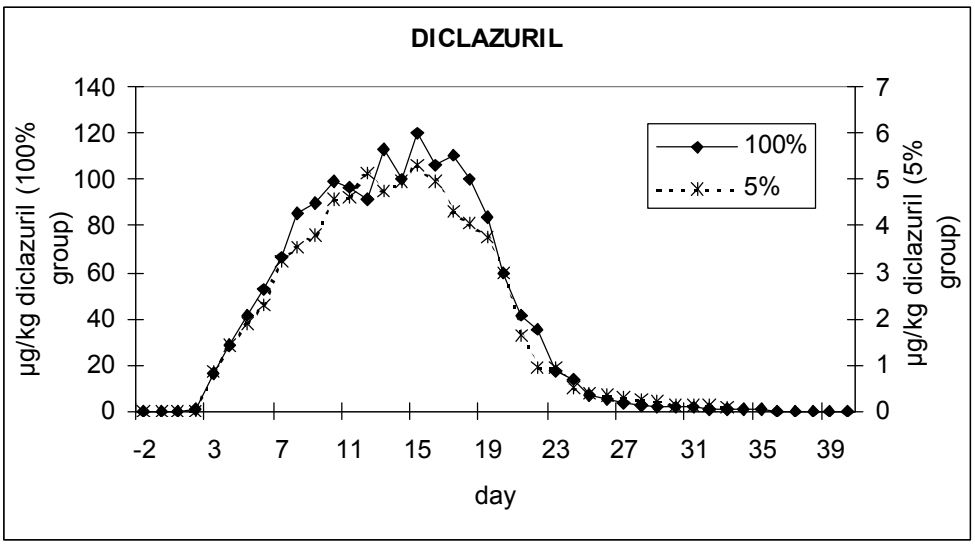

Figure 1 Deposition and depletion curve for diclazuril.

ANALYSIS OF THE EGG YOLK AND ALBUMEN

As mentioned in the animal treatment-section, also some separate yolk and albumen samples were analysed. It has to be noted that the yolk and albumen samples were no pooled samples as was the case with the whole egg samples. So variability between animals is not compensated. Major differences in distribution between the compounds could be observed. Diclazuril, robenidine, dinitrocarbanilide and narasin are mainly present in the egg yolk. For both groups of diclazuril, about 4 times more residues were found in the yolk than in the albumen. For the highest concentration group of dinitrocarbanilide, concentrations up to 10 mg/kg were found in the yolk, while the maximum concentration in the albumen was 120 µg/kg. For the highest concentration group of robenidine, 2300 µg/kg was detected in the yolk on day 13 of the experiment while only 7 µg/kg was detected in the white of the same egg. For narasin, about 5 times more residues are found in the yolk. For as well narasin, dinitrocarbanilide, diclazuril and robenidin, residues disappear much faster out of the albumen than the yolk. This is very clearly shown in figure 2 for the 100% robenidine group.

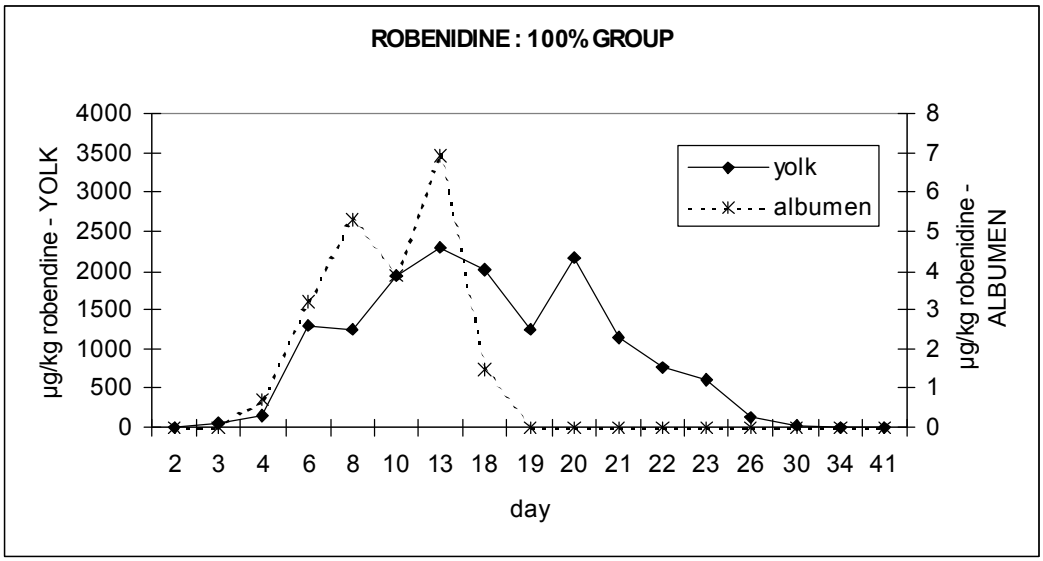

Figure 2 Deposition and depletion curve for robenidine (100% group) for the individual yolk and albumen.

As shown in figure 3, for halofuginone, initially more residues are found in the albumen.

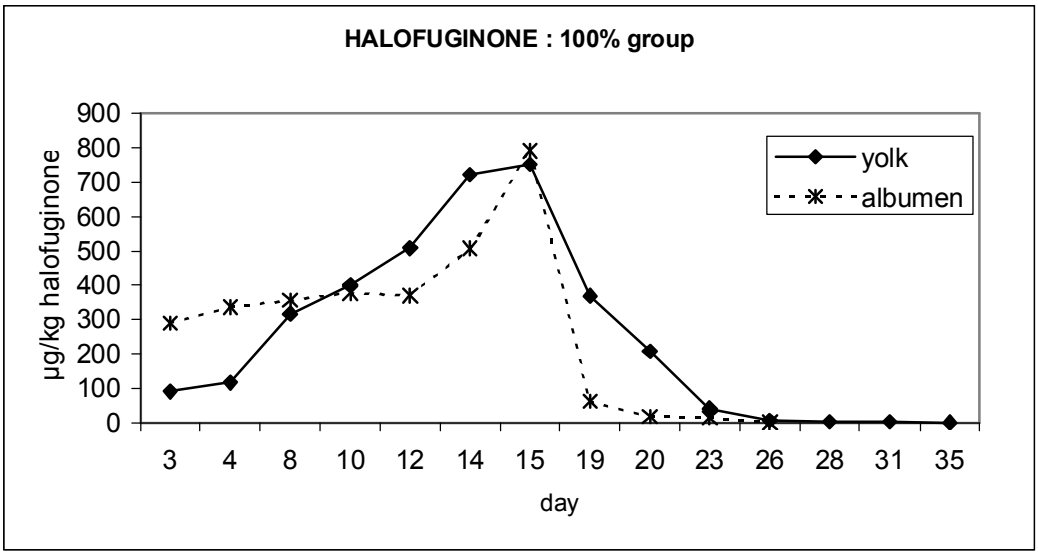

Figure 3 Deposition and depletion curve for halofuginone (100% group) for the individual yolk and albumen.

During the plateau period, halofuginone can be found in both compartments. During depletion, residues are found for a longer period in the yolk. All these observations reflect the process of egg formation in the hen.

For dimetridazole and its metabolite 2-hydroxydimetridazole, a completely different pattern is observed. Dimetridazole concentration is ten times higher in the albumen than in the yolk. Also for the hydroxymetabolite, higher concentrations are found in the albumen.

ZOOTECHNICAL PARAMETERS, YOLK COLOUR AND EGGSHELL QUALITY

As mentioned in the animal treatment-section, the influence on the zootechnical parameters (laying rate, egg weight, daily egg mass, average feed intake and feed conversion) was determined. Also the yolk colour and shell quality were evaluated. The yolk colour was determined using the Roche Yolk Colour Fan. No significant effect could be observed. The shell quality was evaluated using a non-destructive method that measures the deformation of the shell. Again, no differences were measured.

For the egg weight a reduction was noticed in the group receiving the highest Maxiban concentration. This reduction was found to be significant by performing an ANOVA analysis. A downward tendency in the daily egg mass for the highest Maxiban group and in the laying rate for both groups treated with Maxiban was observed.

Average feed intake and feed conversion were not affected by any of the compounds studied.

Conclusion

By performing an animal experiment in which laying hens were administered 6 different anticoccidials, deposition and depletion curves for the whole egg, and separate yolk and albumen could be generated. The experiment revealed that even at concentrations corresponding to 5% carry-over at the feeding mill, considerable amounts of residues can be found in the eggs. Also the egg weight, daily egg mass and laying rate can be affected by some compounds.

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