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Deposition and Depletion of Five Anticoccidials in Eggs

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Anticoccidials are compounds that are widely used as feed additives to prevent and treat coccidiosis. They are licensed for use 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 carry-over at the feeding mill is found to be the main reason for the presence of residues in eggs. An animal experiment was set up to investigate the effect of carry-over at the feeding mill on the presence of residues of anticoccidials in eggs. For the compounds diclazuril, robenidine, halofuginone and nicarbazin in combination with narasin, two concentration levels were tested: the maximum allowed concentration for broilers (100%) and a concentration corresponding to 5% carry-over during feed preparation. Also dimetridazole was included in the experiment but only at one concentration level. Eggs were sampled during treatment (14 days) and for a period of 30 days after withdrawal of the anticoccidial-containing feed. Residues were determined, and deposition and depletion curves were generated. Analyses were performed by ELISA and LC-MS/MS. 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 analyzing both fractions.

KEYWORDS: Anticoccidials; feed; eggs; depletion; residues; LC-MS/MS; ELISA

INTRODUCTION

Anticoccidials are compounds that are widely used to prevent and treat coccidiosis. Coccidiosis is a contagious condition affecting livestock, especially poultry, throughout the world. Particular in warm, humid environments it causes intestinal lesions, which result in diarrhoea and related health problems in the animal. The disease is carried by unicellular organisms belonging to the genus *Eimeria* in the class Sporozoa. In its acute form coccidiosis causes high mortalities, in its subacute form, small numbers of oocysts can adversely effect weight gain, feed conversion and egg production in poultry. Of all domestic animals, industrially bred poultry and rabbits are particularly prone to this disease.

The economic damage caused by coccidiosis in modern poultry production is so serious that practically all poultry farms have resorted to feeding anticoccidial drugs as a feed additive to pullets and broiler breeders for 12 to 16 weeks and to broiler chickens for almost their entire life. Despite the use of anticoccidial drugs, coccidiosis remains one of the biggest causes of losses in poultry production.

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.

According to Regulation 1831/2003/EC, anticoccidials are at the moment licensed as feed additives (*1*). They can be used at a prescribed concentration and during a certain time interval for broilers and pullets but not for laying hens. Hence, no residues of anticoccidials should be present in eggs. However, carry-over in the feeding mill is a major problem and can run up to 15% (*2*). Therefore, anticoccidials can be present in feed intended for laying hens. With a view to a decision on the

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phasing-out of the use of anticoccidials as feed additives by December 31, 2012, the European Commission shall submit to the European Parliament and the Council a report on the use of these substances as feed additives and available alternatives before January 1, 2008. In the meantime, there are no maximum residue levels (MRLs) set for eggs, and thus the compounds may not be present in eggs. Hence the zero tolerance principle has to be applied. In practice however, different EU member states apply another approach. In Belgium, an action limit of 10 μg/kg has been proposed by the scientific committee of the Belgian Food Agency for monensin, salinomycin, diclazuril, lasalocid, maduramycin, narasin, nicarbazin, robenidine and the group of sulfonamides (*3*). In the United Kingdom on the other hand, an action limit of 100 μg/kg for nicarbazin in eggs has been set and in Sweden, action is take when narasin concentrations exceed 5 μg/kg (*4*, *5*).

The occurrence of anticoccidial residues in eggs has been widely reported. In Northern Ireland, 161 eggs were analyzed in 1994 (*6*). Six, two and one of them contained monensin, salinomycin and narasin, respectively. In all cases, the concentrations detected were less than 2.5 μg/kg. Lasalocid was detectable in 107 samples (66.5%) at concentrations ranging from 0.3 to 129 μg/kg. In 1995, a granular formulation of lasalocid was introduced in the United Kingdom. This decreased the carry-over, and six months after the introduction of this granular formulation, the incidence of lasalocid residues in eggs had decreased to 21% (*7*). Also in Northern Ireland, in 1996 a survey was conducted in which 190 egg samples were analyzed on the presence of nicarbazin. In 39 samples (20.5%), nicarbazin could be detected in concentrations varying from 4 to 342 μg/kg (*4*). In Great Britain, the overall incidence of residues of nicarbazin in eggs tested was 10.7% in 1996, 6.8% in 1997 and 4.0% in 1998 (*4*). In 1999, 24 egg samples were analyzed on the presence of narasin in Sweden. Twelve samples (50%) contained between 0.2 and 11 μg/kg narasin (*5*). Also in our lab, residues of anticoccidials were encountered in eggs. In 2002, 232 samples were analyzed on the presence of dimetridazole, diclazuril, robenidine, halofuginone and nicarbazin. Four samples contained nicarbazin in concentrations varying from 3 μg/kg to 197 μg/kg. Three samples were positive on the presence of halofuginone but concentrations did not exceed 3 μg/kg. In 2003, 245 eggs were analyzed. Nicarbazin and robenidine were encountered in two samples each. In 2004, 190 samples were analyzed. For these samples, also the ionophores narasin, salinomycin, lasalocid and monensin were included in the monitoring. Twelve of the 190 samples were positive: robenidine (8 μg/kg), monensin (10 μg/kg), salinomycin (2 and 8 μg/kg) and lasalocid (4 to 90 μg/kg) were found.

In this study an extensive animal experiment was set up to investigate the effect of carry-over at the feeding mill on the presence of residues in eggs. This experiment was carried out in the framework of a project, which had the aim to set up an integrated approach for the detection of residues of anticoccidials in eggs. This integrated approach is based on the use of the pyramid structure: first a screening is carried out so that only positive samples need to be analyzed by the more expensive confirmation methods. To use this approach, it is necessary that the screening method does not produce false negative results. The compounds studied in this project are: diclazuril, dimetridazole, halofuginone, robenidine and nicarbazin. In the first part of the project, immunological screening methods and liquid chromatographic mass spectrometric confirmation methods were developed and validated. An ELISA (enzyme linked immunosorbent assay) was developed for the detection of halofuginone, nicarbazin and the nitroimidazoles (*8*, *9*). Unfortunately, despite many immunization attempts, no ELISA could be developed for diclazuril and robenidine. A liquid chromatographic tandem mass spectrometric (LC-MS/MS) method was developed and validated for the chemical anticoccidials halofuginone, robenidine, diclazuril, nicarbazin and dimetridazole (*10*) and for the ionophoric anticoccidials narasin, salinomycin, monensin and lasalocid (*11*).

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 (*12*). Thus in this experiment, we focused 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 (*13*). 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 performing residue analysis.

In the animal experiment, 10 groups of 12 laying hens were included. For the compounds diclazuril, robenidine, halofuginone and nicarbazin, two concentrations levels were tested, namely the maximum level that can be present in feed intended for broilers or pullets and a lower concentration level, corresponding to 5% carry-over at the feeding mill. Since 2001, nicarbazin may no longer be administered alone but only in combination with narasin as Maxiban, and hence narasin was included in the experiment. Dimetridazole is now listed in Annex 4 of Council Directive 2377/90 (*14*) and is, as a consequence, a banned substance. As a result, carry-over is not likely to occur for dimetridazole. Therefore, only one concentration level was included for this compound. In this way, the use of the ELISA test still could be evaluated. Also a blank control group was included. Residue-containing eggs were sampled during the treatment period and for a period of 30 days after withdrawal of the anticoccidial-containing feed.

MATERIALS AND METHODS

Preparation of Diets. Experimental diets were prepared at the mill of CLO-DVV (Agricultural Research Centre, Department of Animal Nutrition and Husbandry, Melle, Belgium).

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 a premix. Therefore, we used an analytical standard purchased 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 + 40 mg/kg for narasin and nicarbazin. For dimetridazole, a feed containing 200 mg/kg was prepared. For each compound, except dimetridazole, a second concentration corresponding to 5% carry-over, i.e., 50 μg/kg for diclazuril, 150 μg/kg for halofuginone, 1800 μg/kg for robenidine and 2000 + 2000 μg/kg for narasin and nicarbazin, was also prepared.

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	927 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	Maxiban	2000 μg/kg	2114 μg/kg	106
	nicarbazin			2144 μg/kg	107
10	dimetridazole	analytical standard Sigma	200 mg/kg	101 mg/kg	50

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. No pelletation was carried out.

Animal Treatment. Animal experiments were conducted at the poultry experimental facility of CLO-DVV (Agricultural Research Centre, Department of Animal Nutrition and Husbandry). A flock of medium weight laying hens (ISA-brown) was used for the trial during the first 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 complete course of the study. 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 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. Collecting of the eggs was stopped 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 refrigerated. 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. They were stored separately at −18 °C.

Sample Analysis. *Feed Samples.* All feed samples were analyzed by liquid chromatography tandem mass spectrometry. Details on the method were described earlier (*10*).

Briefly, after an extraction with methanol, a fraction of the organic phase was evaporated. The dried extract was redissolved, filtered and finally injected into the LC-MS/MS system.

Egg Samples. The whole egg samples of group 4 and 5 (halofuginone), group 8 and 9 (dinitrocarbanilide) and group 10 (dimetridazole) were analyzed both by LC-MS/MS and by ELISA. The whole egg samples containing diclazuril, robenidine and narasine were analyzed by LC-MS/MS only since no suitable ELISA was available. Also the individual egg white and yolk samples were analyzed only by LC-MS/MS.

The procedure used to perform the screening assays were described elsewhere (*8*, *9*). This ELISA proved to be very specific and had an estimated detection capability (CCβ) below 0.5 μg/kg. A second ELISA was developed for the detection of dinitrocarbanilide. With this test, concentrations below 3 μg/kg can be detected in a specific way. The ELISA, which was developed for the detection of dimetridazole, was able to detect dimetridazole and 2-hydroxydimetridazole at levels below 1 μg/kg and 20 μg/kg, respectively.

The mass spectrometric analyses of the egg samples were performed according to the methods described elsewhere (*10*, *11*). In short, an extraction with acetonitrile was performed, and for some compounds (dimetridazole, 2-hydroxydimetridazole and halofuginone) an additional wash step with hexane was added. The decision limit or CCα was 0.5 μg/kg for diclazuril, 1 μg/kg for dimetridazole, halofuginone, robenidine, narasin and dinitrocarbanilide, and 2 μg/kg for 2-hydroxydimetridazole. For each series of samples analyzed, all criteria set by Commission Decision 2002/657/EC, i.e., ion ratios and relative retention time, were checked (*15*).

Method of Quantification. Analyses performed by LC-MS/MS were quantitative. 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 $1/x$. The results were calculated by the TargetLynx software. 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, two spiked samples were included as a control.

RESULTS AND DISCUSSION

Analyses 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, satisfactory 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. Consequently, for halofuginone, there was a 50% and a 5% group (instead of a 100% and 5% group). 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.

Analyses of the Whole Egg Samples. *Analysis by ELISA.* The incurred samples were extracted and tested using the screening assays for halofuginone, dinitrocarbanilide and dimetridazole together with its main metabolite. For each extraction, one known negative sample and three known negative samples fortified at CCβ level were included to serve

Table 2. Overview of the Main Results of the LC-MS/MS Analyses of the Whole Egg Samples

compound	group	% of maximum allowed concn for broilers	first positive sample (concn (μg/kg))	plateau concn (μg/kg)	no. days withdrawal needed to obtain negative sample[a]
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. concn = 70	13
narasin	8	102.3	day 3 (40)	90	18
nicarbazin		101.5	day 2 (3)	6500	23
narasin	9	5.3	day 3 (1)	6	8
nicarbazin		5.4	day 3 (11)	300	15
dimetridazole	10	50.4	day 2 (676)	650	10
2-hydroxydimetridazole			day 2 (1513)	1700	10

[a] Negative sample = concentration below CCα.

as quality control (halofuginone at 0.5 μg/kg, dinitrocarbanilide at 3 μg/kg and dimetridazole at 1 μg/kg). In addition, a standard curve prepared in egg matrix was extracted simultaneously with the samples. It has to be kept in mind however that the ELISAs were developed to perform a qualitative screening test and not to obtain quantitative results. Therefore, concentrations obtained with the ELISAs should be considered as estimations.

The mean values of the absorbance (450 nm) values obtained for each sample were divided by the absorbance value of the zero standard (Bo) and multiplied by 100. The extraction was considered as valid if two out of three quality control samples gave a binding percentage $\leq$58% for the halofuginone assay, $\leq$74% for the dinitrocarbanilide assay or $\leq$76% for the dimetridazole assay and if the negative control showed a binding percentage superior to these values.

In some cases, concentrations were so high that the mean value of the absorbance fell out of the range of values obtained with the extracted standard curve. In that case, the dilution factor was increased and the samples were again applied on the ELISA plate until the OD values corresponded to the range of OD values obtained with the standard curve after extraction.

Analysis by LC-MS/MS. All samples analyzed from the sampling during the adjustment period, i.e., before the administration of the anticoccidial-containing feed, were blank. Moreover, for the group that received blank feed during the entire experiment, all eggs sampled during the entire experiment were blank. Results of the analyses are presented graphically in **Figures 1**−**6**. In **Table 2**, for each group is mentioned from which day on positive samples were encountered, what was the plateau concentration and how many days after withdrawal of the anticoccidial-containing feed it took to obtain residue-free samples.

1. Diclazuril. The depletion curves of both groups receiving diclazuril are presented in **Figure 1**. Diclazuril was detectable in the eggs from birds fed the 1 mg/kg diet from day 2 onward whereas it was detectable in the eggs from birds fed the 0.05 mg/kg diet from day 3 onward. Concentrations increased until a plateau concentration of about 100 μg/kg for the 100% group and a plateau concentration of about 5 μg/kg for the 5% group was reached at day 10. This plateau was maintained until day 16 for the 5% group and until day 18 for the 100% group. Thereafter, concentrations started to drop until no more residues were found 22 days and 11 days after the end of the treatment for the 100% and 5% group, respectively. For diclazuril, a clear relationship between feed and egg concentration was observed. Taken into consideration the fact that yolk formation takes about 10 days and that a plateau concentration is reached 10 days after start of the treatment, this suggests that residues mainly will be present in the egg yolk. For diclazuril, results of only one study could be found in the literature but the limit of detection of the method used was only 50 μg/kg (*16*).

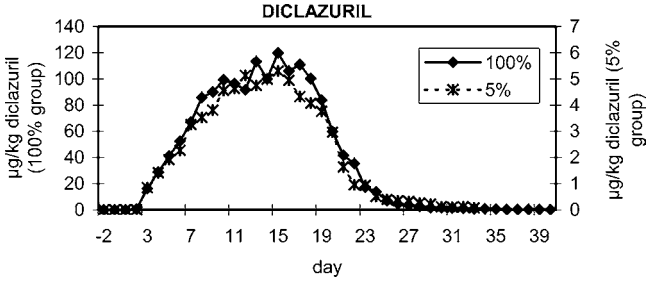

Figure 1. Results of the analyses of the whole eggs of the experimental groups receiving diclazuril.

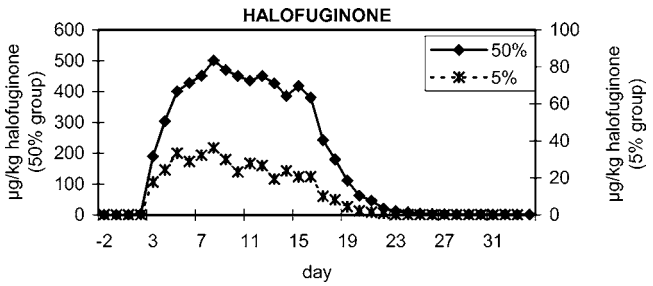

Figure 2. Results of the analyses of the whole eggs of the experimental groups receiving halofuginone.

2. Halofuginone. For halofuginone a similar pattern is observed as for diclazuril. This is shown in **Figure 2**. The first residues appear 2 and 3 days after the beginning of the administration of the halofuginone-containing feed for the 50% and 5% group, respectively. For both groups, a plateau concentration is reached but this happens earlier for the 5% group (day 4) than for the 50% group (day 7). As this plateau is already reached at day 4 and 7, halofuginone residues will probably also be present in the albumen. Plateau concentrations are 450 μg/kg for the highest concentration group and 30 μg/kg for the lowest concentration group. So for halofuginone, the relationship between feed and egg concentration is less obvious. Yakkundi et al. described an animal experiment in which laying hens were fed halofuginone-containing feed in order to establish the relationship between the halofuginone concentration in feed and the residues in eggs. Five groups of six laying hens were fed with halofuginone-containing diets at concentrations ranging between 0.1 and 10% of the therapeutic dose for broilers (3

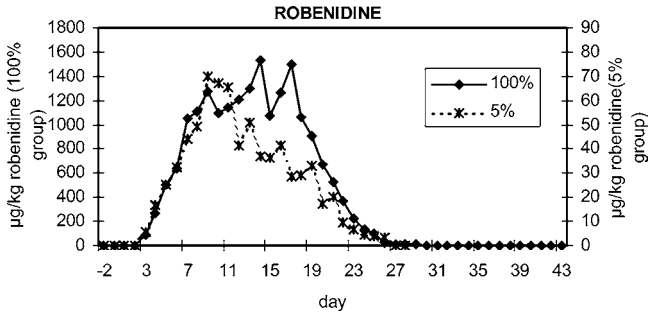

Figure 3. Results of the analyses of the whole eggs of the experimental groups receiving robenidine.

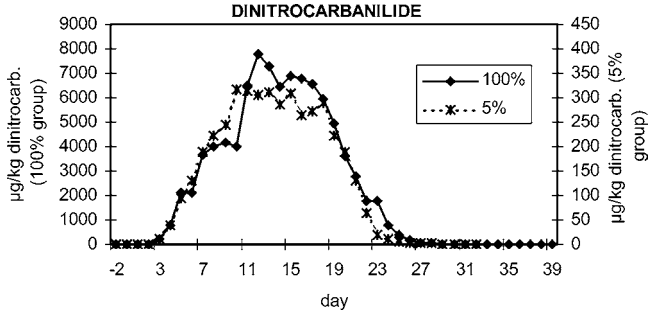

Figure 4. Results of the analyses of the whole eggs of the experimental groups receiving nicarbazin.

mg/kg) for 14 days. The group fed the highest dose was then fed with a halofuginone-free diet for a further 14 days. A plateau concentration of about 40 μg/kg was observed after feeding laying hens feed containing 0.3 mg/kg halofuginone (*17*). Another animal experiment, in which laying hens were fed halofuginone-containing feed, was described by Mulder et al. Twenty ISA brown laying hens were treated with feed containing 3 mg/kg halofuginone for 14 days. Eggs were collected before, during and after treatment. Residue concentrations were determined in whole egg, as well as in the yolk and albumen. Mulder et al. reported a plateau concentration of 450 μg/kg after administrating feed with 3 mg/kg halofuginone during 14 days (*18*).

3. Robenidine. For robenidine (**Figure 3**), only for the 100% concentration group, a plateau is reached, i.e., about 1300 μg/kg. For the 5% group, a maximum value of 70 μg/kg is reached on day 9 of the experiment, and from that point on, a decrease is observed. For both concentration groups, the first robenidine residues were observed on the third day of the experiment. For the 5% group, no more residues were found 13 days after cessation of the treatment. For the 100% group, it took 26 days to obtain concentrations below the CCα value (1 μg/kg), but until day 29, traces of robenidine could still be found. To our knowledge, no experiments with robenidine were published elsewhere.

4. Dinitrocarbanilide. The administration of nicarbazin to laying hens clearly leads to considerable amounts of dinitrocarbanilide in eggs. This is shown in **Figure 4**. For the highest concentration group from day 11 onward, a plateau concentration of 6500 μg/kg was observed. This plateau was maintained until day 18 of the experiment. Thus only 5 days after cessation of the treatment with nicarbazin, dinitrocarbanilide concentrations in the eggs start to drop. Residues can be found more than three weeks (23 days) after nicarbazin-free feed was given. For the 5% group also a plateau is reached: this happens from day 10 until day 18 of the experiment. As was the case with diclazuril, residues are probably present in the egg yolk since

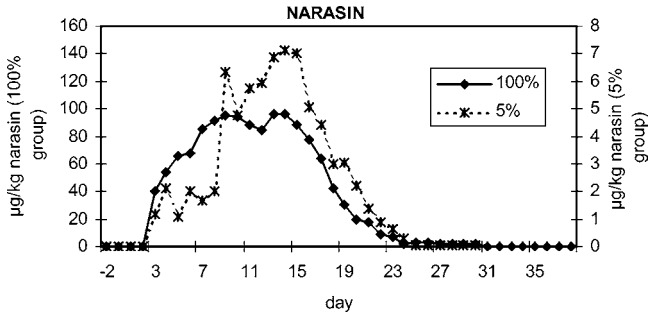

Figure 5. Results of the analyses of the whole eggs of the experimental groups receiving narasin.

it takes 10 days to reach the plateau. For the 5% group, it took 15 days to obtain eggs free of residues of dinitrocarbanilide. Blanchflower et al. described a small feeding trial in which a group of 5 laying birds was fed a ration formulated to contain 10 mg/kg nicarbazin for 9 consecutive days. DNC concentrations continued to rise throughout the experiment, reaching a mean level of 309 μg/kg on day 9 (*19*). Cannavan et al. designed an experiment to establish the relationship between nicarbazin-containing feed and nicarbazin residues in eggs. Five groups of six laying hens received for 16 consecutive days daily 120 g feed containing 0.2, 0.4, 1.3, 3.8 and 12.1 mg/kg nicarbazin, respectively (*4*). Concentrations of dinitrocarbanilide in the whole eggs increased rapidly until about day 6, and then reached a plateau. The plateau concentration was 600 g/kg. Twelve days after withdrawal of the nicarbazin-containing feed, DNC was not longer detected in the eggs.

5. Narasin. Although narasin is administered as Maxiban and thus together with nicarbazin, a different pattern for narasin and dinitrocarbanilide was observed, as shown in **Figure 5**. The plateau concentration of 90 μg/kg is already reached 8 days after start of the treatment. This plateau is maintained until day 15 of the experiment. From day 16 onward, i.e., 2 days after the switchover to Maxiban-free feed, narasin concentrations already start to drop. Concentrations below 1 μg/kg are reached 17 days after this switchover. But until after 24 days, still some narasin was detected in the whole egg samples. For the lowest concentration group, a totally different pattern was observed. From day 3 on, it seems like a plateau is reached of about 2 μg/kg but then suddenly a new plateau of 6 μg/kg is reached that is maintained for 7 days. Eight days after the ending the treatment with Maxiban, concentrations fall below 1 μg/kg. For narasin, only one study with laying hens is described. In an experiment carried out by Kolsters three groups of 4 laying hens were fed narasin-containing feed during 7 days at a concentration of 76 mg/kg and another three groups of 4 laying hens at a concentration of 3 mg/kg (*20*). In this experiment a detection method was used which had a limit of detection of 10 μg/kg. Therefore, it is difficult to compare the results.

6. Dimetridazole. For dimetridazole, only one group was included in the experiment. But as mentioned in the Introduction, when performing residue analysis for dimetridazole, also the main metabolite, 2-hydroxydimetridazole, has to be monitored. The results are presented in **Figure 6**. For both compounds, the first positive samples are encountered from day 2 onward and immediately the plateau concentration is reached. This suggests that residues will mainly be present in the egg white since the egg white concentrations can be considered as a measure for the plasma concentration (*21*). Clearly higher concentrations of the metabolite are found. Immediately after cessation of the treatment, concentrations drop. During the plateau period, the metabolite/parent compound ratio equals 2.6

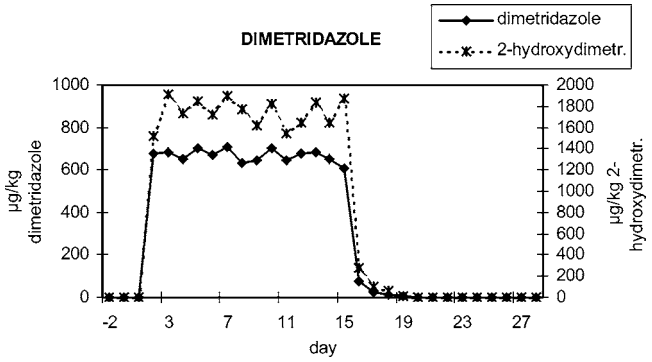

Figure 6. Results of the analyses of the whole eggs of the experimental groups receiving dimetridazole.

± 0.2. This clearly indicates that the hydroxy metabolite must be included when performing residue analysis for dimetridazole.

An animal experiment in which laying hens were fed feed containing 10 mg/kg dimetridazole was described by Cannavan et al. (*22*). In this trial laying hens received daily 120 g feed containing approximately 10 mg/kg dimetridazole for 7 days. Residues of dimetridazole were found in the eggs taken 1 day after commencement of the dimetridazole diet and in all eggs taken thereafter. The mean concentration in eggs taken after 7 days was 21.6 µg/kg. The samples were not analyzed for 2-hydroxydimetridazole.

Comparison of the Results Obtained by ELISA and LC-MS/MS. The main objective of the development of the ELISAs was to reduce the number of samples that need to be analyzed by the more expensive LC-MS/MS methods. Therefore, it is import that the screening method does not produce false negative results. With the incurred samples of the animal experiment, this could be tested. A comparison of the results between both methods is presented in **Table 3**. The ELISA is perfectly capable of identifying the first positive samples as shown in the first part of the table. But the ELISA for the detection of dinitrocarbanilide clearly overestimates the concentrations. During depletion, the ELISAs detect residues somewhat longer, and hence false positive results are generated. Nevertheless, it can be concluded that the ELISAs are perfectly suitable for performing the screening.

Results in the Perspective of the Action Limit Set by the Belgian Food Agency. As mentioned earlier, in December 2004 an action limit of 10 µg/kg was set by the Scientific Committee of the Belgian Food Agency for monensin, salinomycin, diclazuril, lasalocid, maduramycin, narasin, nicarbazin, robenidine and all sulfonamides. Especially for the 5% groups, it is interesting to check when concentrations drop below 10 µg/kg. For the 5% diclazuril group, concentrations never exceed the 10 µg/kg value as the plateau concentration equals 5 µg/kg. For the highest diclazuril concentration group, it would take 11 days to obtain residues below 10 µg/kg. Also for narasin, concentrations of the lowest group never exceed 10 µg/kg. For the 5% groups of robenidine and nicarbazin, it would take 8 and 11 days until concentrations fall below 10 µg/kg. For the highest concentration groups, 15, 8 and 19 days are required to obtain concentrations below the action limit for robenidine, narasin and nicarbazin, respectively. Although not included in the advice, for halofuginone it would take 4 and 10 days for the lowest and highest concentration group, respectively.

Analyses of the Yolk and Albumen Samples. As mentioned in the animal treatment-section, also some separate yolk and albumen samples were analyzed. The same methods as for the whole egg samples were used. 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. Another remark that has to be made is that the egg samples were stored refrigerated for several months before yolk and albumen were separated. This is because the separate analysis of yolk and albumen was originally not planned. Therefore, it cannot be excluded that there was transfer from residues from one egg compartment to the other. However, Kolsters et al. tested this possible transfer for monensin, narasin and salinomycin and no differences in concentration were observed when separating yolk and albumen immediately after laying or after storage (*20*).

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 dinitrocarbanilide, similar results were obtained by Cannavan et al. (*4*). The relatively nonpolar component of nicarbazin, was also found to be almost exclusively contained in the more fatty matrix of the yolk. For the highest concentration group of robenidine, 2300 µg/kg was

Table 3. Comparison of the Analyses of the Whole Eggs Samples by ELISA and LC-MS/MS

		ELISA		LC-MS/MS	
compound	group	first positive sample	concn (µg/kg)[a]	first positive sample	concn (µg/kg)
halofuginone	5%	day 3	15	day 3	17.8
	50%	day 2	3	day 2	3.3
dinitrocarbanilide	5%	day 3	26	day 3	10.7
	100%	day 2	11	day 2	2.7
dimetridazole + 2-hydroxydim.	100%	day 2	926	day 2	676.3 + 1513.1

		day below (deg) concentration		
compound	group	concn (deg) (µg/kg)	ELISA	LC-MS/MS
halofuginone	5%	1	day 25	day 22
	50%		>day 34	day 33
dinitrocarbanilide	5%	1	day 31	day 29
	100%		>day 34	day 37
dimetridazole + 2-hydroxydim.	100%	DMZ: 1 DMZ-OH: 2	day 23	DMZ: day 23 DMZ-OH: day 24

[a] Estimations.

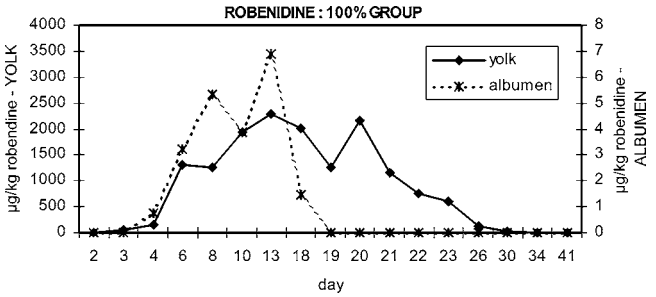

Figure 7. Results of the analyses of the separate yolk and albumen samples for the 100% robenidine group.

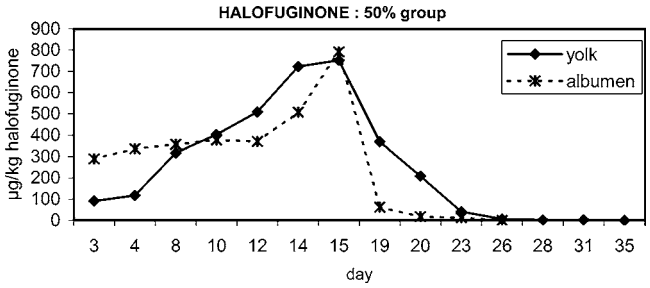

Figure 8. Results of the analyses of the separate yolk and albumen samples for the 50% halofuginone group.

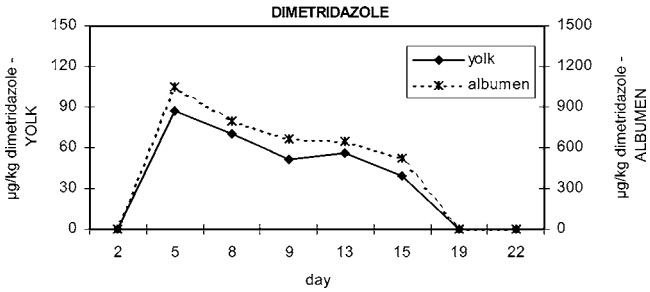

Figure 9. Results of the analyses of the separate yolk and albumen samples for the 100% dimetridazole group: dimetridazole.

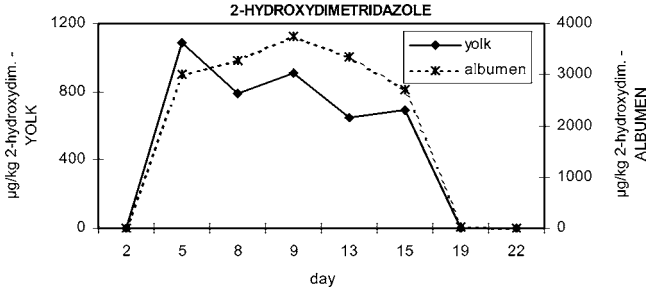

Figure 10. Results of the analyses of the separate yolk and albumen samples for the 100% dimetridazole group: 2-hydroxydimetridazole.

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 robenidine, residues disappear much faster out of the albumen than the yolk. This is very clearly presented for the 100% robenidine group in **Figure 7**.

As shown in **Figure 8**, for halofuginone, initially more residues are found in the albumen. During the plateau period, halofuginone can be found in both compartments. During depletion, residues are longer found in the yolk. All these observations reflect the process of egg formation in the hen. Similar results are obtained for the lowest halofuginone concentration group. These observations are in agreement with those of Yakkundi et al. who reported that the concentrations in the egg yolk were marginally higher than those in the albumen (*17*). Mulder et al. on the other hand, reported that residue concentrations were approximately twice that in albumen (*18*). This ratio remained more or less constant during the medication and post-medication period. This does not seem to agree with the process of egg formation, which predicts residues to last longer in the yolk. They observed substantial variability with respect to the concentrations determined in the individual egg white and yolk samples (relative standard deviation up to 78%) as well as in the distribution ratio (relative standard deviation up to 74%). They suggest that this may be due to differences in metabolism between individual hens but mention also that the method performed less well for the egg white and yolk matrixes than for whole egg.

For dimetridazole and 2-hydroxydimetridazole, a completely different pattern is observed (Figures 9 and 10). Dimetridazole concentration is 10 times higher in the albumen than in the yolk. Also for the hydroxy metabolite, higher concentrations are found in the albumen. These observations are in agreement with the curves observed for the whole egg samples.

It can be concluded that this experiment has shown that carry-over levels of anticoccidials in feed intended for laying hens leads to the presence of residues in eggs. Even with 5% carry-over, it can take up to 15 days to become residue-free eggs. The analyses of the separate yolk and albumen revealed that differences in distribution between the different compounds are big. The ELISAs developed within the framework of the project (i.e. for halofuginone, dimetridazole and nicarbazin) can be used to perform a screening.

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