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SPSD II

INTEGRATED APPROACH FOR THE CONTROL ON RESIDUES OF COCCIDIOSTATS IN EGGS

CP-29

SCIENTIFIC SUPPORT PLAN FOR A SUSTAINABLE DEVELOPMENT POLICY

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INTEGRATED APPROACH FOR THE CONTROL ON RESIDUES OF COCCIDIOSTATS IN EGGS

E. DAESELEIRE, P. DELAHAUT, G. HUYGHEBAERT

PART 1

SUSTAINABLE PRODUCTION AND CONSUMPTION PATTERNS

GENERAL ISSUES

AGRO-FOOD

ENERGY

TRANSPORT

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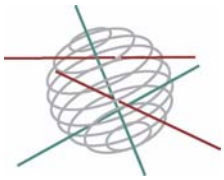
SCIENTIFIC SUPPORT PLAN FOR A SUSTAINABLE DEVELOPMENT POLICY
(SPSD II)



Part 1:
Sustainable production and consumption patterns

FINAL REPORT

INTEGRATED APPROACH FOR THE CONTROL ON RESIDUES OF COCCIDIOSTATS IN EGGS

CP/29

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FINAL REPORT

1. PROJECT TITLE

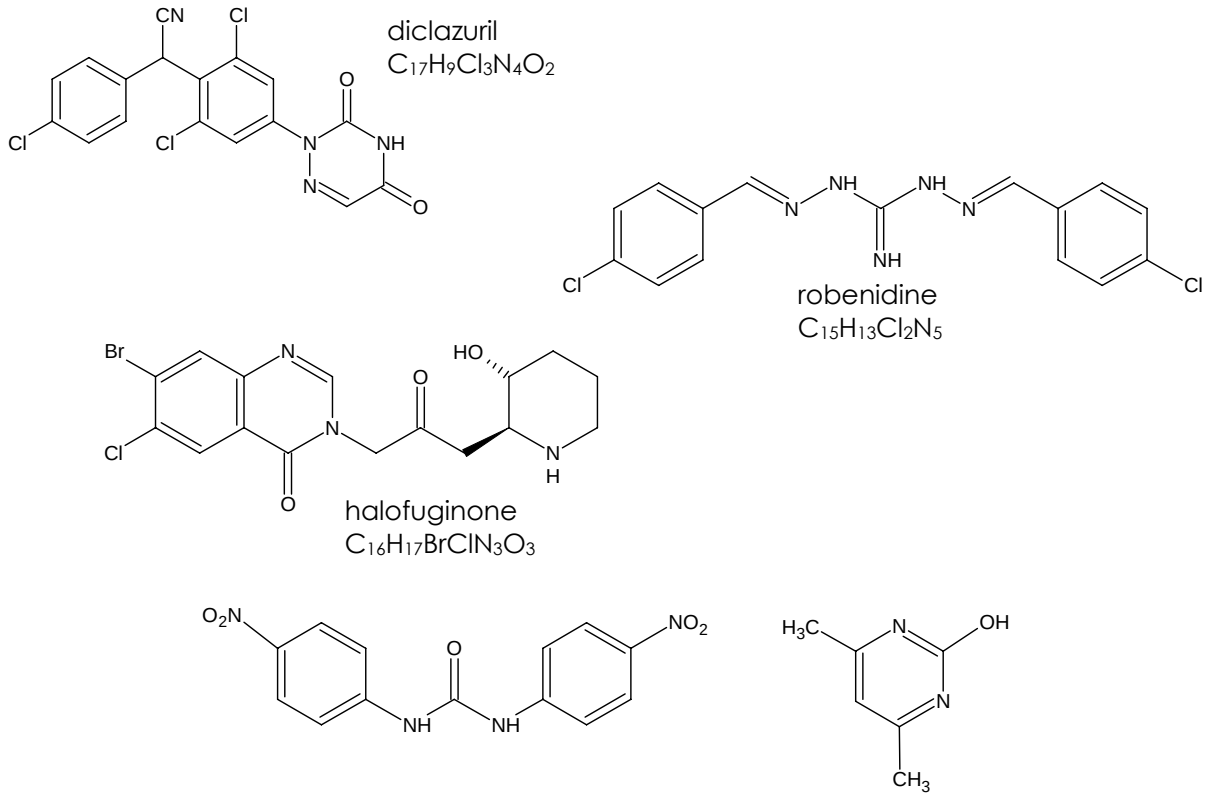
"Integrated approach for the control on residues of coccidiostats in eggs"

2. INTRODUCTION

Since a few decades our society is very much concerned about the microbiological and chemical safety of food. Several crises, mainly of animal origin, (hormones, PCB's, BSE, …) broke out the last fifteen years with a decline in consumption of certain foodstuffs as a consequence. To prevent such crises in the future and to be able to guarantee safe food for the consumers, an effective control is necessary. Also the European Communion is concerned about the health of the consumers and set maximum residue limits (MRLs) in different matrices for a lot of veterinary drugs (Regulation 2377/90/EC). To perform efficient controls on the correct use of veterinary drugs and on the absence of forbidden substances an economical liable approach is necessary. This approach can at first be used by official governmental laboratories and in a later phase in the different sectors performing self-control on the products they supply.

The aim of this project is to set up an integrated approach for the control on the presence of residues of coccidiostats in eggs, to check the applicability of the methods developed on incurred samples and to perform a monitoring plan on Belgian egg samples from different origin.

Coccidiostats are compounds that are widely used to prevent and treat coccidiosis, a contagious amoebic disease affecting livestock, particularly poultry that is associated with warm and humid conditions. These feed additives are licensed in broilers and in pullets but not in laying hens. It was shown in the past that accidental cross contamination of feed could lead to residues of the compounds in eggs. Out of the several compounds that were introduced since 1948, five compounds were chosen to be studied, namely diclazuril, dimetridazole, nicarbazin, halofuginone and robenidine. Their molecular structures are presented in figure 1.



nicarbazin ($C_{19}H_{18}N_6O_6$) (equimolar complex, 1/1 ratio)

=

4,4'-dinitrocarbanilide + 2-hydroxy-4,6-dimethylpyrimidine

$C_{13}H_{10}N_4O_5$ $C_6H_8N_2O$

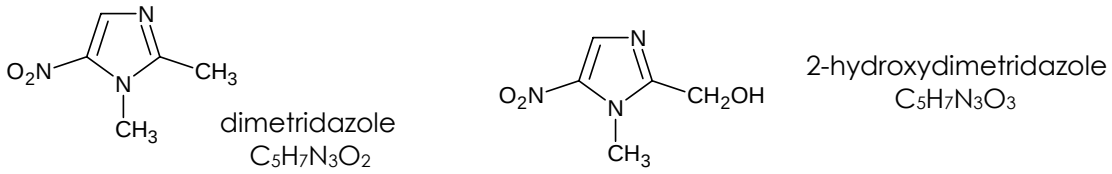


Figure 1 : molecular structures of the compounds studied

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. Thus, for the development of a method to detect residues of nicarbazin in eggs, we focused only on the DNC compound.

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. 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.

The first step in the integrated approach is performing screening analyses by immunological methods. For dimetridazole, nicarbazin and halofuginone antibodies were already developed in the framework of a European project "Poultry check". For diclazuril and robenidine the antibodies will be raised in rabbits after appropriate conjugation with a carrier. With all the available antibodies ELISAs will be developed with the aim of obtaining a detection limit as low as possible. The possibility to develop multi-residue ELISAs, to reduce the number of tests, will be explored. An appropriate and as simple as possible cleanup of the egg samples will be developed to reduce the noise level caused by the matrix. For confirmation purposes liquid chromatographic – mass spectrometric methods, based on molecular structure identification, will be developed for the different compounds. Also here different clean-up procedures will be tested on their usefulness with liquid chromatographic – mass spectrometric detection.

The immunological and mass spectrometric methods will be validated according to the criteria set by the revision of Decision 93/256/EC (since 1 September 2002 : 2002/657/EC). Parameters such as specificity/selectivity, analytical limits, precision, trueness and robustness will be determined.

The applicability of the methods on incurred samples will be tested because in the past it was shown that in spiked samples factors like protein binding are not covered. Therefore laying hens will be fed feed containing the compounds studied at levels that are currently used in practice and at levels that could originate from carry-over in the feed mills. Eggs will be gathered before and after the treatment and will be analysed by the methods developed. Excretion curves and the time for complete removal of the residues in the eggs will be determined. Also the influence on the zootechnical parameters (laying rate, egg weight, feed intake and feed conversion), on the yolk colour and on the shell quality will be checked.

In the last stage of the project Belgian eggs obtained from different sources (supermarket, retail trade, farmers, bio-farmers, packing stations…) will be examined so that the incidence of positive cases in Belgian eggs can be determined.

The aim of this project is to develop an integrated approach for the detection of residues of coccidiostats in eggs. This includes the development of immunological screening methods, the development of mass spectrometric confirmation methods, the validation of these methods according to the most recent European legislation, the applicability of the methods to incurred eggs samples and finally the monitoring of commercially available egg samples.

3. RESULTS, CONCLUSIONS AND RECOMMENDATIONS

The results, conclusions and recommendations are presented according to the different tasks of the project.

3.1. TASK A : DEVELOPMENT OF IMMUNOLOGICAL SCREENING METHODS

task A.1 : Literature study

- *partner(s) responsible :* CER
- *objective :* By carrying out a thorough literature study, the knowledge of the immunological analysis of coccidiostats is actualised. The objective is to know the marker metabolites and to determine the best way to synthesize haptens
- *results :* Few publications concerning the determination of residues of coccidiostats by immunological methods were found. No publication was found to help us to synthesize haptens for diclazuril and robenidine.
- *conclusions :* The literature study was regularly reviewed (even if the task is completed) to obtain more information about the five studied coccidiostats, their stability, their detection, their extraction from eggs...

task A.2 : Purchase of standard material

- *partner(s) responsible :* CER
- *objective :* Standards of the five compounds have to be gathered. When they are not available at commercial suppliers, contact has to be taken with the

pharmaceutical companies distributing the drugs or with the authorized European Reference Laboratories.

- *results :* In table 1, an overview is given of the standards, their origin and their solvent. For each compound, a solution of 1 mg/ml was prepared in the adequate solvent; then, a solution of 1 µg/ml was made in ethanol by serial dilution (10 x). The latter solution was used to prepare the standards curve in the buffer used to dilute antibodies and conjugates.

Table 1 : overview of the standards

Compound	Standard	Supplier	Solvent
Halofuginone	Halofuginone	Intervet, Mechelen, Belgium	Water
Nicarbazin	Nicarbazin	Sigma, St Louis, MO, USA	Dimethylsulfoxide (DMSO)
	Dinitrocarbanilide	Sigma, St Louis, MO, USA	Dimethylsulfoxide (DMSO)
Nitroimidazoles	Dimetridazole	Sigma, St Louis, MO, USA	Ethanol
	Hydroxydimetridazole	B.g.V.V., Berlin, Germany	Ethanol
	Ipronidazole	B.g.V.V., Berlin, Germany	Ethanol
	Hydroxyipronidazole	B.g.V.V., Berlin, Germany	Ethanol
	Ronidazole	Sigma, St Louis, MO, USA	Ethanol
	Metronidazole	Sigma, St Louis, MO, USA	Ethanol
	Hydroxymetronidazole	B.g.V.V., Berlin, Germany	Ethanol
Diclazuril	Diclazuril (R064433)	Janssen, Beerse, Belgium	Dimethylformamide (DMF)
Robenidine	Robenidine	Alpharma, Technical Center, Willow Island, USA	Ethanol

- *conclusions :* For each compound, a suitable standard was found and stock solutions were made.

task A.3 : Synthesis of antigen and conjugate (diclazuril and robenidine)

- *partner(s) responsible :* CER
- *objective :* In order to elicit an immune response in the host animal, the coccidiostats must be chemically conjugated to a large carrier protein. For the conjugates, the immunogenic carrier protein has been replaced by HRP.

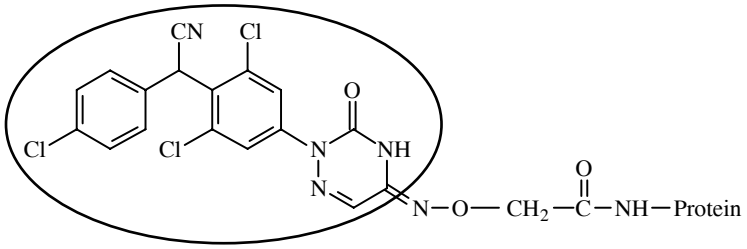
- *results :*

A.3.1. Diclazuril

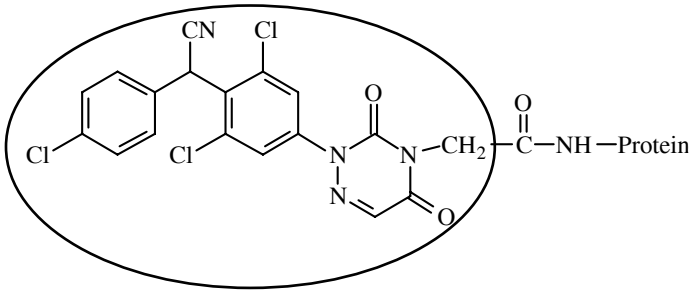
Fifteen diclazuril antigens were produced for this project: (1-2) Diclazuril-Carboxymethyl oxime (CMO) either conjugated to Human Serum Albumin (HSA) or to Bovine Thyroglobulin (BTG); the first reaction was done between diclazuril and carboxymethoxylamine hemihydrochloride, then the conjugation with carrier protein was done by using Ethyl Dimethylaminopropyl Carbodiimide (EDC) and N-Hyrdroxysuccinimide (NHS) reaction. (3-4) Carboxydiclazuril-BTG prepared either via mixed anhydride or via EDC/NHS reaction. (5) Carboxydiclazuril-HSA prepared via mixed anhydride. (6-7) Diazo-diclazuril-HSA and diazo-clazuril-HSA, the first step was the synthesis of diazotised aminobenzoic acid then the addition of diclazuril or clazuril and finally the EDC/NHS reaction to conjugate the carrier protein. (8-9) Diclazuril carboxylic acid either conjugated to HSA or to BTG; these antigens were prepared by using the EDC/NHS reaction on the acid and reacting the resultant intermediate with free amines on the proteins. (10-11) Diclazuril carboxylic acid (HSA or BTG), these antigens were prepared via carbonyldiimidazole (cbmi). (12-13) Azauridine (HSA or BTG), azauridine contains the heterocyclic ring of diclazuril and so it may produce an antibody that will be able to bind it. (14-15) Amino diclazuril (HSA or BTG), these antigens were prepared by diazotising of the aromatic amine followed by reaction with free amines on the proteins. The last compounds were kindly provided by Janssen Animal health (Beerse, Belgium).

Tracer conjugates were synthesized using the same haptens but replacing carrier protein (HSA or BTG) by an enzyme Horseradish Peroxidase (HRP). In figure 2 the molecular structures of these chemical syntheses are given (the parts similar to diclazuril are surrounded).

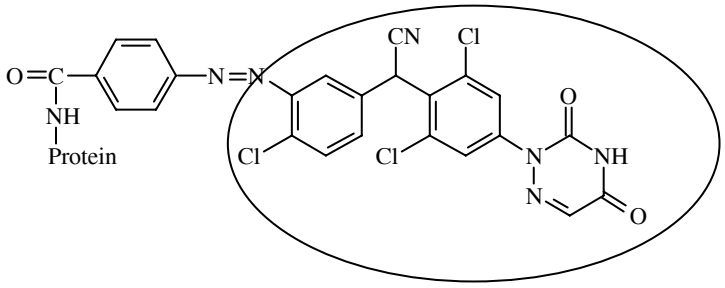
(1-2) diclazuril-CMO-HSA or BTG
diclazuril-CMO- HRP



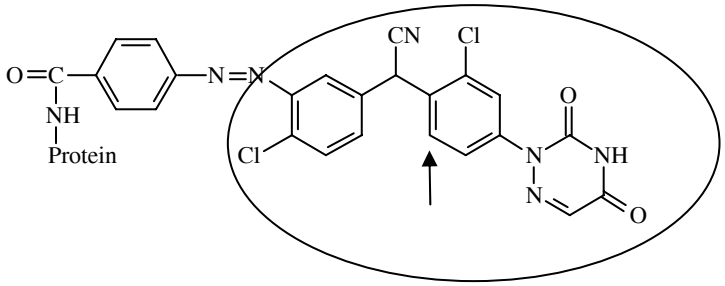
(3-4) Carboxydiclazuril-BTG via mixed anhydride and via EDC/NHS
Carboxydiclazuril-HSA via mixed anhydride
Carboxydiclazuril-HRP via mixed anhydride and via EDC/NHS



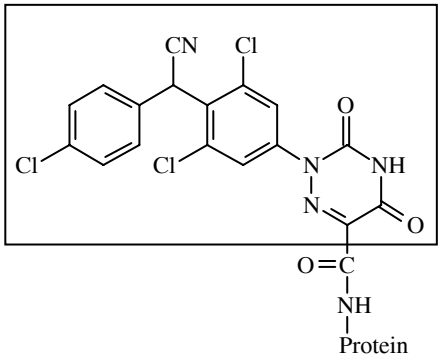
(6) diazo-diclazuril-HSA
diazo-diclazuril-HRP



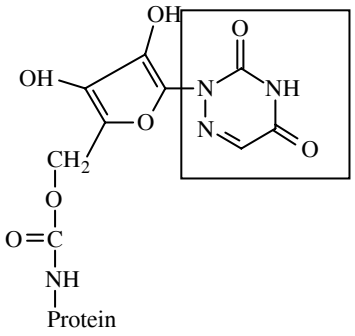
(7) diazo-clazuril-HSA
diazo-clazuril-HRP



(8-9-10-11) diclazuril-carboxyl acid-HSA or BTG (via EDC/NHS or via cbmi)
diclazuril-carboxyl acid-HRP (via EDC/NHS or via cbmi)



(12-13) azauridine-HSA or BTG
azauridine-HRP



(14-15) aminodiclazuril-HSA/BTG
aminodiclazuril-HRP

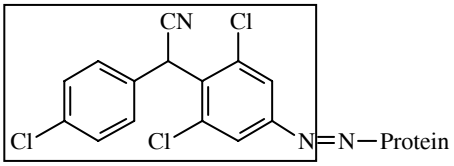


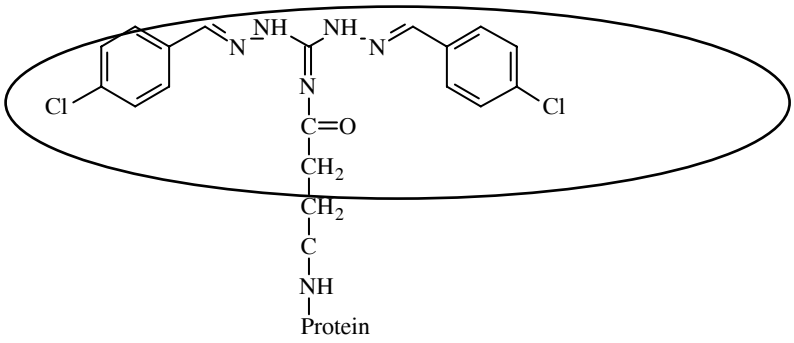
Figure 2: chemical structures of diclazuril antigens injected on the rabbits in order to induce a production of the polyclonal antibodies. Enzyme labelled were synthesized using the same method but replacing carrier protein by an enzyme HRP

Rabbits were immunised every 28 days with the different antigens and blood samples were taken 10 days after each immunisation (from the third immunisation onward). The immunisation results for each diclazuril antigen are presented in A.4.1.

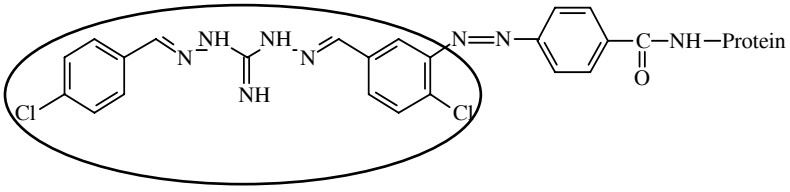
A.3.2. Robenidine

Eight robenidine antigens were produced for this project: (1-2) Robenidine-hemisuccinate-HSA or BTG, the succinylation site at –NH becoming COOH can be conjugated to the carrier protein by using EDC/HNS. (3-4) Diazo-robenidine-HSA or BTG, the first step was the synthesis of diazotised aminobenzoic acid then the addition of robenidine and finally the diazotised derivaties were conjugated to the carrier protein via a NHS enhanced, carbodiimide-mediated coupling reaction. (5-6) Diazo-chloroanilide-HSA or BTG, the sub-structure marked by a box is similar to part of the robenidine structure except that the C=N has been replaced by N=N (figure 3). If antibodies are created against the diazo-chloroanilide, there is a possibility that these antibodies will bind free robenidine. (7-8) Diazo-chloroanilide-HSA or BTG prepared as explained above (second trial).

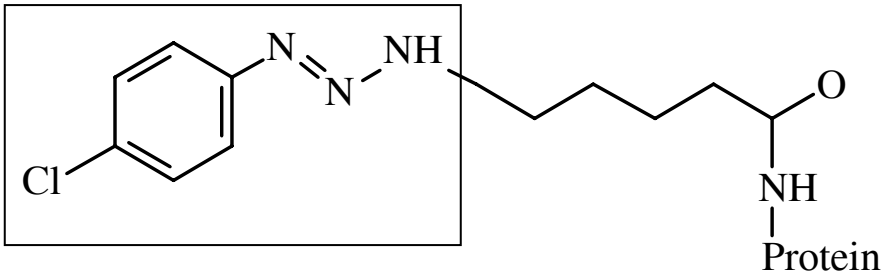
(1-2) robenidine-hemisuccinate-HSA or BTG
robenidine-hemisuccinate-HRP



(3-4) diazo-robenidine-HSA or BTG
diazo-robenidine-HRP



(5-6-7-8) diazo-chloroanilide-HSA or BTG
diazo-chloroanilide-HRP



robenidine

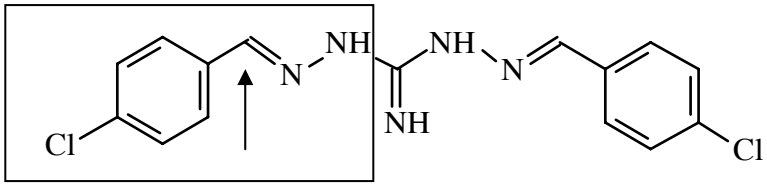


Figure 3: chemical structures of robenidine antigens injected on the rabbits in order to induce a production of the polyclonal antibodies. Their corresponding conjugates are also presented.

The robenidine conjugates were produced using the same way but HRP replaced the carrier protein. In figure 3 the molecular structures of these chemical syntheses are given, the parts similar to robenidine are surrounded. The immunisation results for each robenidine antigen are presented in A.4.2.

- ***conclusions*** *:* Small molecules like coccidiostats must be chemically conjugated to carrier proteins prior to immunization. This is a critical step which greatly influences the successful production of antibodies and their quality. Basically, the position on the molecule through which linkage occurs, the length of the linker arm and the chemistry of the linkage are critical factors. The chemical syntheses presented in figure 2 and 3 showed that we tested these different parameters. Moreover, an immunisation program takes time and we must wait five to six months before knowing if injected rabbits give an immune response or not. Eight robenidine antigens + four robenidine conjugates and fifteen diclazuril antigens + nine diclazuril conjugates were produced for this project. Forty rabbits were immunized with robenidine antigens and seventy five rabbits were immunized with diclazuril antigens.

task A.4 : Production of the polyclonal antibodies (diclazuril and robenidine)

- *partners responsible :* CER
- *objective :* The rabbits were injected subcutaneously with the immunogens every 28 days. During the immunization, the titer is checked in order to know if the rabbits were able to produce antibodies against diclazuril or robenidine. The titer helps to determine the ideal period to proceed to the final bleeding if an immune response occurred.
- *results :*

A.4.1. Diclazuril

Five rabbits were injected with each immunogen presented above (figure 2). Diclazuril antisera were characterized with regards to titer using all enzyme labelled drugs synthesized for the diclazuril compound (table 2).

Table 2*:* summary of immunisation results obtained with all diclazuril antigens. Each antisera has been tested with all diclazuril conjugates that we had in our possession since the beginning of the project.

Antigen	Rabbits	Conjugates	Immune response	Competition
Diclazuril-CMO-HSA	M 113 to M 117	a) carboxydiclazuril-HRP (EDC/N b) carboxydiclazuril-HRP (mixed anhydride) c) diclazuril-CMO-HRP d) diazo-diclazuril-HRP e) diazo-clazuril-HRP f) diclazuril carboxylic acid-HRP (EDC/NHS) g) diclazuril carboxylic acid-HRP h) aminodiclazuril-HRP i) azauridine-HRP	No	No
Diclazuril-CMO-BTG	M 118 to M 122	a, b, c, d, e, f, g, h, i	M 120, M 121, M 122 with c	No
Carboxydiclazuril-BTG (EDC/NHS)	M 260 to M 264	a, b, c, d, e, f, g, h, i	No	No
Carboxydiclazuril-BTG (mixed anhydride)	M 265 to M 269	a, b, c, d, e, f, g, h, i	No	No
Carboxydiclazuril-HSA (mixed anhydride)	M 270 to M 274	a, b, c, d, e, f, g, h, i	No	No

Diazo-diclazuril-HSA	M 295 to M 299	a, b, c, d, e, f, g, h, i	No	No
Diazo-clazuril-HSA	M 300 to M 304	a, b, c, d, e, f, g, h, i	No	No
Diclazuril carboxylic acid-HSA (EDC/NHS)	M 415 to M 419	a, b, c, d, e, f, g, h, i	M 417 with c	No
Diclazuril carboxylic acid-BTG (EDC/NHS)	M 410 to M 414	a, b, c, d, e, f, g, h, i	M 413 with c	No
Diclazuril carboxylic acid-HSA (cbmi)	M 450 to M 454	a, b, c, d, e, f, g, h, i	No	No
Diclazuril carboxylic acid-BTG (cbmi)	M 445 to M 449	a, b, c, d, e, f, g, h, i	No	No
Azauridine-HSA	M 420 to M 424	a, b, c, d, e, f, g, h, i	All rabbits with i	No
Azauridine-BTG	M 415 to M 419	a, b, c, d, e, f, g, h, i	All rabbits with i	No
Aminodiclazuril-HSA	M 400 to M 404	a, b, c, d, e, f, g, h, i	No	No
Aminodiclazuril-BTG	M 405 to M 409	a, b, c, d, e, f, g, h, i	No	No

No titer was obtained either for rabbits injected with diclazuril-CMO-HSA (1). The bleeding results of rabbits M 120, M 121 and M 122 (diclazuril-CMO-BTG (2)) showed a production of antibodies using conjugate diclazuril-CMO-HRP. The bleeding results of rabbits immunized with carboxydiclazuril derivatives (3-4-5) or with diazo-(di)clazuril-HSA (6-7) didn't show any production of antibodies. No antibody was obtained for rabbits injected with antigens diclazuril carboxylic acid-HSA/BTG via EDC/NHS (8-9) except the rabbit M 413 (diclazuril carboxylic acid-BTG) and rabbit M 417 (diclazuril carboxylic acid-HSA) showing a production of antibodies with the conjugate diclazuril-CMO-HRP. No titer was obtained for rabbits injected with the antigen diclazuril carboxylic acid-HSA/BTG via cbmi (10-11). All rabbits injected with the antigens azauridine-HSA/BTG (12-13) showed an immune response when working with conjugate azauridine-HRP. No detectable immune response was obtained with the antigens aminodiclazuril-HSA/BTG (14-15) even if the antisera were tested with all available conjugates.

Fifteen out of seventy five rabbits presented an immune response. Working with these antisera, several conditions were tested in order to observe a displacement by a standard curve (until 1 μg/ml of diclazuril) but all failed. Among these parameters, the incubation temperature (4°C and 37°C), the time incubation (overnight and two hours), the assay format (the usual format of this work presented in figure 4 and another one called B) were tested.

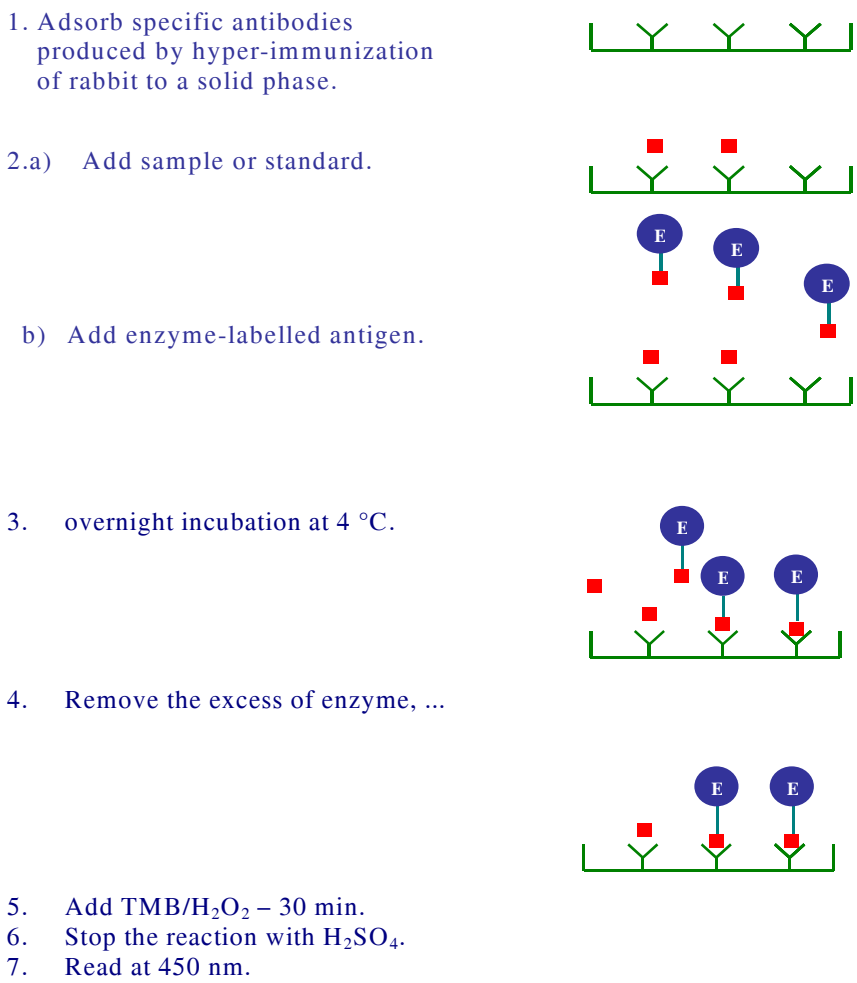


Figure 4 : schematic representation of the competitive ELISA used in our work

Format B: a 96-wells plate was first coated with sheep antibodies raised against rabbit IgGs, while antibodies from rabbits M 120 to M 122, M 413, M 417 and M 415 to M 424 and the tracer/analyte were added in solution.

We also tried to purify the antibody M 122 on protein A Sepharose before being coated on microwells plate. Two different incubation times (two hours and overnight) were compared for purified antibody M 122 as well as two different temperatures (4°C and room temperature). Even purified, the antibody was unable to create any displacement.

The antibodies were unable to create any displacement, any competition in spite of all these trials. Due to unwanted cross-reactivities towards the region between diclazuril and the carrier protein, there is no recognition with free diclazuril.

A.4.2. Robenidine

Five rabbits were injected subcutaneously with each robenidine antigen presented in figure 3. Robenidine antisera were characterized with regards to titer using all enzyme labelled drugs synthesized for the robenidine compound (table 3).

Table 3: summary of immunisation's results obtained with the all robenidine antigens. Each antisera has been tested with all robenidine conjugates that we had in our possession since the beginning of the project.

Antigen	Rabbits	Conjugates	Immune response	Competition
Robenidine-hemisuccinate-HSA	M 143 to M 147	a) robenidine-hemisuccinate-HRP b) diazo-robenidine-HRP c) diazo-chloroanilide-HRP d) diazo-chloroanilide-HRP (second trial)	No	No
Robenidine-hemisuccinate-BTG	M 148 to M 152	a, b, c, d	No	No
Diazo-robenidine-BTG	M 250 to M 254	a, b, c, d	No	No
Diazo-robenidine-HSA	M 255 to M 259	a, b, c, d	No	No
Diazo-chloroanilide-BTG	M 275 to M 279	a, b, c, d	No	No
Diazo-chloroanilide-HSA	M 280 to M 284	a, b, c, d	M 281 M 284 with a	No

Diazo-chloroanilide-BTG (second trial)	M 339 to M 343	a, b, c, d	M 340 with d	No
Diazo-chloroanilide-HSA (second trial)	M 344 to M 348	a, b, c, d	No	No

No detectable immune response was obtained neither for rabbits injected with robenidine-hemisuccinate derivatives (1-2) nor with diazo-robenidine derivatives (3-4) nor with diazo-chloroanilid-BTG (6). The bleeding results of rabbits immunized with diazo-chloroanilide-HSA (second trial) (7) didn't show any production of antibodies.

The bleeding results of rabbits M 281, M 284 (diazo-chloroanilide-HSA (5)) and M 340 (diazo-chloroanilide-BTG, second trial (8)) showed a response with the conjugates robenidine-hemisucinate-HRP and the diazo-chloroanilide-HRP (second trial) respectively. No detectable immune response was obtained with the other rabbits belonging to the same group. These antibodies were tested for further development such as the competition with free robenidine. Several conditions were tested to observe a displacement by a standard curve (until 1 μg/ml of robenidine) but all failed. Among these parameters, the incubation temperature (4°C and 37°C), the time incubation (overnight and two hours), the assay format (see explanation above) were tested. These antibodies were unable to create any displacement, any competition. The problem is that the structure is not easy to conjugate because of a lack of reactive functional groups. In a case like this, the chemists usually looks for a compound that shares a sufficient part of the structure but also contains a suitable functional group; then they can raise antibodies to it that will hopefully bind the target drug. Without success against the free robenidine.

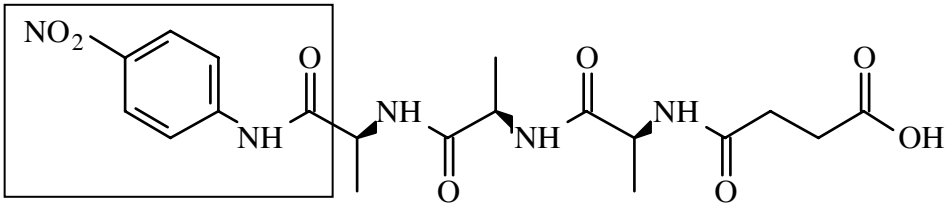
- *conclusions :* Fifteen rabbits produced antibodies raised against diclazuril but these antibodies were unable to recognize free diclazuril. Three rabbits produced antibodies raised against robenidine but these antibodies were unable to recognize free robenidine. Without antibodies able to recognize free coccidiostat, no further development is possible; so no screening assays for diclazuril and robenidine were developed. Nevertheless, no publication or commercial ELISA kit are available, until now, for these two residues.

task A.5 : Development of enzyme linked immunosorbent assays (nicarbazin, nitroimidazole and halofuginone)

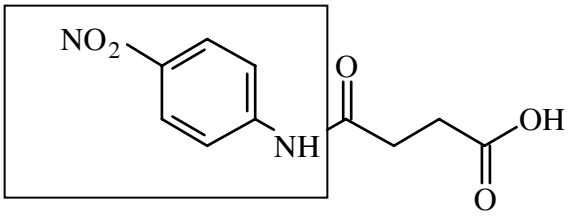
- *partners responsible :* CER
- *objective :* The best conjugate and the best antibodies were selected and their dilution rates were evaluated. ELISA tests were examined according to several parameters in order to be optimal.
- *results :* In order to optimise the assay with regards to sensitivity, specificity and precision; several key parameters were studied: the format of the assay, the choice of antibody and conjugate, the amounts of coating antibody and conjugate, the incubation time and temperature. For the format of the ELISA, it was decided to adopt the competitive assay (figure 4). Under this configuration, the specific antibodies were directly coated onto the solid phase of the 96 well plate for 72 hours; followed by a blocking procedure with 25 g/L casein hydrolysate solution (pH 7) for two hours at room temperature. The plates were washed and filled with 1% saccharose then completely emptied and stored at 4°C. The coated well was filled with standard or sample and peroxidase conjugate was added for one night at 4°C under agitation. After three washes, antibody-bound conjugate was measured with chromogen TMB and enzyme substrate H_2O_2. The optical density is read at 450 nm.
  On one hand, microtitre plates were coated with two specific antibodies (M 187 recognizing DNC and M 98, halofuginone); unpurified antisera were both diluted 1/48,000 in the same well. On the other hand, 96-well microtitre plates were coated by adding diluted (1/15,000) antibodies PC 107 raised against metronidazole.
  The best immunogen/conjugate and antibody for dinitrocarbanilide, halofuginone and nitroimidazole family were summarized in this paragraph.

  ELISA for nicarbazin (DNC)

  N-succinyl-L-alanyl-L-alanyl-L-alanine 4-nitroanilide-HSA was used to immunize rabbit M 187 and the enzyme labelled drug was nitrosuccinanilic acid–HRP. Indeed these compounds share a common substructure with dinitrocarbanilide (DNC) and allow an optimal mimicking of the target analyte (figure 5).



N-succinyl-L-alanyl-L-alanyl-L-alanine 4-Nitroanilide (SAN



Nitrosuccinanilic acid (NSA)

Figure 5: chemical structures of two DNC-mimicking compounds used as immunogen and conjugate

The method used to form these chemical syntheses has been described in detail by Connolly *et al.* (2002). Moreover, we confirmed the finding of this author that such antibodies work best when different compounds are used to synthesize the immunogen and the peroxidase conjugate (heterologous assay format).

ELISA for halofuginone

Rabbit M 98 was immunized with halofuginone-hemisuccinate-HSA and the halofuginone-hemisuccinate-HRP was synthesized adopting the same strategy as for the immunogen (figure 6).

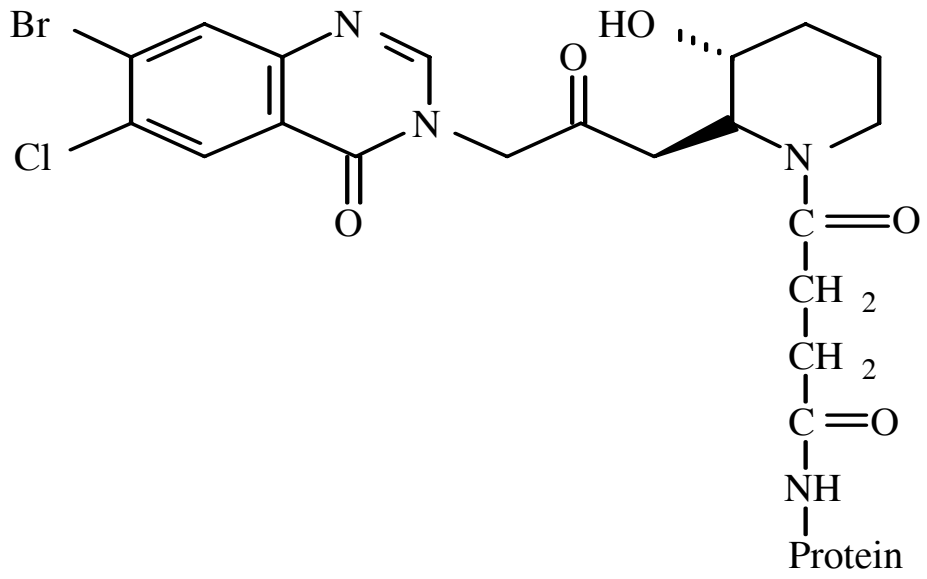


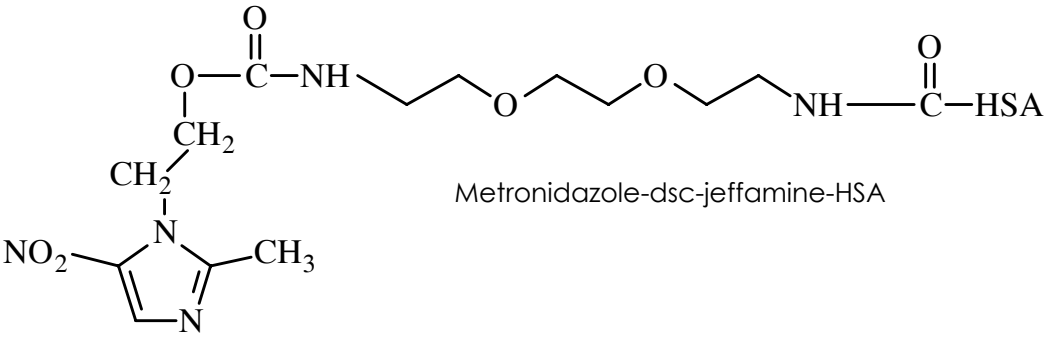
Figure 6: chemical structures of immunogen and conjugate used to develop the ELISA for halofuginone

The strategy has been described in detail by Rowe *et al.* (1993). Briefly, *N*-(trimethylsily)-imidazole was used to protect the HFG hydroxyl group during reaction of the piperidyl nitrogen with succinic anhydride. The succinyl derivative was conjugated to HSA via N-hydroxy-succinimide enhanced, carbodiimide-mediated coupling reaction.

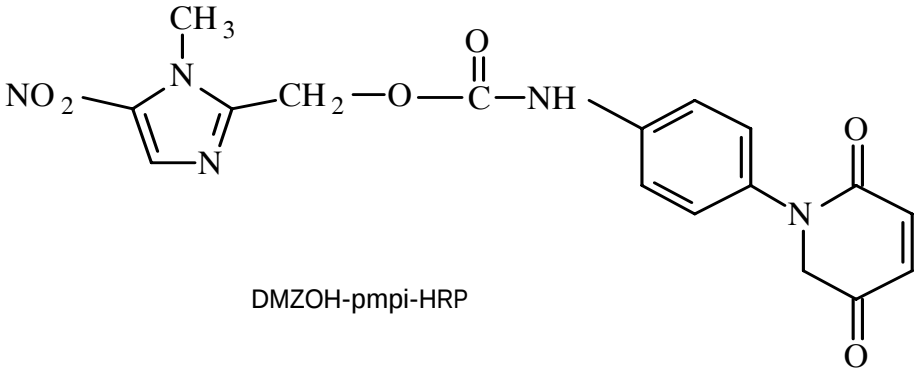
ELISA for nitroimidazoles

The nitroimidazole compounds: dimetridazole (DMZ) and ronidazole (RNZ) are metabolized to hydroxydimetridazole (DMZOH) while metronidazole (MNZ) is metabolized to hydroxymetronidazole (MNZOH) and ipronidazole (IPZ) becomes hydroxyipronidazole (IPZOH). These drugs are suspected to be genotoxic, carcinogenic and mutagenic, as their hydroxy metabolites have retained the original nitroimidazole ring. The dimetridazole has been the most frequently used compound of the group of nitroimidazoles to treat coccidiosis. Moreover, DMZ is rapidly metabolized and DMZOH is present at higher concentrations in tissues and eggs emphasizing the need for a method that not only detects DMZ but also the other nitroimidazoles. In order to screen for a large number of samples by immunoassay for the presence of these family of drugs and metabolites, it was necessary to produce an antibody with a broad-spectrum of recognition.

Metronidazole-Disuccinimidyl Carbonate-jeffamine-HSA was used to immunize rabbit PC 107; a jeffamine spacer was used to extend the distance between the drug and the carrier protein. For the enzyme labelled drug, hydroxydimetridazole-p maleimidophenyl isocyanate–HRP was chosen (figure 7).



Metronidazole-dsc-jeffamine-HSA



DMZOH-pmpi-HRP

Figure 7: chemical structures of immunogen and conjugate used to develop the ELISA for nitroimidazoles

Briefly, the isocyanate end of p maleimidophenyl isocyanate was allowed to react with hydroxyl group of DMZOH. The maleimide end of the hapten was then allowed to react with amines and sulfhydryls on the protein. Further practical details about the chemical synthesis are available in the reference of Fodey *et al.* (2003).

The optimisations of the screening method conditions were summarized in table 4: the assay sensitivity was calculated as 50 % inhibition deviation (ID 50), the concentration of residue necessary to cause 50 % antibody binding.

Table 4: summary of best conditions for the development of three ELISA screening test for dinitrocarbanilide, halofuginone and nitroimidazoles

ELISA assay	Antibodies	Conjugate	Dilution rate conj.	ID50 (ng/ml)	Incubation
Dinitrocarbanilide	M 187 (1/48,000) + M 98 (1/48,000)	Nitrosuccinanilic acid-HRP	1/6,000	2.5	overnight 4°C agitation
Halofuginone	M 187 (1/48,000) + M 98 (1/48,000)	Halofuginone hemisuccinate-HRP	1/60,000	0.08	overnight 4°C agitation
Nitroimidazole	PC 107 (1/15,000)	DMZOH-pmpi-HRP	1/65,000	1.3 (DMZ)	overnight 4°C agitation

The mean values were 2.5 ng/ml, 0.08 ng/ml and 1.3 ng/ml for DNC, HFG and DMZ respectively (figures 8 and 9).

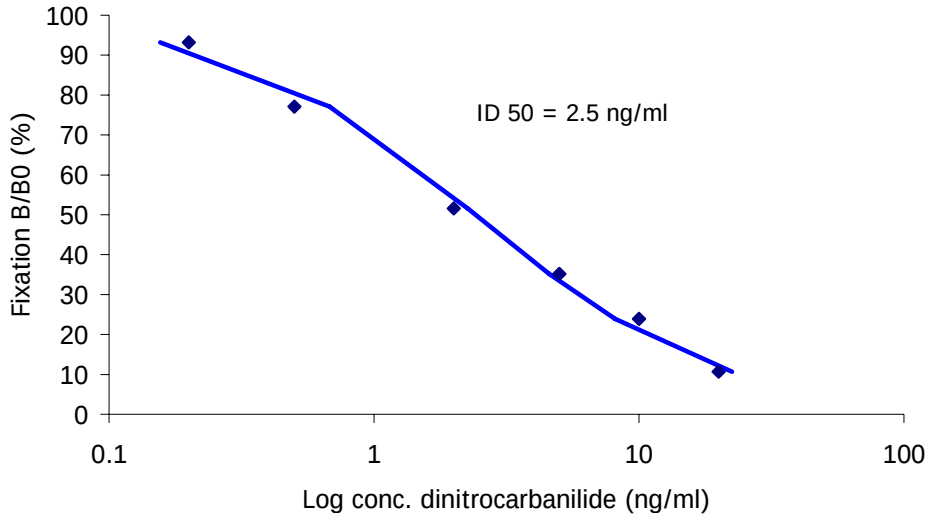


Figure 8: The ability of anti-DNC polyclonal antibodies to bind free DNC was evaluated using a standard curve analysis. The sensitivity was calculated using ID 50 value

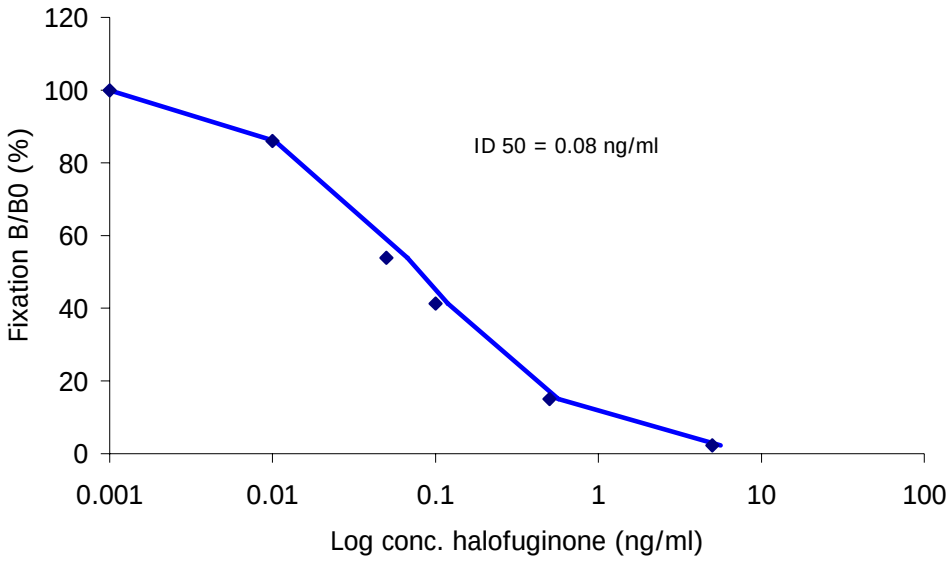


Figure 9: The ability of anti-HFG polyclonal antibodies to bind free HFG was evaluated using a standard curve analysis. The sensitivity was calculated using ID 50 value

The specificity of different antibodies was demonstrated by studies on the cross-reactivity profile. Since antibodies to DNC and HFG were present in the same well, it was essential to check for cross-reactivity; it was calculated by means of the formula 100 X (ID 50 of compound used to raise the antibody).

(ID 50 of the competing compound)

Cross-reactions were calculated for halofuginone, dinitrocarbanilide, robenidine, diclazuril and a range of nitroimidazoles. The M 98 antibody proved HFG-specificity and the M 187 antibody proved DNC-specificity; they showed insignificant reactivity to any other coccidiostats.

The degree of antibody specificity within the nitroimidazoles was estimated for PC 107 antibody. Cross-reactions were observed with DMZ, DMZOH, RNZ, IPZ and MNZ (table 5).

Table 5: comparison of ID 50 values and cross-reactivity profiles of anti-metronidazole antibody (PC 107) with a range of nitroimidazoles

Nitroimidazole	**MNZ**	**RNZ**	**DMZ**	**DMZOH**	**IPZ**
ID50 (ng/ml)	17.5	15.3	1.3	24.4	17.6
Cross-reactivity (%)	100	114	1346	72	99

No significant cross-reactivity was found with any of the following other coccidiostats (halofuginone, dinitrocarbanilide, diclazuril and robenidine). Table 5 shows also the 50 % inhibition deviation (ID 50) values obtained with these different nitroimidazoles. The chosen antibody showed the highest affinity for DMZ and an equal affinity for all other compounds (RNZ, DMZOH, MNZ and IPZ) except IPZOH and MNZOH for which the affinity was quite low. The highest and lowest affinities observed with antibody suggest an effect of steric hindrance on antibody binding. The chemical structure of DMZ is the simplest, making antibody binding easier. The electron density, greater with other nitroimidazoles, makes antibody access for binding more difficult and might explain the differential antibody binding results. Moreover, the antibody binding is influenced by the presence or absence of a single hydroxyl group and by its position on the imidazole ring.

Beside the individual screening test described just above, the feasibility of multiresidue ELISA was proposed and tested. This system aimed at reducing the number of tests by sample and consequently leading to a considerable gain of time and money. It was demonstrated that the use of three antibodies per well in conjunction with a mixture of three enzyme labelled drugs was impossible since no displacement (no competition) occurred.

- *conclusions :* The ELISA conditions are fully optimised. We have successfully developed three competitive immunoassays capable of detecting halofuginone, dinitrocarbanilide and five nitroimidazoles including dimetridazole and its metabolite.

task A.6 : Optimalisation of the clean up of the egg matrix for ELISA detection

- *partners responsible :* CER
- *objective* : After homogenisation of the sample an appropriate clean up has to be performed which makes the sample suitable for the developed assays. The aim of this clean up is to obtain clean extracts with a high recovery of the analytes of interest so that a low detection limit can be obtained.
- *results* : After mixing the whole egg, 2g homogenized egg was weighed into a 50-ml Falcon tube. At this stage, a few blanks were spiked with appropriate amount of standards. The sample was vortex mixed and allowed to stand for 10 minutes. Acetonitrile (8 ml) was added to each sample and the mixtures were immediately vortexed for 1 minute and placed in an ultrasonic bath for five minutes. All tubes were centrifuged for 10 minutes at 4000 rpm. Supernatants were transferred to 10-ml tubes

and evaporated to dryness under nitrogen at 40°C. Hexane (1 ml) was added to each sample and mixed, then 1 ml methanol:water (3:1) was added. Samples were vortexed for 10 seconds and allowed to stand in a water bath for 5 minutes at 40°C. After centrifugation at 2000 rpm for 5 minutes, the hexane layer and any traces of emulsion at the interface were delicately removed with a Pasteur pipette. The remaining solution was evaporated to dryness under nitrogen at 40°C and the dried material was dissolved in assay buffer containing 5% methanol. As samples must be well cooled before use in the nitroimidazoles ELISA, they were kept on ice during the preparation of test. Sometimes, a dilution of the reconstituted sample with assay buffer is necessary to obtain Optical Density (OD) values for blank-sample near the OD values for Bo (zero standard).

- *conclusions :* The clean-up procedure with ACN extraction and de-fatted by washing with hexane is fully validated and can be used for routine analysis of egg samples.

task A.7 : Development of immunoaffinity columns

- *partners responsible :* CER
- *objective* : Prior to inject the samples into the LC-MS-MS instrument, it is possible to purify them through an immunoaffinity column for a very specific and selective purification. This additional step may improve the detection limit.
- *results* : Three immunoaffinity chromatography (IAC) columns were produced, each column is filled with a gel containing specific antibodies able to recognize halofuginone, dinitrocarbanilide and nitroimidazoles respectively; further practical details are available in task B.5. The gel capacity was determined for each specific gel IAC: 1 ml of gel is able to retain 200 ng of HFG or 125 ng of DNC or 150 ng of MNZ. Several washing solutions and eluents were tested and the best results were obtained with a mixture of methanol and water (10/90, v/v) as washing solution and a mixture of ethanol and water (80/20, v/v) brought at pH 3 with HCl as eluent for two gels: halofuginone and dinitrocarbanilide. Nevertheless, we have some problems with the IAC for the nitroimidazoles, the elution is incomplete. Theoretically, elution strategies can be divided into: use of extreme pHs, use of chaotropic salts, changes in ionic strength, use of denaturants, use of organic solvents,... Several of these strategies were tested without interesting results.

  The beneficial effects of using IAC during clean-up is demonstrated for halofuginone in task B.5. Indeed, IAC offers the advantage over other clean-up techniques that it allows a very selective extraction and concentration and thus results in a very low

detection limit. Unfortunately the use of IAC has also some disadvantages. As well the development as the application in routine analysis is very time consuming and labour-intensive. Taken into consideration that the extra specificity is not really necessary since LC-MS/MS gives already an unambiguous identification and that already low detection limits with the simple ACN-extraction were obtained. It was decided that the use of IAC would not be further investigated for the other compounds.

- *conclusions :* We produced the IAC for HFG, DNC and nitroimidazoles. The washing and elution solutions were established for halofuginone and nicarbazin IAC. However, it is important to be aware that the use of IAC during clean-up takes such a long time, it is difficult to use it routinely. It is the reason why the use of IAC would not be further investigated.

3.2. TASK B : DEVELOPMENT OF LIQUID CHROMATOGRAPHIC – MASS SPECTROMETRIC CONFIRMATION METHODS

task B.1 : literature study

- *partner(s) responsible* : DVK
- *objective* : By executing a thorough literature study, the most recent data concerning the mass spectrometric conditions, clean-up of the matrix and metabolisation of the compounds in order to determine the marker compounds were gathered.
- *results* : In November 2001, a detailed and thorough literature study was performed. This was done by searching on the internet and gathering the publications in different libraries. During the further course of the project, this literature study was updated on a regular base.
- *conclusions* : By carrying out a detailed literature study in the beginning of the project and making regularly updates, we could determine the marker compounds and are always aware of the most recent scientific developments and publications concerning the determination of residues of coccidiostats.

task B.2 : choice and purchase of the internal standard

- *partner(s) responsible* : DVK

- *objective* : The use of an internal standard is indispensable when performing residue analysis with mass spectrometric equipment. To obtain such an internal standard, the availability of deuterated analogues must be checked and when those are not available, other compounds having a similar molecular structure as the molecules studied, must be searched for.
- *results* : It was decided to use an internal standard for both ionisation modes used : one for the electrospray positive and one for the electrospray negative ionisation mode. For the positive ionisation mode, we were able to obtain deuterated dimetridazole. For the negative ionisation mode, we could not lay hands on a deuterated compound but a structural analogue of diclazuril was found to be very suitable as internal standard. In figure 10 the molecular structures of both internal standards are presented.

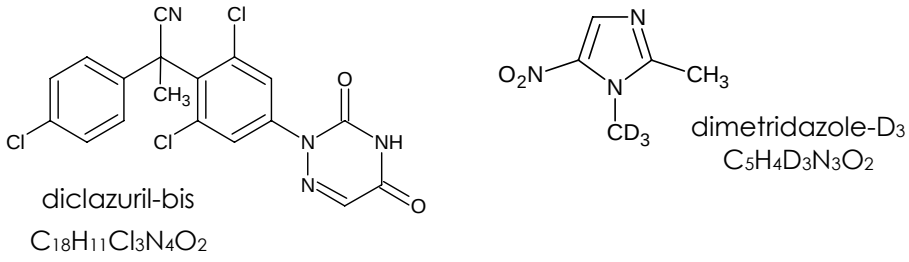


Figure 10 : molecular structures of the internal standards

- *conclusions* : For each ionisation mode a suitable internal standard was found which allowed a robust and quantitative analysis.

task B.3 : optimisation of the mass spectrometric conditions

- *partner(s) responsible* : DVK
- *objective* : For each compound studied, the optimal mass spectrometric conditions must be determined. For each molecule, the precursor ion and the accompanying product ions must be established.
- *results* : The main part of the analyses were performed on the LC-MS/MS system that consists of a 2695 Alliance LC coupled to a Waters Micromass Quattro Ultima Pt mass spectrometer equipped with a Z-spray system. The system is controlled by version 4.0 of the Masslynx software. The optimum mass spectrometric conditions were determined by tuning individual standard solutions using a T-piece. To be able to fulfil

the criteria set by Commission Decision 2002/657, at least 2 product ions for each compound must be generated. Results of the optimisation are presented in table 6.

Table 6 : overview of the main mass spectrometric conditions

compound	ionisation mode	m/z precursor ion	cone voltage (V)	RF lens 1 (V)	capillary (kV)	m/z product ion	collision energy (eV)
diclazuril	ES -	404.9 406.9	50	50	3.0	334.1 336.1	16 16
dimetridazole	ES +	142.1	50	35	1.0	96.1 81.2	12 20
2-hydroxy dimetridazole	ES +	158.1	40	30	1.0	140.1 55.2	9 14
dinitrocarbanilide	ES -	301.1	35	35	2.5	137.1 107.1	8 30
halofuginone	ES +	416.0	50	40	3.2	100.2 120.1	20 18
robenidine	ES +	334.1	50	40	3.2	155.1 138.1	18 24
dimetridazole-D_3	ES +	145.0	50	30	1.0	99.2	12
diclazuril-bis	ES -	419.0	50	50	3.2	321.0	25

- *conclusions* : The mass spectrometric conditions were optimised for all compounds. For each compound at least 2 product ions could be generated so it is possible to comply with the most recent European legislation concerning residue analysis.

task B.4 : optimisation of the chromatography

- *partner(s) responsible* : DVK
- *objective* : Before the sample is coming into the mass spectrometer a separation is performed on a chromatographic column to increase selectivity. The ideal composition of the solvents, the gradient program, the injection volume and column temperature must be determined.
- *results* : As we wanted to develop a multi-residue method, it had to be taken into consideration that all compounds belong to a different compound group and

therefore require other optimal chromatographic conditions. Hence, compromises had to be made.

Based on a literature search, about 20 different chromatographic conditions were tested. A lot of the tested conditions were found to be suitable for one or sometimes two compounds. But since it was the aim to develop a multi-residue method, only those conditions were taken in further consideration that gave good results for all compounds.

Finally two LC-conditions were found to be suitable. Those two conditions were evaluated by comparing linearity, recovery and repeatability. One condition clearly gave better results and was hence used for further analyses. These final chromatographic conditions are presented in table 7.

Table 7 : final chromatographic conditions

	FINAL CHROMATOGRAPHIC CONDITIONS
column	Waters Symmetry C_{18}, 5 µm, 150 mm x 2.1 mm i.d.
precolumn	Altech, Alltima C_{18}, 5 µm, 7.5 mm x 2.1 mm i.d.
eluent A	H_2O/ACN (95/5) + 0.1% HCOOH
eluent B	ACN + 0.1% HCOOH
column temperature	35°C
flow rate	0.25 ml/min
injection volume	10 µl
gradient	0 '- 0.5' : 100/0 A/B 0.5 '- 0.6' : 55/45 A/B 0.6 '- 8.5' : 35/65 A/B 8.6' - 9.6' : 0/100 A/B 9.8 '- 17' : 100/0 A/B

- *conclusions :* The liquid chromatographic conditions were fully optimised. All 5 coccidiostats, the metabolite of dimetridazole and both internal standards can be determined in one run.

task B.5 : optimisation of the clean-up of the egg matrix for mass spectrometric detection

- *partner(s) responsible* : DVK
- *objective* : After homogenisation of the sample an appropriate clean-up has to be performed which makes the sample suitable for injection into the LC-MS instrument. The goal of this clean-up is to obtain clean extracts with a high recovery of the analytes of interest so that a low detection limit can be obtained.

results : Based on literature, a number of methods using solid phase extraction with different types of columns were tested. None of them gave satisfying results. Good results were however obtained applying a simple clean-up procedure that consists of an extraction with acetonitrile. This method was found to be suitable to perform a multi-residue analysis. But the main drawback of this procedure is that this clean-up procedure is very rudimentary and therefore very polluting for the mass spectrometer. Therefore an additional washing step with hexane was added to the procedure. This method was found to be suitable for dimetridazole, 2-hydroxydimetridazole en halofuginone. For dinitrocarbanilide, robenidine and diclazuril on the other hand, this method could not be used since these compounds are lipid soluble and as a result would be removed together with the hexane layer. So for these compounds, an alternative way for reducing source contamination was used. Splitting off the flow can also reduce pollution of the mass spectrometer. A rheodyne 6-way valve was installed. In this way it can be programmed that the flow only enters the mass spectrometer when the compounds are expected to elute. The effect of splitting of the flow was demonstrated by comparing the pollution on baffle and cone after 15 consecutive injections with and without splitting. Pollution was clearly less when the flow is splitted off. Hence, for all compounds studied, a satisfying clean-up procedure was developed. In table 8, details on the procedures are given.

Table 8 : clean-up procedure for eggs

simple procedure with acetonitrile	**procedure with additional hexane washing step**
weigh 10 g of homogenised egg in centrifuge tube	
add internal standard and, after thoroughly vortex mixing, allow to stand for 10 min	
add 10 ml acetonitrile	
vortex mix for 1 min	
place in ultrasonic bath for 5 min	
centrifuge : 10 min / 3000 rpm	
concentrate ACN-layer to a volume of 4 ml under N_2 in waterbath (60°C)	transfer 10 ml supernatant in tube
filtrate remaining extract	add 10 ml hexane
inject into LC-MS/MS system	gently shake for 3 min
	remove hexane layer
	concentrate ACN-layer to a volume of 1ml under N_2 in waterbath (60°C)
	filtrate remaining extract
	inject into LC-MS/MS system

Also the use of immunoaffinitychromatography (IAC) was tested. IAC offers the advantage over other clean up techniques that it allows a very selective extraction and concentration and thus results in a very low detection limit. The principle of IAC is schematically presented in figure 11.

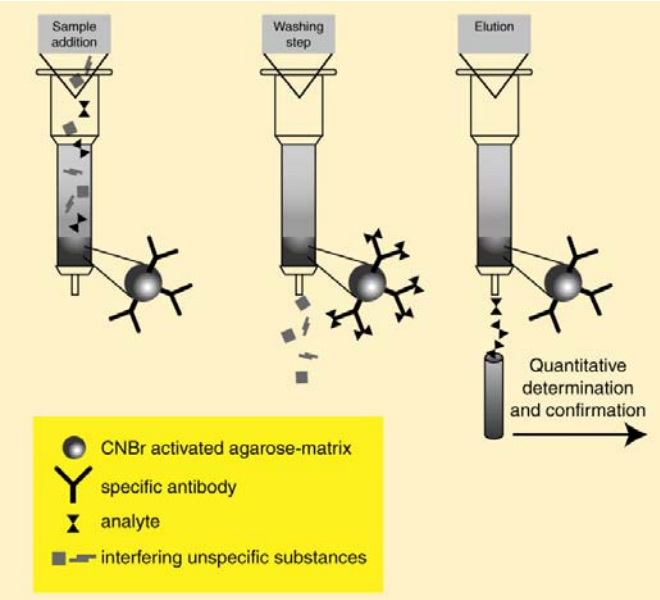


Figure 11 : schematic presentation of the principle of IAC

A column is filled with a gel containing a specific antibody. After applying the sample, a washing step is performed to remove interfering substances. Then the sample is eluted with a suitable eluent and can be further analysed by LC-MS/MS. A method was developed for the detection of halofuginone in eggs with IAC. First the gels were prepared. The IgG fractions of the collected antisera were purified on protein A sepharose 4 fast flow, dialysed in a $NaHCO_3$ (0.1M) buffer at pH 8.4 and then quantified according to Lowry's method. The IgG was bound to cyanogen bromide-activated sepharose gel. The remaining active groups were blocked with coupling buffer. Finally the gel was washed. The gel was kept at 4°C in PBS buffer (phosphate buffer saline) with 0.1% azide. One ml of gel was brought into an Econo Column® (Bio-rad). The gel capacity was determined to be 200 ng HFG/ml. Several washing solutions and eluents were tested and the best results were obtained with a mixture of methanol and water (10/90, v/v) as washing solution and a mixture of ethanol and water (brought at pH 3 with HCl) (80/20, v/v) as eluent. Then a suitable clean-up was developed. After mixing the eggs with an ultra-turrax, 5 g was weighed in a centrifuge tube. Spiking was performed at this stage if necessary by adding an

appropriate amount of standard solution in water. After 10 min, 5 ml of acetonitrile was added and the sample was vortex mixed (30 s) and placed in an ultrasonic bath for 5 min. The sample was then centrifuged during 10 min at 3000 rpm. The supernatant was transferred into a graduated tube and was concentrated to a volume of 1 ml under nitrogen in a water bath at 60°C. After addition of 9 ml PBS, the sample was filtered to make it suitable for application on the IAC gel. First the gels were allowed to adjust to room temperature for 10 min. Before loading the first sample, the gel was equilibrated by applying consequently 5 ml PBS, 5 ml eluent, 5 ml water, 5 ml PBS and 3 times 5 ml water. After loading 1 ml water, the sample was applied. The gel was then washed with 5 ml water and 5 ml MeOH/H_2O (10/90, v/v). The sample was eluted with 15 ml eluent. Before applying the next sample the gel was rinsed with 10 ml water, 5 ml eluent, 5 ml water, 5 ml PBS buffer and 3 times 5 ml water. After the last sample, the gel was rinsed with 20 ml eluent, 10 ml water and 20 ml PBS buffer. The column was filled with PBS buffer and put in the refrigerator. The eluent was concentrated to a volume of 5 ml under nitrogen in a water bath at 60°C and transferred into a vial. Ten µl was injected on the LC-MS/MS system. In figure 12 a chromatogram of an egg spiked at 1 µg/kg is shown.

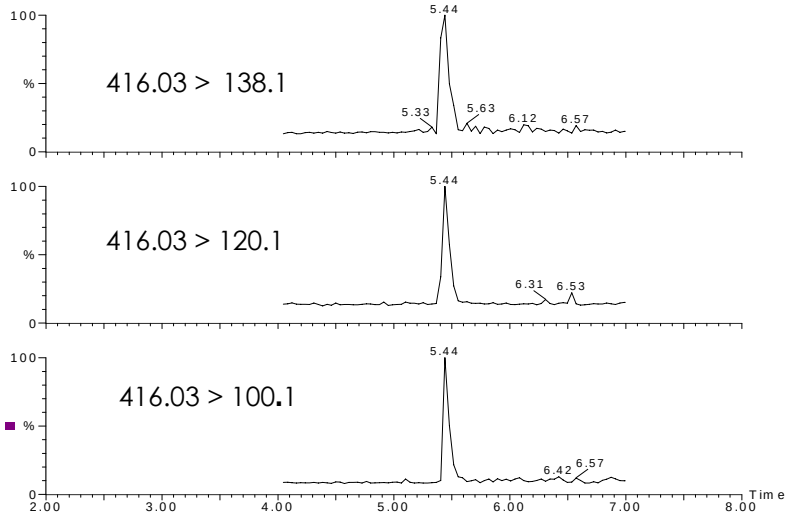


Figure 12 : chromatogram of an egg spiked at 1 µg/kg

This method for the determination of halofuginone with IAC was validated. Results of the validation study are presented in table 9.

Table 9 : results of the validation of the method for the determination of halofuginone with IAC

LINEARITY	
standard calibration curve (3 curves, concentration range 0 – 50µg/kg)	$R^2 \geq 0.9989$
matrix calibration curve (concentration range 0- 20 µg/kg)	$R^2 = 0.9978$
TRUENESS	
→ recovery (6 samples spiked at 5 µg/kg and quantified with a calibration curve in the matrix)	mean recovery = 101%
PRECISION	
→ repeatability (6 samples spiked at 5 µg/kg and quantified with a calibration curve in the matrix)	intra-laboratory CV = 6%
SPECIFICITY	
MS-response (analysing standard solutions of other coccidiostats on the presence of halofuginone)	no interference
IAC (determination of cross-reactivity to a range of other coccidiostats)	ID50 < 0.01%
CCα/CCβ The decision limit (CCα) was determined by fortification of blank material. The acquired signal was plotted against the added concentration. The corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the intercept equals CCα. The detection capability (CCβ) was calculated by adding 1.64 times the standard deviation of the intercept to the value of CCα. When a calibration curve in the concentration range 0-20 µg/kg was used, 1.26 µg/kg and 2.16 µg/kg were obtained as CCα and CCβ respectively. But when a calibration curve in the range 0-3 µg/kg was used, lower values were found for CCα and CCβ. This shows that determination of CCα and CCβ with calibration curves is not unambiguous. Alternatively, the signal-to-noise ratio (S/N) was determined for spiked samples. For an egg spiked at 0.5 µg/kg , a S/N of 10 was obtained which indicates that a CCα value of 0.5 µg/kg or even lower is possible.	

The effect of using IAC during clean up was demonstrated by comparing a chromatogram of an egg spiked at 2 µg/kg. This is shown in figure 13.

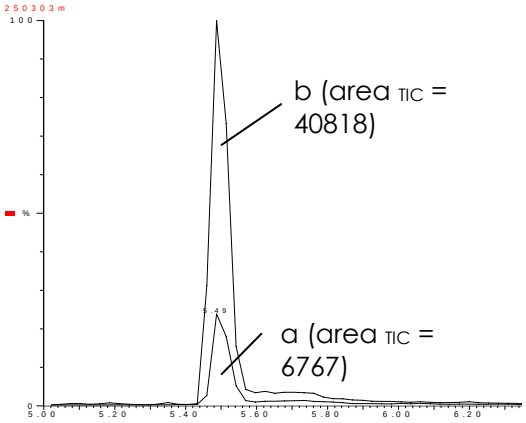


Figure 13 : comparison of the chromatogram of an egg spiked at 2 µg/kg : (a) clean-up with ACN-extraction, (b) clean-up with IAC

When using IAC the respons is almost 7 times higher. Unfortunately the use of IAC has also some disadvantages. As well the development as the application in routine analysis is very time consuming and labour-intensive. Taken into consideration that the extra specificity is not really necessary since LC-MS/MS gives already an unambiguous identification and that already low detection limits with the simple ACN-extraction were obtained, it was decided that the use of IAC would not be further investigated for the other compounds. But it was undeniable proven that IAC can offer the solution when very low levels must be determined or when very specific results must be obtained.

- *conclusions* : A multi-residue clean-up procedure consisting of a simple extraction with acetonitrile has been optimised. To overcome the problem of source pollution, a rheodyne divert valve was installed. Additionally, a more extensive clean-up including a washing step with hexane was optimised and can be applied for the detection of dimetridazole, 2-hydroxydimetridazole and halofuginone. In addition, a clean-up procedure with immunoaffinitychromatography for the detection of halofuginone has been developed and optimised.

3.3. TASK C : VALIDATION OF THE METHODS DEVELOPED ACCORDING TO REVISION OF COMMISSION DECISION 93/256/EC

Since 1 September 2002 validation of methods has to be done according to Commission Decision 2002/657/EC instead of revision of Commission Decision 93/256/EC.

task C.1 : validation of the ELISA's

- *partner(s) responsible* : CER
- *objective* : For screening methods, 4 parameters have to be determined according to Commission Decision 2002/657/EC, namely detection capability, decision limit (optional), selectivity/specificity and applicability/ruggedness/stability.
- *results* :

task C.1.1 : Detection capability

The detection capability (CCβ) is defined as the smallest concentration of analyte that can be identified and/or quantified in a sample with an error probability of β. The β error should be less than or equal to 5%. In the case of substances without an established MRL (Maximum Residue Limit), the CCβ is the lowest concentration at

which a method can detect truly contaminated samples with a statistical certainty of 1-β.

Theoretically, if 19 of the 20 fortified samples were declared non-compliant, then CCβ = level of fortification. If 18 or less of the fortified samples were declared non-compliant, then CCβ > level of fortification. If all of the fortified samples were declared non-compliant, then CCβ < level of fortification. In practice, the levels of fortification for each analyte were chosen so as to ensure that all of the fortified samples were declared non-compliant. This decision should avoid the problem of false negatives; we kept in mind, however, that the level of fortification must be as low as possible.

CCβ was determined by analysing 20 blank eggs fortified with the analytes at the lowest level of fortification. Table 10 shows the following detection capabilities for egg : DNC < 3ppb, HFG < 0.5ppb, DMZ < 1ppb, DMZOH and RNZ < 20ppb, MNZ < 10ppb and IPZ < 40ppb.

Table 10: validation data of the screening methods. Detection capabilities (CCβ) of halofuginone, dinitrocarbanilide and nitroimidazoles in egg matrix. The average (n=20) binding ratio B/Bo (%) was calculated ± two standard deviations for all negative samples (blank) and spiked samples (test).

Coccidiostat	**Blank** **Binding B/Bo(%)**	**CCβ (ppb)**	**test** **Binding B/Bo(%)**
Dinitrocarbanilide	81 - 101	< 3	53 - 74
Halofuginone	62 - 114	< 0.5	45 - 58
Dimetridazole	80 - 103	< 1	59 - 76
Hydroxydimetridazole	80 - 103	< 20	43 - 61
Ronidazole	80 - 103	< 20	33 - 61
Metronidazole	80 - 103	< 10	42 - 65
Ipronidazole	80 - 103	< 40	40 - 69

All of the fortified samples were declared non-compliant; thus, CCβ < level of fortification. The highest CCβ recorded for ipronidazole is probably due to a low recovery of this residue.

Table 10 summarises the results obtained after processing the biological matrix. Beside of the determination of CCβ, 20 known negative samples were extracted and applied to an ELISA plate. The mean value of the absorbance values obtained for each sample (fortified or not) is divided by the mean value absorbance values of the

zero standard (Bo) and multiplied by 100. The average of ratio B/Bo (%) for each group of 20 samples is calculated with ± two standard deviations. Egg samples were declared non-compliant if the binding percentage was inferior to 74 %, 58 % and 76 %.

task C.1.2 : Decision limit (optional)

The decision limit or CCα is the concentration level at which and beyond which we can conclude with an error probability of α that a sample is non-compliant. The α error is the probability that a tested sample is compliant, even if the result is non-compliant. CCα was determined by analysing 20 blank eggs: the concentration corresponding to the mean response for negative samples minus three times the standard deviations. The results estimated the following decision limits: DNC 0.5ppb, HFG 0.1ppb and nitroimidazoles 0.3ppb.

task C.1.3 : Selectivity/specificity

In analytical methods, the ability of separation between the analyte and related substances (isomers, metabolites, degradation products, compounds of matrix) is essential.

The specificity of developed assays was demonstrated by the cross-reactivity profiles explained in the task A.5 and further details were given in the previous reports. Each ELISA format was analysed in the presence of other coccidiostats. No significant interference between the different compounds was detected.

task C.1.4 : Applicability/ruggedness/stability

Factors such as the analyst, the solvents supply, the age of reagents or not, the temperature,… may influence the results. These parameters were tested by introducing some minor changes in the procedure.

Real-time stability data have been generated from the developed ELISAs. The results showed that coated microwells plates, standard solutions (in buffer EIA) and the conjugates (in stabilgen solution + 10% foetal calf serum) are stable for at least one year at 4°C without any change in the performance of the assays. The performances of halofuginone, dinitrocarbanilide and dimetridazole screening tests were evaluated according to the sensitivity of each assay. The stock standard solutions (1mg/ml) were prepared in an appropriate solvent and were stored at -20°C. Under these conditions, these solutions were also stable for at least one year.

We are not obliged to apply reconstituted samples the day of the extraction procedure, we can wait a few days at 4°C or applied them even if they were frozen.

- *conclusions :* The three immunological tests are all validated according to Commission Decision 2002/657/EC. The validation was done on the clean-up procedure involved an ACN-extraction and a de-fatted by washing with hexane. The results of the validation realized for the three screening assays were summarized in table 11.

Table 11: results of the validation of the screening methods

ELISA assay	CCβ (ppb)	CCα (ppb)	specificity	Stability/ ruggedness
Dinitrocarbanilide	< 3	0.5	OK	At least 1 year at 4°C
Halofuginone	< 0.5	0.1	OK	At least 1 year at 4°C
Nitroimidazole	< 1 (DMZ) < 10 (MNZ) < 20 (DMZOH) < 20 (RNZ) < 40 (IPZ)	0.3	OK	At least 1 year at 4°C

task C.2 : validation of the mass spectrometric methods

- *partner(s) responsible* : DVK
- *objective* : For confirmation methods 6 parameters have to be determined according to Commission Decision 2002/657/EC, namely detection capability, decision limit, trueness, precision, selectivity/specificity and applicability/ruggedness/stability. Also the performance criteria and other requirements specific for mass spectrometric detection must be fulfilled.

- *results* :

task C.2.1 : detection capability

The detection capability or CCβ is the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. The β-error should be less than or equal to 5%. In the case of banned substances, such as the coccidiostats studied in the project, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1-β. CCβ was determined by analysing 20 blank eggs fortified

with the analytes at the decision limit (CCα, see below). The value of the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility of the measured content equals the detection capability.
CCβ can also be determined by fortification of blank material. When the acquired signal is plotted against the added concentration, CCβ is calculated by adding 1.64 times the standard deviation of the intercept to the value of CCα.

task C.2.2 : decision limit

The decision limit or CCα is in the case of banned substances the lowest concentration level, at which a method can discriminate with a statistical certainty of 1-α whether the identified analyte is present. In the case of substances for which no permitted limit has been established the α-error equals 1%. CCα is determined by analysing 20 samples spiked at a concentration for which a signal-to-noise ratio of 3 was obtained.
CCα can also be determined by fortification of blank material. When the acquired signal is plotted against the added concentration, the corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the intercept equals CCα.

task C.2.3 : trueness

Trueness is one compound of accuracy. Trueness can be established by analysing certified reference material. For the detection of coccidiostats in eggs, no certified reference material is available. Trueness can than be assessed through recovery of additions of known amounts of the analytes to blank matrix. At least 6 measurements per concentration level have to be performed. The amount of material that is recovered has to be within certain values, depending on the concentration level. Recovery was determined by analysing 6 fortified samples and quantified with a calibration curve in the matrix.

task C.2.4 : precision

Precision means the closeness of agreement between independent test results obtained under stipulated conditions. The measure of precision usually is expressed in terms of imprecision and computed as a standard deviation of the test results. Less precision is determined by a larger standard deviation. Precision was expressed as the intra-laboratory CV for the analysis of 6 spiked samples.

task C.2.5 : selectivity/specificity

Specificity means the ability of a method to distinguish between the analyte being measured and other substances. It was tested by analysing a number of blank samples and checking for any interference in the region of interest where the target analyte is expected to elute. The specificity was also tested by injecting standard solutions of the individual compounds (dimetridazole, halofuginone, robenidine, nicarbazine and diclazuril) and analysing them on the presence of the other compounds. No interferences were observed. Finally, specificity was evaluated by analysing an egg spiked at 50 µg/kg of other compounds (e.g. ionophoric coccidiostats, avermectins, β-lactam antibiotics,…). Also here, no interferences were observed.

task C.2.6 : applicability/ruggedness/stability

Ruggedness means the susceptibility of an analytical method to changes in experimental conditions such as sample material, storage conditions and temperature. The stability of the analyte or matrix constituents in the sample during storage or analysis may affect the outcome of the result of analysis. Therefore analyte stability must be characterised under various storage conditions. Stability was tested and it was proven that standard stock solutions are stable at –18°C for at least 6 months.

The results of the validation of the different methods developed according to the procedures described above are presented in table 12 to 14

Table 12 : Detailed results of the regression analysis of the standard calibration curves and calibration curves in the egg matrix.

	matrix	mean slope ± standard deviation	mean R^2	number of data points	concentration range
diclazuril	standard	1.15 ± 0.02	0.998	8	0 – 100 µg kg^{-1}
	egg	0.71 ± 0.07	0.998	10	0 – 200 µg kg^{-1}
dimetridazole	standard	9.35 ± 0.41	0.999	8	0 – 100 µg kg^{-1}
	egg	1.02 ± 0.05	0.999	10	0 – 200 µg kg^{-1}
2-hydroxy dimetridazole	standard	0.52 ± 0.03	0.998	8	0 – 100 µg kg^{-1}
	egg	0.60 ± 0.10	0.997	10	0 – 200 µg kg^{-1}
dinitrocarbanilide	standard	16.40 ± 0.40	0.998	6	0 – 20 µg kg^{-1}
	egg	27.06 ± 5.57	0.993	8	0 – 75 µg kg^{-1}
halofuginone	standard	0.75 ± 0.01	0.999	8	0 – 100 µg kg^{-1}
	egg	0.60 ± 0.09	0.993	10	0 – 200 µg kg^{-1}
robenidine	standard	1.52 ± 0.06	0.999	8	0 – 100 µg kg^{-1}
	egg	1.29 ± 0.03	0.994	10	0 – 200 µg kg^{-1}

Table 13 : Summary of CCα and CCβ values

	egg	
compound	CCα (µg kg^{-1})	CCβ(µg kg^{-1})
diclazuril	0.5	0.6
dimetridazole	1	1.1
2-hydroxydimetridazole	2	2.2
dinitrocarbanilide	1	1.2
halofuginone	1	1.1
robenidine	1	1.2

Table 14 : Detailed results of the inter- and intra-day precision and recovery experiments

	concentration A*		concentration B*		concentration C*	
	mean CV (%)	mean rec (%) ± stdev	mean CV (%)	mean rec (%) ± stdev	mean CV (%)	mean rec (%) ± stdev
diclazuril	4.9	95.5 ± 4.4	3.8	108.2 ± 0.6	3.8	108.1 ± 1.4
dimetridazole	2.4	101.4 ± 1.2	2.1	101.8 ± 2.3	1.5	99.5 ± 2.2
2-hydroxy dimetridazole	5.1	87.7 ± 10.2	3.7	93.5 ± 15.6	3.4	93.7 ± 7.5
halofuginone	6.5	96.8 ± 2.4	3.8	105.0 ± 2.6	2.0	100.4 ± 1.0
robenidine	7.4	104.9 ± 2.3	4.9	100.6 ± 3.4	3.6	101.1 ± 1.0
dinitrocarbanilide	6.2	101.2 ± 6.7	6.3	99.9 ± 4.8	4.7	97.1 ± 5.4

* : for diclazuril, dimetridazole, 2-hydroxydimetridazole, halofuginone and robenidine : conc. A = 5 µg kg^{-1}; conc. B = 50 µg kg^{-1}; conc. C = 100 µg kg^{-1}and for dinitrocarbanalide : conc. A = 5 µg kg^{-1}; conc. B = 10 µg kg^{-1}; conc. C = 50 µg kg^{-1}.

According to Commission Decision 2002/657/EC some specific criteria for mass spectrometric detection, such as relative retention time and ion ratios, must be fulfilled. When samples are analysed, these criteria werre checked every time.

Chromatograms of eggs spiked at CCα levels are presented in figures 14-16.

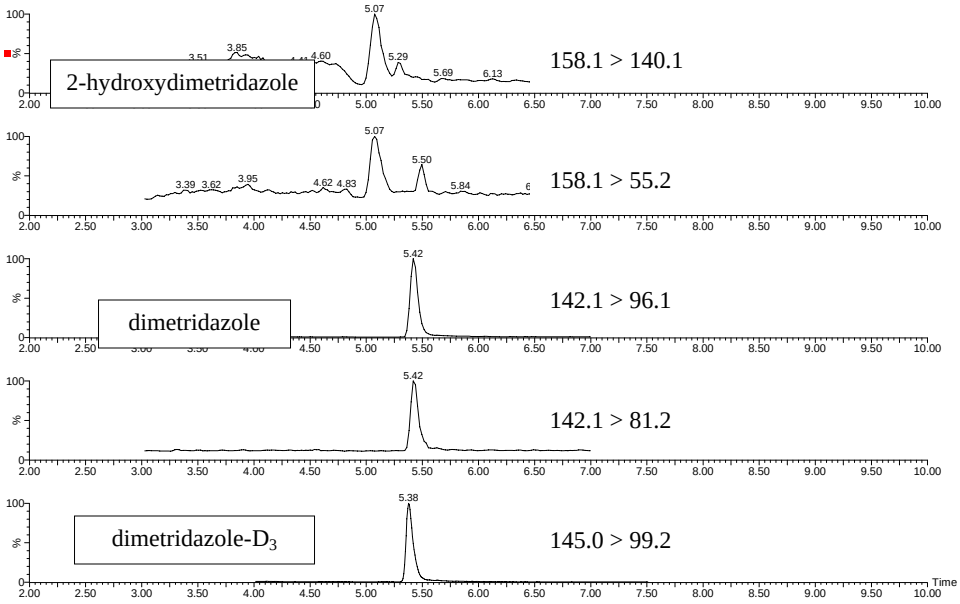


Figure 14 : chromatogram of an egg spiked at 2 µg kg^{-1} 2-hydroxydimetridazole, 1 µg kg^{-1} dimetridazole and 10 µg kg^{-1} dimetridazole-D_3 (= internal standard)

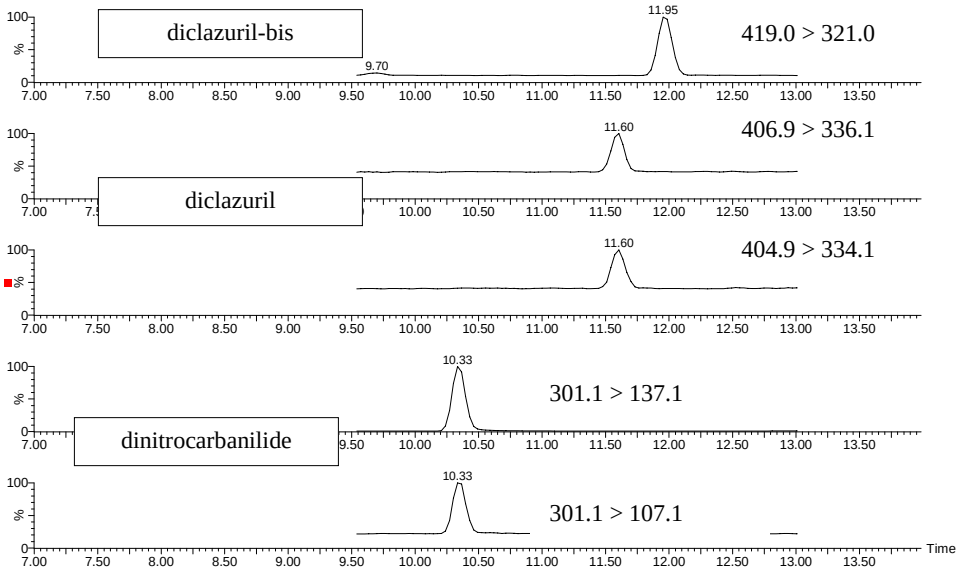


Figure 15 : chromatogram of an egg spiked at 0.5 µg kg^{-1} diclazuril, 1 µg kg^{-1} dinitrocarbanilide and 5 µg kg^{-1} diclazuril-bis (= internal standard)

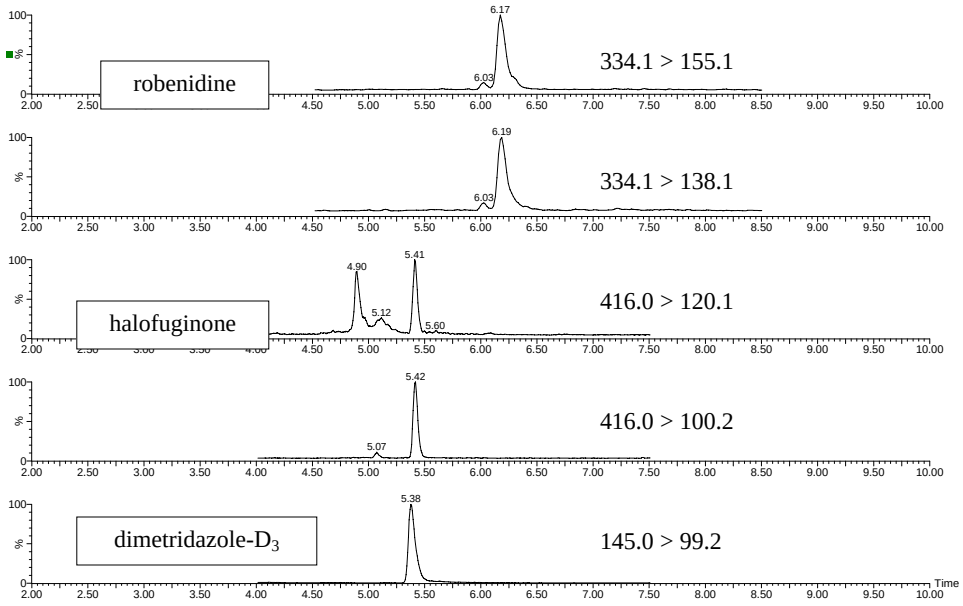


Figure 16 : chromatogram of an egg spiked at 1 µg kg^{-1} robenidine and halofuginone and 10 µg kg^{-1} dimetridazole-D_3 (= internal standard)

- ***conclusions*** : All methods were validated based on the most recent European legislation concerning residue analysis i.e. Commission Decision 2002/657/EC.

3.4. TASK D : APPLICABILTIY OF THE METHODS FOR INCURRED SAMPLES

The applicability of the methods was tested on incurred samples. Laying hens were fed feed containing the compounds studied at the maximum level that can be used for broilers and at a level (5%) that could originate from carry-over during feed preparation. Eggs were gathered before and after the treatment and were analysed with the methods developed. Excretion curves and the time for complete removal of the residues in the eggs were determined. Also the influence on zootechnical parameters, yolk colour and eggshell quality was evaluated.

task D.1 : treatment of the animals – influence on zootechnical parameters, yolk colour and shell quality

- *partner(s) responsible* : DVK + DVV
- *objective* : An animal experiment with laying hens has to be carried out in which the effect of carry-over during feed preparation is studied. The influence of the coccidiostats studied on zootechnical parameters, yolk colour and eggshell quality also have to be checked.
- *results* :

A) Feed

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 and 100 mg/kg for dimetridazole. 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.

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.

All feed samples were analyzed by liquid chromatography tandem mass spectrometry. The results of the analyses of the feed samples are presented in table 15.

Table 15 : results of the analysis 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

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. 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.

B) Animal treatment

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.

During the experiment, the following conventional zootechnical data were recorded : average feed intake (g/day), laying percentage, egg weight (g), daily egg mass (g/hen) and feed efficiency (feed intake/egg mass). 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.

C) Effect on zootechnical parameters, yolk colour and eggshell quality

- Zootechnical parameters

a) Average egg weight

In figure 17 the daily average egg weight is presented. Each day, before breaking the eggs to prepare the pooled sample, each egg was individually weighed. Figure 17 shows that for one group, the 100% nicarbazin + narasin group, the egg weight seems to change during the experimental period. Therefore, the results for that group are presented separately in figure 18.

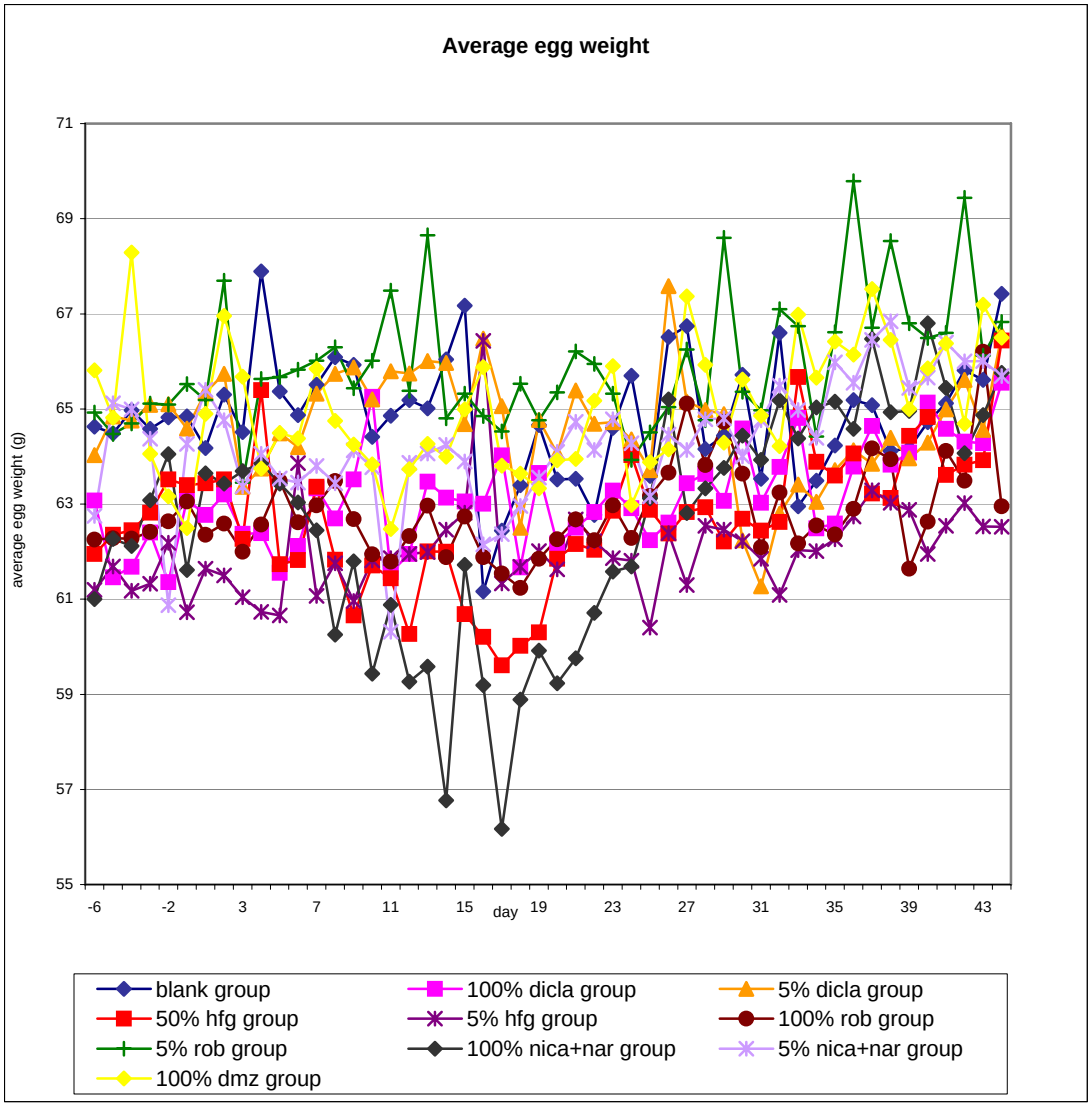


Figure 17 : average egg weight for the 10 experimental groups

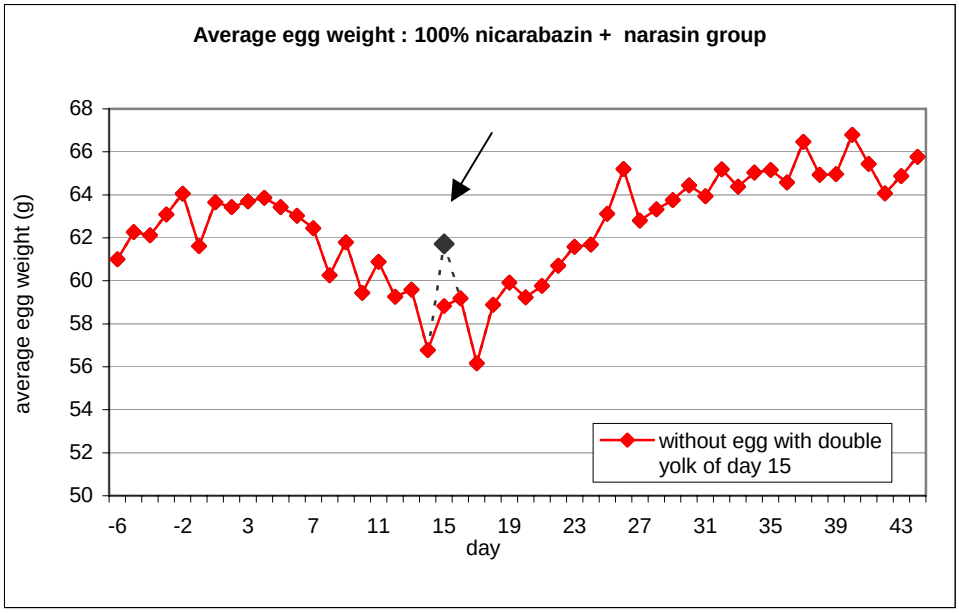


Figure 18 : average egg weight of the 100% nicarbazin + narasin group

A decrease in the average egg weight during the administration of Maxiban® is observed. One data point, however, seems to disagree with this observation i.e. day 15. But on that day, for the 100% Maxiban® group, there was one egg with a double yolk and hence, this made the average weight increase significantly. Therefore, the average egg weight was recalculated without the weight of that particular egg. The red curve in figure 18 represents then the course of the average egg weight. In Statistica 7 a bygroup analysis was performed on these data. This analysis compared the daily average egg weight of the blank group with that of the test group (100% Maxiban®-group) by means of a t-test. It could be concluded that from day 10 until day 21 (=7 days after cessation of the treatment) the difference in the average egg weight between the blank and test group was significant. Fitting the data to a multiphase linear model indicated that the decrease in average egg weight for the test group started 5 days after the treatment was started.

Therefore it can be concluded that the average egg weight is only influenced when rather high concentrations of Maxiban® are administered. For the other compounds tested, no influence was observed.

b) Laying percentage

As mentioned before, for the evaluation of the zootechnical parameters, the experimental period is subdivided into three 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.

The laying percentage is defined as :

$$\text{laying percentage} = \left[\frac{\text{number of eggs}}{\text{maximum number off eggs}}\right] \times 100$$

In figure 19 the laying percentage for the ten experimental groups during the three periods is presented. A downwards tendency in the laying percentage for as well the 100% as 5% nicarbazin + narasin group in the second period, i.e. when the Maxiban® is administered, is noticeable. For the 100% diclazuril group, a decrease in the third period is observed.

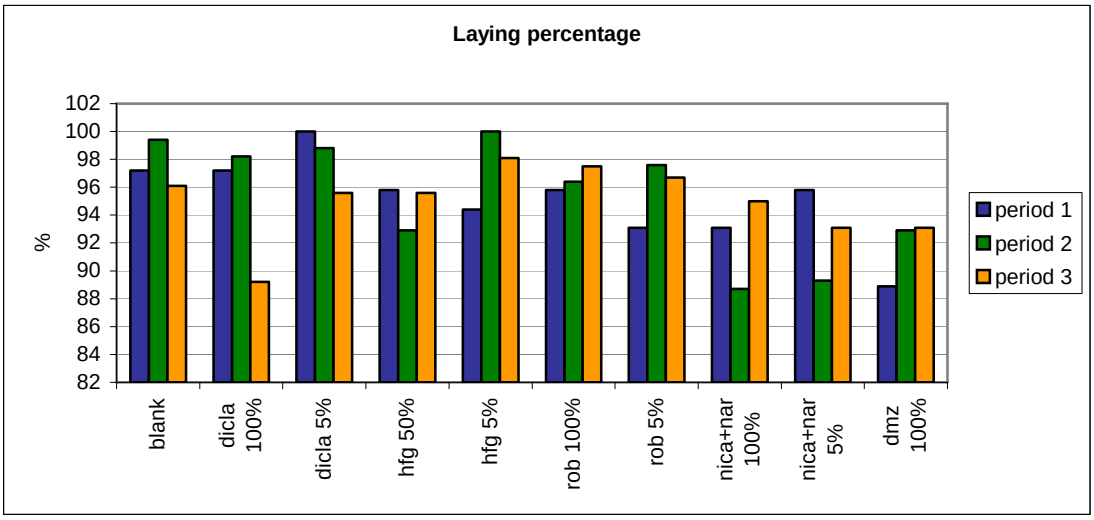


Figure 19 : average laying percentage

c) Daily egg mass

Also for the evaluation of the daily egg mass, the experiment was subdivided into three periods.

The daily egg mass is defined as :

$$\text{daily egg mass} = \text{average egg weight} \times \text{laying percentage}$$

This value can be considered as "the average egg produced daily by one hen". Since as well the average egg weight as the laying percentage is adversely affected for the

100% Maxiban®-group, also the daily egg mass is lower for that group during the second period. Results are presented in figure 20.

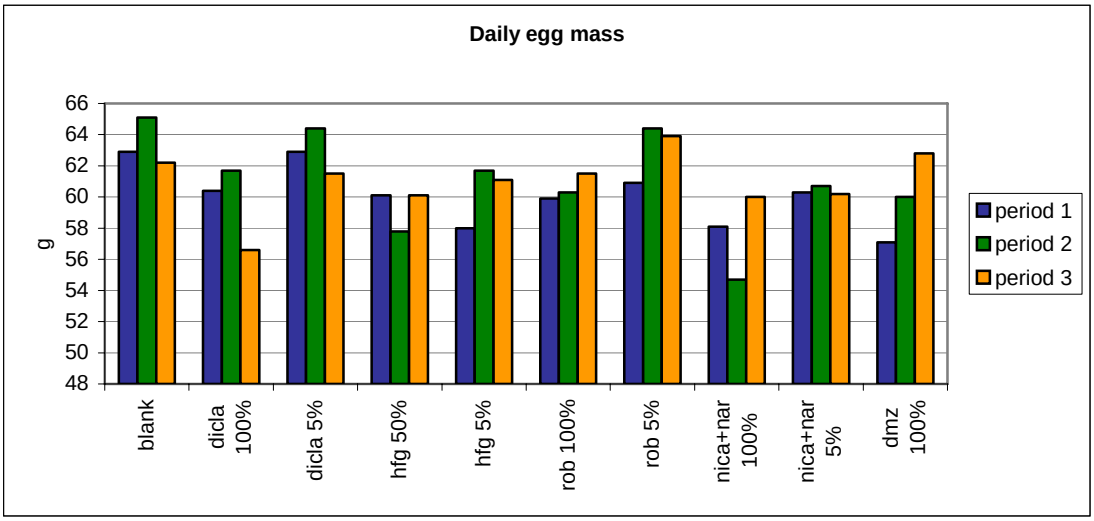


Figure 20 : daily egg mass

d) Feed intake

The feed intake is expressed as g feed consumed per animal per day. The feed intake of the hens of the experimental groups during the three periods is presented in figure 21. No remarkably effects could be observed.

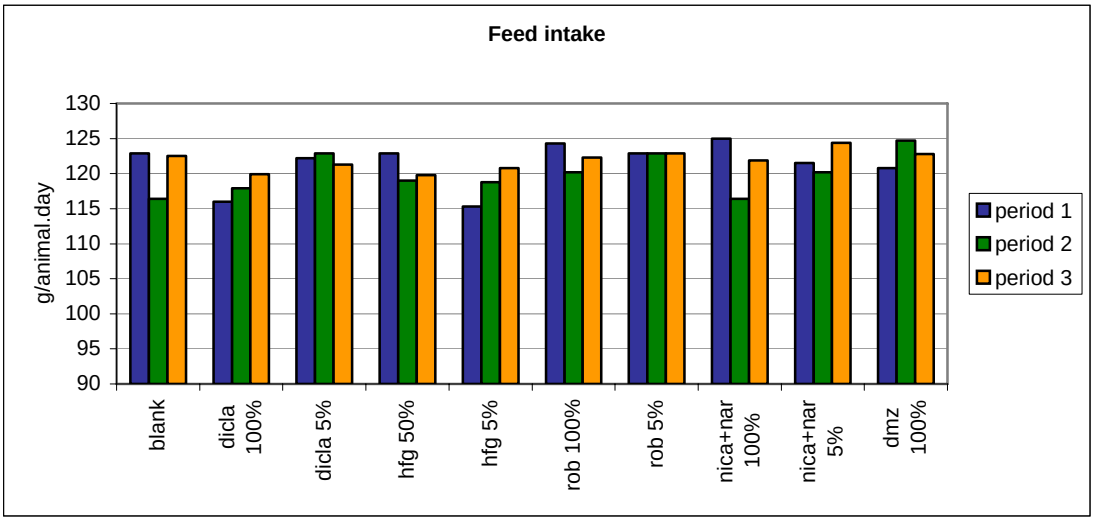


Figure 21 : average feed intake

e) Feed efficiency

The feed efficiency is defined as :

$$\text{feed efficiency} = \frac{\text{feed intake}}{\text{daily eggmass}}$$

As a consequence, the lower the feed efficiency, the higher the production ('less feed is needed to produce more'). This definition is currently used in Western Europe. In the USA on the other hand, usually feed efficiency is defined inversely and hence, in that case, the higher the feed efficiency, the higher the production. It is thus important to know how feed efficiency is defined before evaluating the results.

The feed efficiency of the different groups during the three periods is presented in figure 22. No effects could be observed.

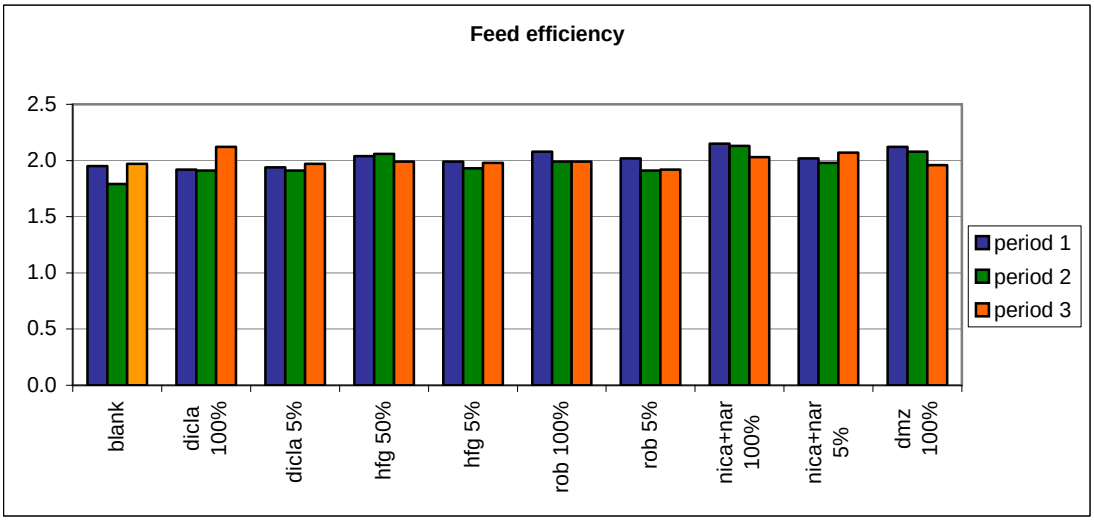


Figure 22 : feed efficiency

- Yolk colour

During the entire experimental period, once a week the colour of the yolk was measured (8 measurements in total). This was done with the Roche yolk colour fan as presented in figure 23. On each measurement day, each egg of each group was broken and the content was brought into a little plate. The colour was evaluated with the colour fan independently by at least two persons. Lighting conditions were as reproducible as possible between different measurement days.

Figure 23 : measurement of the yolk colour with the Roche yolk colour fan

No difference in yolk colour between the individual groups was noticed.

- Eggshell quality

As was the case with the yolk colour, the eggshell quality was determined once a week on all egg samples. The shell quality was evaluated using a non-destructive method that measures the deformation of the shell under the influence of a 500 g weight. This is shown in figure 24. After an appropriate conversion, the measurements result in a µm deformation-value that is inversely proportional with the eggshell quality.

Figure 24 : measurement of the eggshell quality

No remarkably differences between the individual groups could be observed.

- *conclusions* : Eggshell quality and yolk colour did not seem to be affected by one of the anticoccidials used. Also the zootechnical parameters feed intake and feed efficiency were not influenced. The average egg weight, laying percentage and as a consequence the daily egg mass on the other hand are adversely affected when Maxiban® is administered at rather high concentrations.

task D.2 : analysis of the egg samples

- *partner(s) responsible* : DVK + CER
- *objective* : By analysing the egg samples, excretion curves and the time for complete removal of the residues are determined. Analysing the egg samples with as well ELISA as LC-MS/MS allows the evaluation of the suitability of the ELISA as screening assay.
- *results* :

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 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 was also extracted which allowed estimating

the concentration of anticoccidials present in the incurred 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 ≤ 58% for the halofuginone assay, ≤ 74% for the dinitrocarbanilide assay or ≤ 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 *:* Analyses performed by LC-MS/MS were quantitative. 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 25 to 30. In table 16, 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.

Table 16 : overview of the main results of the analyses of the whole egg samples

compound	group	% of maximum allowed concentration for broilers in feed	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	5.3	day 3 (1)	6	8
nicarbazin		5.4	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

1. Diclazuril

The depletion and excretion curves of both groups receiving diclazuril are presented in figure 25.

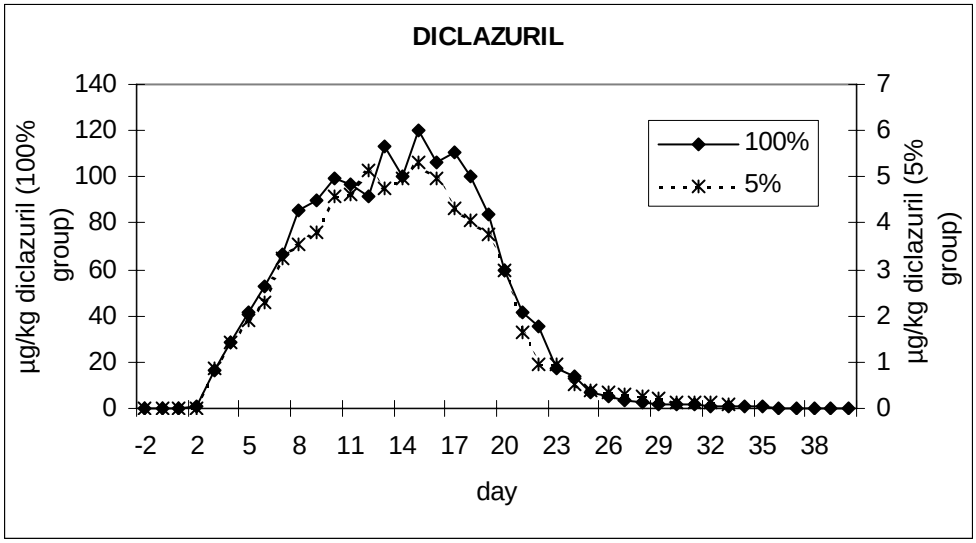


Figure 25 : results of the analyses of the whole eggs of the experimental groups receiving diclazuril

Diclazuril was detectable in the eggs from birds fed the 1 mg/kg diet from day 2 onwards whereas it was detectable in the eggs from birds fed the 0.05 mg/kg diet from day 3 onwards. 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, suggest that residues mainly will be present in the egg yolk.

2. Halofuginone

For halofuginone a similar pattern is observed as for diclazuril. This is shown in figure 26.

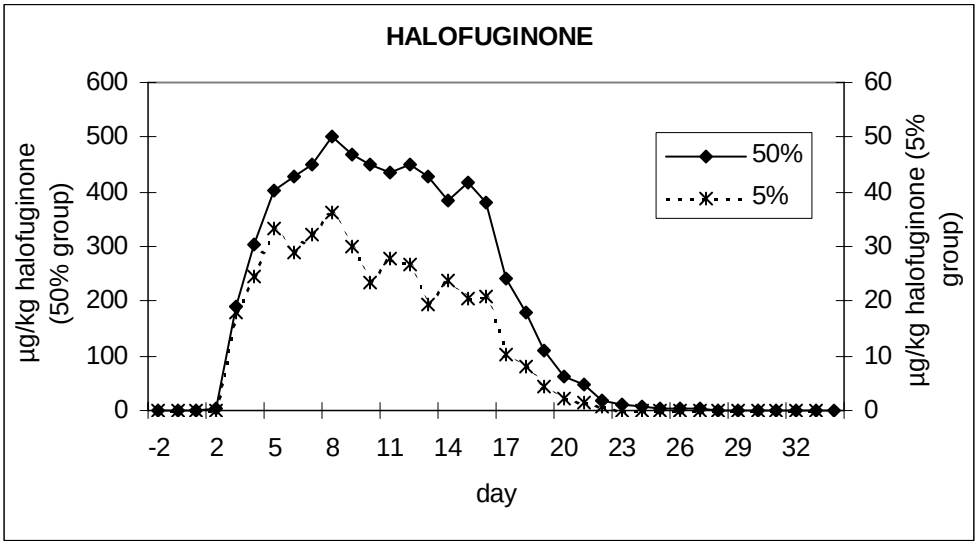


Figure 26 : results of the analyses of the whole eggs of the experimental groups receiving halofuginone

The first residues appear two and three 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.

3. Robenidine

For robenidine (figure 27), 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.

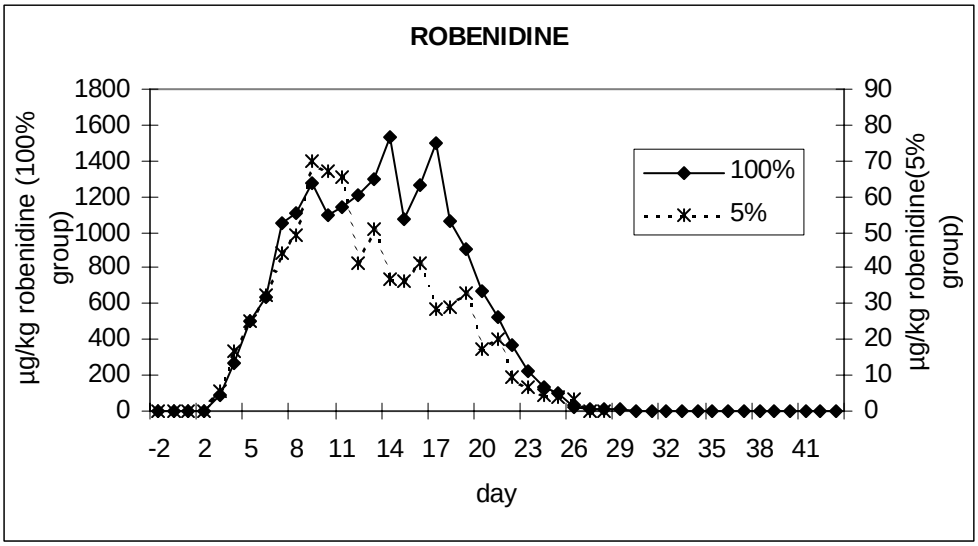


Figure 27 : results of the analyses of the whole eggs of the experimental groups receiving robenidine

4. Dinitrocarbanilide

The administration of nicarbazin to laying hens clearly leads to considerable amounts of dinitrocarbanilide in eggs. This is shown in figure 28. For the highest concentration group from day 11 onwards, 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 it takes ten days to reach the plateau. For the 5% group, it took 15 days to obtain eggs free of residues of dinitrocarbanilide.

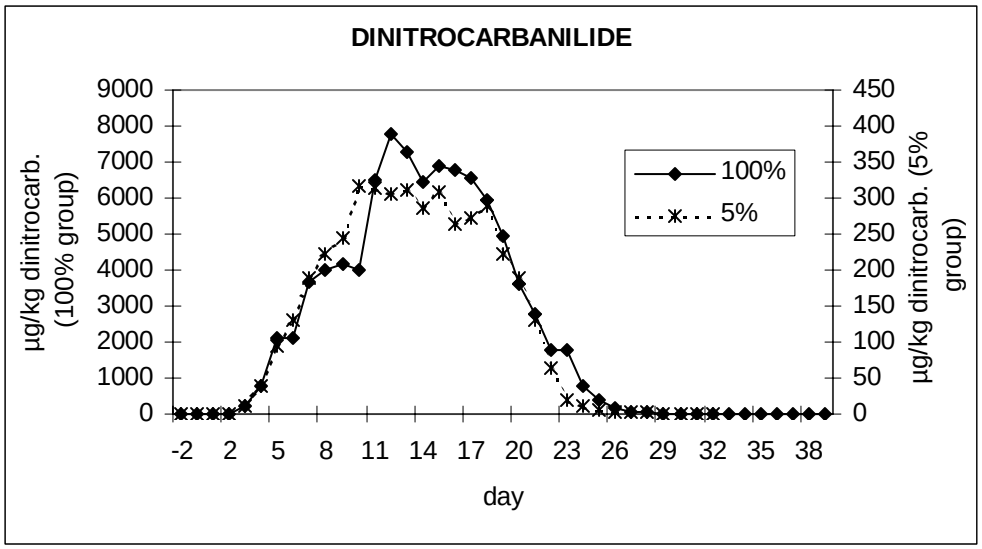


Figure 28 : results of the analyses of the whole eggs of the experimental groups receiving nicarbazin

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 29. 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 onwards, 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.

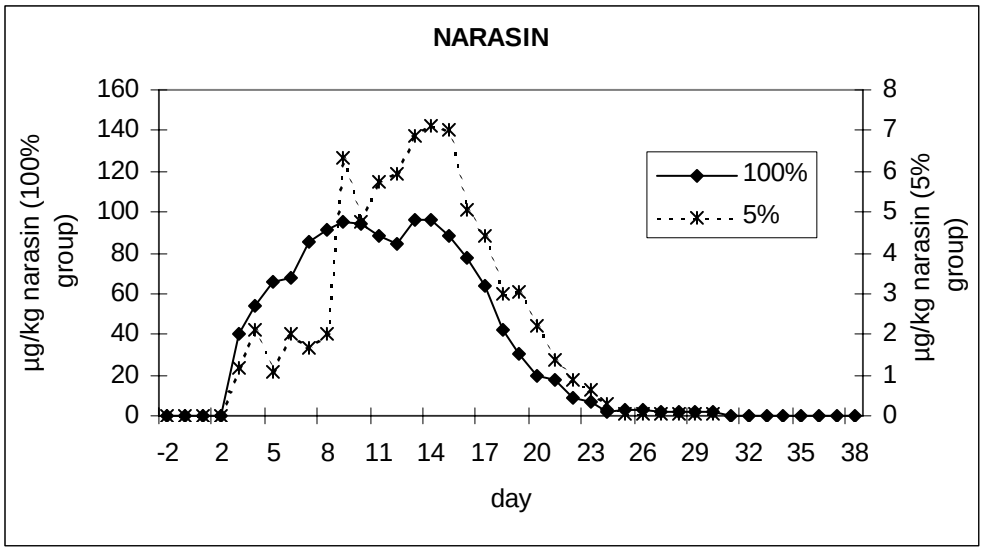


Figure 29 : results of the analyses of the whole eggs of the experimental groups receiving narasin

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 30.

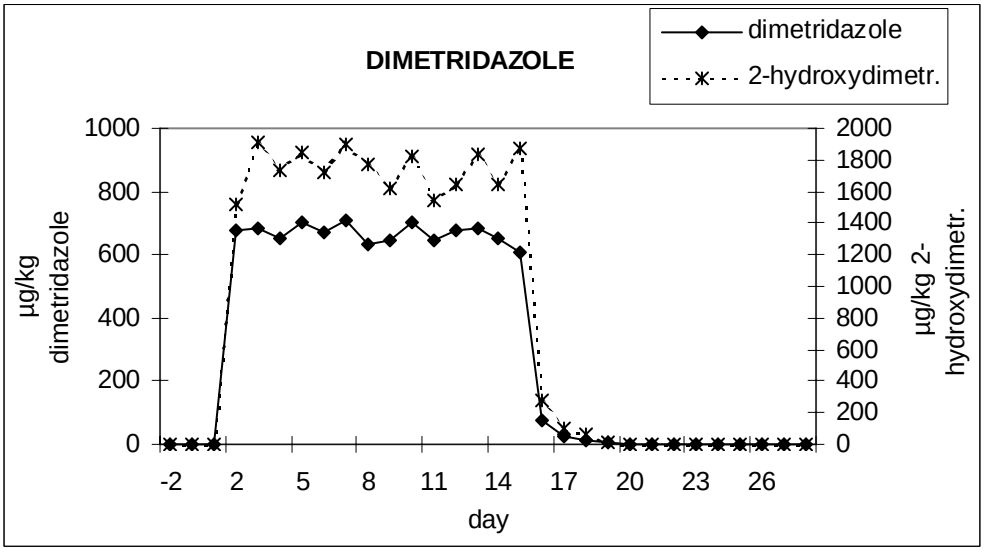


Figure 30 : results of the analyses of the whole eggs of the experimental groups receiving dimetridazole

For both compounds, the first positive samples are encountered from day 2 onwards 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. 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 ± 0.2. This clearly endorses that the hydroxymetabolite must be included when performing residue analysis for dimetridazole.

Comparison of the results obtained by ELISA and LC-MS/MS : The main objective of the development of the ELISAs was to enable to perform a screening in order 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 17.

Table 17 : comparison of the analyses of the whole eggs samples by ELISA and LC-MS/MS

compound	group	ELISA		LC-MS/MS	
		first positive sample	conc. (µg/kg)*	first positive sample	conc. (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

* : estimations

compound	group	day below (*) concentration		
		conc. (*)(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

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 : 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 sulphonamides. 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.

Analysis of the separate yolk and albumen samples : The results of the separate yolk and albumen samples showed great differences between the compounds studied.

Dimetridazole and its hydroxymetabolite are mainly present in the albumen while halofuginone is distributed between as well albumen as yolk. As well robenidine, nicarbazin, narasin as diclazuril on the other hand are mainly present in the yolk.

- *conclusions* : 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 obtain residue-free eggs. The analyses of the separate yolk and albumen revealed that differences in distribution between the different compounds are big.
  It has also been proven that the ELISAs are perfectly capable of identifying positive samples.

3.5. TASK E : MONITORING OF COMMERCIALLY AVAILABLE EGGS

To obtain a picture of the current situation regarding the occurrence of residues of coccidiostats in eggs, a monitoring was carried out. It was the aim to gather at least 300 different egg samples from different sources over the country. On demand of the users committee, also eggs from other European countries were included.
As no ELISA was available for all compounds, all eggs were analysed by as well ELISA (for dimetridazole, halofuginone and nicarbazin) as by LC-MS/MS (for dimetridazole, halofuginone, nicarbazine, diclazuril, robenidine, narasin, salinomycin, monensin and lasalocid). The ionophores, which were originally not included in the project, were also searched for since a multi-residue LC-MS/MS method was available.

task E.1 : sampling

- *partner(s) responsible* : DVK + CER
- *objective* : At least 300 different egg samples originating from as many as possible different places had to be collected.
- *results* : The eggs were gathered by ourselves with the help of colleagues, relatives and friends. Three hundred and twenty egg samples were collected at supermarkets, markets, a packaging station,…but also with private breeders. On commercially available eggs, a code must be printed. The EU has introduced new legislation that makes it easy for consumers to identify exactly where each egg they bought comes from and how it was produced. Every egg is now individually stamped with a code,

which makes it fully traceable back to the farm where it was produced. The code consists of three parts : (1) a number (0, 1, 2 or 3) referring to the farming method or housing facility of the hens, (2) two letters referring to the country of origin (e.g. BE for Belgium, NL for the Netherlands, IE for Ireland,...) and (3) the registration code of the producer. Four farming methods can be distinguished : organic eggs (code 0), free range eggs (code 1), barn eggs (code 2) and cage eggs (code 3). The composition of the 320 egg samples according to the farming method was as follows : 10.9% organic eggs, 21.9% free range eggs, 5.3% barn eggs, 39.1% cage eggs and 22.8% of the samples had no code. The eggs with a code were purchased in 8 different European countries : 78.6% was bought in Belgium, 8.1% in Luxemburg, 3.7% in The Netherlands, 3.2% in France, 2.8% in Austria, 2% in Germany, 1.2% in Switzerland and 0.4% was bought in Sweden. A different distribution is obtained however when the composition according to the country of origin (as mentioned in the egg code) is looked at. In Belgium, 52.7% of the samples was produced while 29.6% originated from France, 10.9% from The Netherlands, 2.8% from Austria, 1.6% from Luxemburg, 1.2% from Switzerland and 0.4% from Sweden, Italy and Germany.

- *conclusions* : It is clearly shown that we succeeded in collecting a wide variety of egg samples.

task E.2 : screening by immunological methods

- *partner(s) responsible* : CER
- *objective* : All 320 samples that were collected were analysed by ELISA on the presence of dimetridazole, halofuginone and nicarbazin.
- *results* : The aim of a screening method is to obtain no false negative results and to obtain as less as possible false positive results.

  The concentration from which the ELISA generates a positive result is 0.5 μg/kg for halofuginone, 1 μg/kg for dimetridazole and 3 μg/kg for nicarbazin (dnc).

  No sample was found to be positive on the presence of dimetridazole by LC-MS/MS analysis. The ELISA analysis revealed two positive samples which are, as a consequence, false positive samples. Those two samples represent only 0.6% of the samples analysed. No false negative samples were obtained.

  The one sample that was found to be positive on the presence of halofuginone by LC-MS/MS tested also positive by ELISA. So for halofuginone, neither false positive neither false negatives results were obtained.

Out of the 13 samples that were found to contain nicarbazin (dnc) by LC-MS/MS, only one sample had a concentration above 3 µg/kg. The ELISA declared 3 samples positive which were also confirmed by the LC-MS/MS analysis. Ten samples were not found positive by the ELISA but all concentrations were below 3 µg/kg. The results of five samples (1.6%), which were declared positive by ELISA, were not confirmed by LC-MS/MS and were hence false positive samples.

- *conclusions :* It can be concluded that all three ELISA's are very suitable as screening method.

task E.3 : confirmation by mass spectrometric methods

- *partner(s) responsible* : DVK
- *objective* : All 320 samples that were collected were analysed by LC-MS/MS. Results were evaluated with respect to compound, concentration, country of origin,…
- *results* : Out of the 320 samples that were analysed, 114 samples (35.6%) were found to contain one or more of the nine anticoccidials in concentrations ranging from 0.1 µg/kg to 63 µg/kg. Four samples contained three compounds while 2 different compounds were found in 15 other samples. When each compound is counted individually, a total of 137 positive samples was achieved. The ionophoric and chemical anticoccidials account for 66.4% and 33.6% of the positive samples, respectively.

  In figure 31 an overview is given of the distribution of the positive samples according to the compound. Salinomycin and lasalocid account for more than 60% of all positive samples. No sample was found to be positive on the presence of dimetridazole and only one sample contained halofuginone.

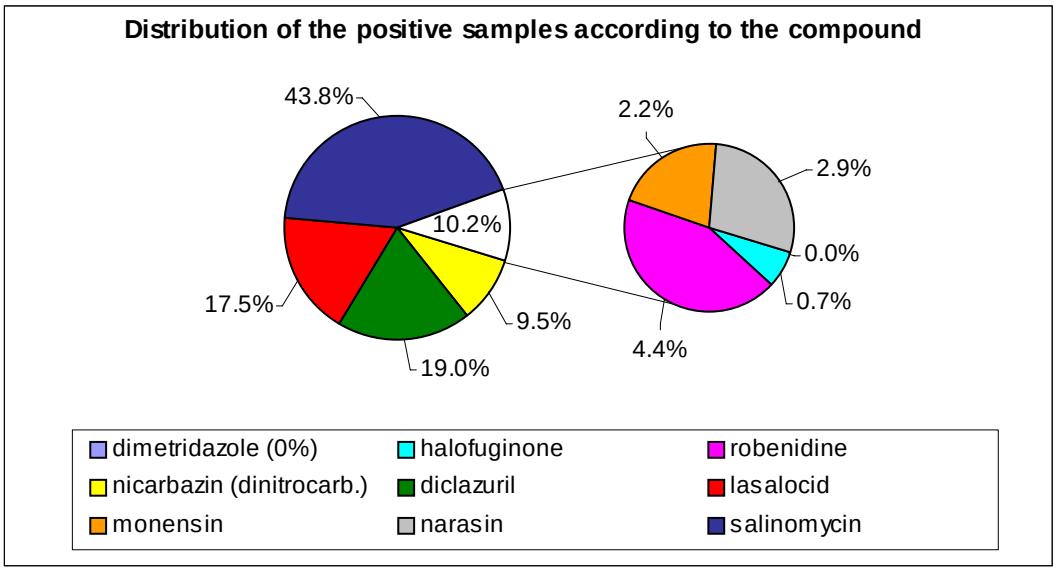


Figure 31: overview of the distribution of the positive samples according to the compound

In figure 32 the distribution of the positive samples according to the concentration level is presented. Almost 90% of all positive samples contained less then 2 µg/kg. Moreover, 77% contained even less then 1 µg/kg. Out of the seven samples with concentrations between 10 and 50 µg/kg, five of them were positive on the presence of lasalocid, and one on the presence of robenidine and nicarbazin. The highest concentration found was 63 µg/kg salinomycin.

With regard to the action limit of 10 µg/kg, which was set by the scientific committee of the Belgian Food Agency, 5.8% of the samples analysed, would give rise to action.

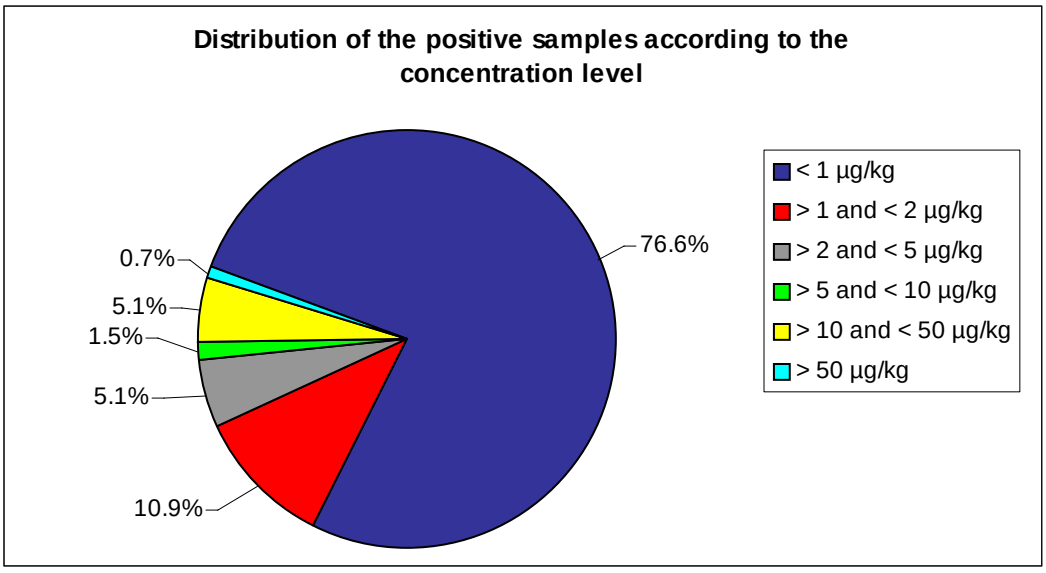


Figure 32 : overview of the distribution of the positive samples according to the concentration level

The results were also put into the perspective of the farming method. Thirty-five organic samples were analysed. Six of them contained lasalocid in concentrations ranging from 0.37 µg/kg to 44 µg/kg. Diclazuril and salinomycin were encountered in one sample each. In the barn eggs mainly residues of the ionophoric anticoccidials were found. As well narasin, monensin, salinomycin as lasalocid were encountered in one or more samples but concentrations did not exceed 1.4 µg/kg. One sample contained as well salinomycin (0.71 µg/kg) as dinitrocarbanilide (0.18 µg/kg). Thirty-two free range samples were found positive. Salinomycin was found in twenty cases but concentrations did not exceed 1.9 µg/kg. Two of the 6 samples that contained lasalocid had levels above 10 µg/kg, namely one sample contained 31 µg/kg and another contained 40 µg/kg. Fifteen of the 320 samples analysed were found positive on two different anticoccidials. Seven of these samples were free range eggs. Three of the samples containing two compounds were cage eggs and in three cage eggs residues of three different products were found. Eighteen cage eggs were found positive on the presence of salinomycin but concentrations did not exceed 1.1 µg/kg. Lasalocid concentrations were remarkably higher : 5.6 µg/kg, 5.9 µg/kg, 27 µg/kg and 49 µg/kg lasalocid were measured. The other lasalocid concentrations ranged between 0.21 µg/kg and 2.5 µg/kg. Six samples contained dinitrocarbanilide of which one contained 10 µg/kg. Robenidine was found in three cage samples. The maximum concentration was 3.1 µg/kg.

Seven different compounds were found in the eggs without code. The only sample found positive on the presence of halofuginone was an egg from a farmer in Austria. One egg was obtained which was not intended for human consumption but originated from breeders. This sample contained 0.11 µg/kg narasin and 2.6 µg/kg salinomycin. Ten samples were found positive on the presence of diclazuril in concentrations ranging from 0.31 to 3.1 µg/kg. Three different types of eggs (from three different types of laying hens) were gathered from the same private breeder. All three samples contained diclazuril. This may indicate that diclazuril-containing feed was used to feed the laying hens, especially since this private breeder also breeds broilers that are fed diclazuril-containing feed. Possible causes are the use of the same barrel to store the two types of feed or the use of remainder of the broiler feed for the laying hens. One sample contained 12 µg/kg robenidine. Talking to the private breeder revealed that the laying hens were held in a meadow on which a rabbit hutch is placed. When cleaning the feeding bins of the rabbits, the remainder of the content is emptied out in the meadow, and hence the laying hens eat this feed. Robenidine is licensed for use in rabbits. This probably explains the presence of the robenidine in the eggs. Only one sample contained lasalocid (1 µg/kg). This is in contrast with the other farming methods where a big part of the positive samples is attributed to lasalocid. In two home bred egg samples concentrations above 10 µg/kg were found : one sample contained 63 µg/kg salinomycin, which is the highest concentration found in all 320 samples, and the other is the sample that contained 12 µg/kg robenidine.

Results were also evaluated with regard to the country of origin. Results for Sweden, Luxemburg, Italy, Germany and Switzerland have to be taken with a pinch of salt since less than five samples of these countries were investigated and moreover, concentrations were all below 2 µg/kg. In the samples originating from Switzerland only small amounts were encountered : 0.30 µg/kg and 0.34 µg/kg monensin and narasin were found. Also in one egg from Austria 0.34 µg/kg monensin was found. Moreover, only in eggs originating from Austria and Switzerland monensin was present while it was not found in the eggs from the other countries. Dinitrocarbanilide was found in one egg sample from Austria at a concentration level of 10 µg/kg. This was the highest dinitrocarbanilide concentration found in all eggs analysed. Almost all positive Dutch eggs contained salinomycin but concentrations were not higher than 1.6 µg/kg. Besides salinomycin, also lasalocid, robenidine, dinitrocarbanilide and diclazuril were present in one sample each. Also in the positive Belgian samples,

mainly salinomycin was found and here also concentrations were really low : maximum 1.9 µg/kg was found. The highest concentration found in Belgian eggs was 2.6 µg/kg lasalocid. Two samples contained traces of robenidine (0.65 µg/kg) and diclazuril (0.13 µg/kg). In contrast to the results obtained by analysing Belgian and Dutch eggs, much higher concentrations were found in the French eggs. All five samples that contained the highest levels of lasalocid (27 to 49 µg/kg) originated from France. Moreover, in French eggs mainly lasalocid and diclazuril are encountered. This is in contrast with the Belgian and Dutch eggs where mainly salinomycin was found. Also traces of robenidine, salinomycin and dinitrocarbanilide were present in the French eggs.

In table 18, a detailed overview is given of the samples positive on the presence of diclazuril, nicarbazin, salinomycin and lasalocid. As there were too little positive samples for the other compounds, it seemed unnecessary to give such a detailed distribution.

Table 18 : detailed overview of the samples positive on the presence of diclazuril, nicarbazin, salinomycin and lasalocid

DICLAZURIL	
number of positive samples	26
% of positive samples	8.1%
distribution according to the concentration levels	
< 1 µg/kg	92.3%
> 1 and < 2 µg/kg	3.8%
> 2 µg/kg	3.8%
distribution according to the farming method	
without code	38.5%
organic	3.8%
free range	23.1%
cage	34.6%
distribution according to the country of origin	
number of positive samples with code	16
Belgium	6.3%
France	87.5%
The Netherlands	6.3%

NICARBAZIN (DINITROCARB.)	
number of positive samples	13
% of positive samples	4.1%
distribution according to the concentration levels	
< 1 µg/kg	92.3%
> 10 µg/kg	7.7%
distribution according to the farming method	
without code	15.4%

organic	7.7%
free range	23.1%
cage	53.8%
distribution according to the country of origin	
number of positive samples with code	11
Belgium	9.1%
France	63.6%
The Netherlands	9.1%
Austria	9.1%
Germany	9.1%

LASALOCID	
number of positive samples	24
% of positive samples	7.5%
distribution according to the concentration levels	
< 1 µg/kg	20.8%
> 1 and < 2 µg/kg	33.3%
> 2 and < 10 µg/kg	25.0%
> 10 µg/kg	20.8%
distribution according to the farming method	
without code	4.2%
organic	25.0%
free range	25.0%
barn	4.2%
cage	41.7%
distribution according to the country of origin	
number of positive samples with code	23
Belgium	13.0%
France	82.6%
The Netherlands	4.3%

SALINOMYCIN	
number of positive samples	60
% of positive samples	18.8%
distribution according to the concentration levels	
< 1 µg/kg	88.3%
> 1 and < 2 µg/kg	8.3%
> 2 and < 5 µg/kg	1.7%
> 50 µg/kg	1.7%
distribution according to the farming method	
without code	28.3%
organic	1.7%
free range	33.3%
barn	6.7%
cage	30.0%
distribution according to the country of origin	
number of positive samples with code	43
Belgium	53.5%
France	4.7%
The Netherlands	30.2%

Austria	4.7%
Italy	2.3%
Luxemburg	2.3%
Germany	2.3%

- *conclusions :* Performing this monitoring programme made it possible to estimate the current situation on the presence of residues of anticoccidials in eggs. By including eggs gathered in other European countries it was possible to put the Belgian situation into perspective. Major differences between eggs originating from Belgium, The Netherlands and France were noticed : in Belgium and The Netherlands mainly very low concentrations of salinomycin were encountered while French eggs contain higher levels of lasalocid. The two most encountered compounds are clearly lasalocid and salinomycin. For both compounds very few residues were found in barn eggs.

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5. PUBLICATIONS

- *Simultaneous detection of five coccidiostats in eggs by liquid chromatography – tandem mass spectrometry*
  L. Mortier, E. Daeseleire and P. Delahaut
  Analytica Chimica Acta, 2003, 483, 27-37

- *Determination of halofuginone in eggs by liquid chromatography – tandem mass spectrometry after cleanup with immunoaffinity chromatography*
  L. Mortier, E. Daeseleire, A.-C. Huet, P. Delahaut and C. Van Peteghem
  Rapid Communications in Mass Spectrometry, 2004, 18, 1817-1820

- *Development of an ELISA screening test for nitroimidazoles in egg and chicken muscle*
  A.-C. Huet, L. Mortier, E. Daeseleire, T. Fodey, C. Elliott and P. Delahaut
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- *Screening for the coccidiostat halofuginone and nicarbazin in egg and chicken muscle : development of an ELISA*
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- *Determination of the ionophoric coccidiostats narasin, monensin, lasalocid and salinomycin in eggs by liquid chromatography – tandem mass spectrometry*
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- *Determination of the coccidiostat diclazuril in poultry feed and meat by liquid chromatography – tandem mass spectrometry*
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- *Detection of residues of the coccidiostat diclazuril in poultry tissues by liquid chromatography – tandem mass spectrometry after withdrawal of medicated feed*
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- *Liquid chromatographic tandem mass spectrometric determination of 5 coccidiostats in poultry eggs and feed*
  L. Mortier, E. Daeseleire and C. Van Peteghem
  Journal of Chromatography B, 2005, 820, 261-270

- *Deposition and depletion of five anticoccidials in eggs*
  L. Mortier, A.-C. Huet, E. Daeseleire, G. Huyghebaert, T. Fodey, C. Elliott, P. Delahaut and C. Van Peteghem
  submitted to Journal of Agricultural and Food Chemistry

- *Incidence of residues of nine anticoccidials in eggs*
  L. Mortier, A.-C. Huet, C. Charlier, E. Daeseleire, P. Delahaut and C. Van Peteghem
  Food Additives and Contaminants, accepted for publication

- *Detection of anticoccidials in eggs, poultry tissues and feed by liquid chromatography – tandem mass spectrometry*
  PhD thesis Leen Mortier

6. GENERAL CONCLUSIONS

ELISA screening assays were developed and validated for the detection of halofuginone, nicarbazin and dimetridazole. Despite many immunisation attempts, no suitable antibodies for diclazuril and robenidine could be obtained. As well the analyses of the samples originating from the experiment with laying hens as from the monitoring have

shown that the ELISAs are perfectly suitable as screening assay when applying a pyramid structure.

A multiresidue LC-MS/MS method was developed and validated for the 5 compounds studied. Additionally, also a multiresidue LC-MS/MS method was developed and validated for 4 ionophoric coccidiostats : narasin, lasalocid, monensin and salinomycin.

The experiment with laying hens has clearly shown that even 5% carry-over during feed preparation can lead to the presence of considerable amounts of residues in eggs. Results were also evaluated in the perspective of the currently applied action limit of 10 µg/kg in Belgium.

In the experiment with laying hens, also some egg and zootechnical parameters were evaluated. Eggshell quality and yolk colour did not seem to be affected by one of the coccidiostats used. Also the zootechnical parameters feed intake and feed efficiency were not influenced. The average egg weight, laying percentage and as a consequence the daily egg mass on the other hand are adversely affected when Maxiban® is administered at rather high concentrations.

By performing the monitoring, we obtained a picture of the current situation of the incidence of residues of coccidiostats in eggs. By analysing eggs originating from other European countries, we were able to evaluate the Belgian situation. A difference was observed in eggs originating from Belgium and The Netherlands on the one hand and France on the other hand : in Belgian and Dutch eggs mainly low levels of salinomycin were found while French eggs contained higher levels of lasalocid.

The two most encountered compounds were clearly salinomycin and lasalocid. It was also interesting to analyse samples from private breeders. Results revealed that in some cases rather high levels could be found.

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