SCIENTIFIC SUPPORT PLAN FOR A SUSTAINABLE DEVELOPMENT POLICY

Intermediary report - January 2003

INTEGRATED APPROACH FOR THE CONTROL ON RESIDUES OF COCCIDIOSTATS IN EGGS
CP-29

CLO - CER

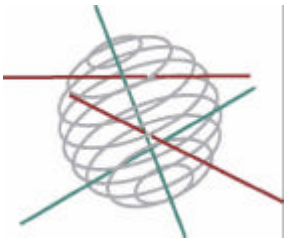
SPSD II

PART 1
SUSTAINABLE PRODUCTION AND CONSUMPTION PATTERNS

- GENERAL ISSUES
- AGRO-FOOD
- ENERGY
- TRANSPORT

This research project is realised within the framework of the Scientific support plan for a sustainable developmentpolicy (SPSD II)

Part I "Sustainable production and consumption patterns"



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INTERMEDIARY SCIENTIFIC REPORT

year 1 : 15/11/2001 – 14/11/2002

1. Project title

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

2. Introduction

2.1. Context and summary

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 (Directive 96/23/EC) 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 young chickens but not all of them 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. 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 an 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 ELISA's will be developed with the aim of obtaining a detection limit as low as possible. The possibility to develop multi-residue ELISA's, 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. A triple quadrupole instrument with Z-spray is available for this purpose. 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 with commercially available medicated 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.

2.2. Objectives

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.

2.3. Expected outcomes

By developing immunological screening methods and mass spectrometric confirmation methods, Belgium will have the ability to perform monitoring on eggs for the presence of these 5 unwanted compounds in a reliable and economic liable way. Until now no immunological methods are commercially available for the detection of coccidiostats. In this project ELISA's for nicarbazin, dimetridazole, halofuginone, robenidine and diclazuril will be developed and when possible even a

multi-residue ELISA will be made available. Positive results have to be confirmed by an independent physico-chemical technique. Liquid chromatographic-mass spectrometric methods will be developed for the compounds studied. The aim is to determine all the compounds in one single chromatographic run. By using these fully validated methods in monitoring programmes, the faith of the consumer in the quality of his food can be renewed and/or strengthened.

By treating the laying hens with 2 different concentrations of each compound, the following research results will be obtained: (1) the influence of the compounds on the zootechnical parameters of the laying hens, on the yolk colour and on the shell quality, (2) the concentrations of the compounds present in the eggs, (3) excretion curves of the compounds in eggs, (4) determination of the time to get residue free eggs and (5) formulation of recommendations concerning carry-over of coccidiostats in feeding mills. By analysing Belgian egg samples from different origin with the methods developed, we will get a picture of the contamination level of eggs with coccidiostats. In case when problems will be brought to light, the cause of the contamination can be searched for and corrective measures can be taken.

3. Detailed description of the scientific methodology

To perform the control in an economical liable way it is obvious to make use of the well-known pyramid structure. This means that initially the samples are screened on the presence of residues by screening methods, which are most of the time immunological methods. These latter have the advantage that a big number of samples can be analysed at the same time at a reasonable price. The disadvantage of these methods is the occurrence of cross-reactivity with matrix and other components so that false positive results can be generated. Therefore confirmation of a positive result is always necessary making use of a physico-chemical technique that is more expensive. These methods have to be able to identify and, in case of molecules with an MRL value, to quantify the residue in a reliable way. At this moment mass spectrometry is the method of choice for this task. Due to the fact that only suspect samples have to be analysed in this way, such a sophisticated and expensive technique is justified.

3.1. Immunological screening methods

3.1.1) introduction

ELISA (Enzyme Linked Immunosorbent Assay) techniques offer the advantage of being rapid, cheap and easy to use for most laboratory personnel. Substances with a Maximum Residue Limit (MRL) require the assay to be quantitative. The EU Commission Decision (2002/657/CE) gives instructions about the validation of immunological screening methods.

The key elements of the development of an immunoassay are the antibody and the conjugate (labelled tracer). Immunogens required for the coccidiostats assays were produced by conjugating haptens to a carrier protein using well established procedures such as carbodiimide, mixed anhydride reactions and NHS (N-hydroxysuccinimide) ester cross linker. Carrier proteins used are Human Serum Albumin (HSA) and Bovine ThyroGlobulin (BTG). Tracer conjugates were synthesized using the same haptens but replacing carrier protein by an enzyme: Horseradish Peroxidase (HRP).

The antibodies for all the compounds included in this project were generated using high volume polyclonal production techniques (hyper-immunization of rabbits). These antibodies were fully characterized with regards to titer, sensitivity and cross-reactivity.

Titers were determined first by evaluating the optimal dilution of the anti-serum to give an OD (Optical Density) varying between 1 and 1,5 and secondly, by checking the value of the non-specific reading. If the latter is higher than 20 % (relative value), the enzyme tracer is purified on a PD10 column. The Non Specific Binding (NSB) is used to characterize the specificity of conjugate. It is determined by reading the optical density given by incubations into wells with no antibodies present in the solution.

The sensitivity of the antibodies was calculated using "Inhibition experiments" and by evaluating the ID 50 (Inhibition Deviation 50) which corresponds to the concentration at the midpoint of the curve.

Cross-reactivity represents the specificity of the antibodies and was calculated as

$$100 * \left(\frac{\text{ID 50 of compound used to raise the antibody)}}{\text{(ID 50 of the competing compound)}} \right).$$

Another important characteristic in an ELISA methodology is the binding obtained for a zero standard (B0). It actually represents the "100 % activity" of the antibody.

The optimal dilution of the antibody and the tracer conjugate were considered when the value of the optical density for B0 is set between 1 and 1,5 with the NSB value lower than or equal to 0,1.

The first year development program was devoted to the development of an ELISA methodology for nicarbazin, halofuginone and dimetridazole. The ELISAs for diclazuril and robenidine including the optimisation of the clean-up of egg matrices will be envisaged in the next periods of the project development.

3.1.2) format of the assays

For the molecules studied in this project: nicarbazin, halofuginone, dimetridazole, robenidine diclazuril, the "sandwich" configuration of an ELISA is not possible. Assay formats were limited to direct and indirect competitive configurations. For the indirect format used, 96 plate wells were

first coated with sheep antibodies raised against rabbit IgGs (1 µg/well), while the raised specific antibodies and the tracer/analyte were added in solution. This type of assay was tested but was found unsatisfactory for some of the molecules. The sensitivity was found to be worse than that obtained for by the direct assay. The objective being to have a common format for all studied molecules, it was decided to adopt the direct competitive assay for all the developments. Under this configuration, the specific antibodies were coated directly onto the solid phase of the 96 well plate *(figure 1)*.

The first antibodies that were tested, were produced as part of the EC project "Poultry-check" QLK1-CT-1999-00313. In order to determine the best conditions for saturation, it was necessary to let the antibodies adsorb onto the plates during 72 hours followed by a blocking procedure with a Casein hydrolysate set at 25 g / L (pH 7). Three different times of saturation have been examined: 30, 60 and 120 minutes. A 120 minutes saturation procedure was finally adopted. Bovine Serum Albumin (BSA) and gelatine were also tested to saturate the wells but the optical densities values for NSB and B0 were not as satisfactory.

3.2. Liquid chromatographic – mass spectrometric confirmatory methods

The development of mass spectrometric confirmation methods is aimed for by the implementation and the carrying out of 5 tasks *(table 1)*. In task B.1 a thorough literature study was carried out in order to gather the most recent data concerning the mass spectrometric conditions, clean-up of the matrix and metabolisation of the compounds so that the marker compounds could be determined.

Task B.2 consisted of the purchase of the standards of the 5 compounds to be studied and the choice and purchase of the internal standards. The use of an internal standard is indispensable when performing qualitative and quantitative residue analysis with mass spectrometric equipment. By using an internal standard that is taken through the whole procedure the ruggedness and quantification of the method is enhanced. The availability of deuterated analogues was checked with pharmaceutical companies, the European reference laboratories and commercial suppliers. If no deuterated analogues are available, the use of other compounds, having a similar molecular structure as the molecules studied, must be examined. But these other compounds may not be used in veterinary medicine.

In task B.3 the mass spectrometric conditions were optimised. The mass spectrometer used is a triple quadrupole instrument (Quattro LC, Micromass) controlled by version 3.3 of the Masslynx software. Ionisation takes place in the source at atmospheric pressure. The ions are sampled through a series of orifices into the first quadrupole where they are filtered according to their mass to charge ratio (m/z). The mass separated ions then pass into the hexapole collision cell (second quadrupole) where they undergo collision induced decomposition (CID). The fragment ions are

then mass analysed by the third quadrupole. Finally the transmitted ions are detected by a conversion dynode, phosphor and photomultiplier detection system. The output signal is amplified, digitised and presented to the data system. This operation mode is called Multiple Reaction Monitoring (MRM) and is schematically presented in *figure 2*. The optimisation of the mass spectrometric conditions was done by direct infusion of the standard solutions in the mass spectrometer. The techniques electrospray and atmospheric pressure chemical ionisation were tested and the technique generating the highest abundance was selected for the definitive method. The distance between the probe and the cone and the voltage of the cone was optimised per compound. For each molecule the precursor ion and the accompanying product ions (preferably 2) were determined. When all these parameters were known, methods were programmed in which the mass spectrometer follows the transition of the precursor ion to the product ions which guarantee specificity. The possibility of a multi-residue method, in which all the molecules studied are detected in one single run, was examined.

Task B.4 contained the optimisation of the chromatography. The HPLC system consisted of a Kontron system (ternary pump 325, vacuum degasser degasys DG 1310) and autosampler (Biotech Instruments, Milan, Italy). Before the sample is coming into the mass spectrometer a separation is performed on a chromatographic column to increase selectivity. On base of the literature available an universal C_{18} column was tested. When coupling a liquid chromatographic system to a mass spectrometer one is limited with respect to the choice of buffers. Non-volatile buffers are advised against since they can block the capillary in the probe. The ideal composition of the solvents, as well as the gradient program, the injection volume, column temperature and the split ratio were determined. This could all be done by the use of standard solutions. Here again the possibility of analysing all compounds with the same chromatographic conditions in one run was examined.

In the last subtask, task B.5, the clean-up of the egg matrix for mass spectrometric detection is optimised. 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. Due to the specificity of the detector, sample clean-up can in theory be kept as simple as possible. In practice however this approach has to be examined by determining the robustness of the method. If the instrument has to be cleaned for e.g. every 10 injections, the method is not suitable for routine analysis. As the egg matrix contains a lot of proteins a deproteinisation has to be carried out. Several solvents must be tested for their efficiency. After centrifugation the extract contains coextractives that can increase the background noise of the detector. Therefore three different approaches have to be tested for their ability to generate a clean extract, namely, liquid-liquid partitioning, solid phase extraction and immunoaffinity clean-up by using the antibodies made in task A of the project. 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.

3.3. Validation of the methods developed

The immunological screening methods and mass spectrometric confirmation methods will be validated according to the most recent European legislation concerning residue analysis. Since 1 September 2002 Commission Decision 2002/657/EC concerning the performance of analytical methods and the interpretation of results has to be applied. The validation includes the determination of the detection capability, precision, selectivity/specificity and applicability/ruggedness/stability for screening methods and the determination of the detection capability, decision limit, trueness, precision, selectivity/specificity and applicability/ruggedness/stability for confirmation methods.

3.4. Applicability of the methods for incurred samples

Laying hens selected for egg production and body weight will be treated, after an adjustment period of 4 weeks, with medicated feed during 14 days. The hens will be fed with a balanced mash ration. Feed and water will be provided *ad libitum*. The feed consumption will be determined during the whole experiment per hen. Per compound 2 concentration levels will be given, namely the maximum (recommended) level in medicated feed according to the Belgian legislation and a lower concentration level, comparable with levels that could occur due to carry-over in the feeding mills. For the dietary treatment of each compound and level, 12 laying hens will be housed in 3 cages of 4 each, in a three-tier battery under conventional conditions of ventilation, temperature and lighting. Blank eggs will be collected before receiving the medicated feed. Influence on the zootechnical parameters, on the yolk colour and on the shell quality will be checked. The eggs will be refrigerated until analysis. As stipulated by EU legislation, ten eggs per day have to be determined. All egg samples will be analysed with the ELISA's and with the mass spectrometric methods developed for the compounds. With the concentration levels found, excretion curves will be established for each compound. The time necessary to get residue free eggs will be determined for each compound.

3.5. Monitoring of commercially available eggs

Three hundred egg samples from different sources, such as supermarket, retail trade, packing stations, farmer, bio-farmer, market place, etc., from different parts over the country will be gathered. Screening of these eggs on the presence of coccidiostats will be performed with the immunological methods and in case of a positive result confirmation will be done by the liquid chromatographic – mass spectrometric methods developed.

4. Detailed description of the intermediary results, preliminary conclusions and recommendations

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

4.1. TASK A : DEVELOPMENT OF IMMUNOLOGICAL SCREENING METHODS

task A.1 : Literature study

- *execution period :* November 2001 –December 2003
- *current status :* in progress
- *partners responsible :* CER
- *objective :* By carrying out a thorough literature study the knowledge about 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 :* Little 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.
- *preliminary conclusions and recommendations :* The literature study is reviewed regularly to obtain more information about the five studied coccidiostats, their stability, their detection, their extraction from eggs, …

task A.2 : Purchase of standard material

- *execution period :* November 2001 – June 2002
- *current status :* completed
- *partners responsible :* CER
- *results :* In *table 2* standards and their origin were presented. For each compound, a solution of 1 mg/ml was prepared in the adequate solvent and 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 also used to dilute antibodies and conjugate.
- *preliminary conclusions and recommendations :* For each compound, a suitable standard was found and stock solutions were made.

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

- *execution period :* January 2002 – December 2002

- *current status :* in progress
- *partners responsible :* CER
- *objective :* To elicite an immune response in the host animal, the coccidiostats must be conjugated to a large carrier protein. For the conjugates, the immunogenic carrier protein has been replaced with HRP.
- *results :*

  a) Diclazuril

  Two diclazuril immunogens were produced in two steps : the preparation of diclazuril-Carboxymethyl Oxime (CMO) and then the conjugation of diclazuril-CMO to HSA or BTG. Diclazuril was conjugated to the enzyme label HRP using the same method as for the immunogens (diclazuril-CMO - HRP). Several conditions were tested to observe a displacement by a standard curve but all failed (see results A.4.a.).

  b) Robenidine

  Two steps were also necessary to synthesize the immunogens : the preparation of robenidine-hemisuccinate and conjugation of robenidine-succ. to HSA or BTG activated by addition of carbodiimide and NHS. The conjugate robenidine-succ. - HRP was produced using the same way. The two immunogens produced failed to render a detectable immune response.

  Another idea was suggested for the synthesis of new immunogens of robenidine. The chemical called 1-(2,6-dichlororbenzildeneamino)-guanidine is similar to half of the robenidine structure *(figure 3)*. Unfortunately, the position of the two chlorines is not preferable but a similar compound with a single chlorine at position 4 would be useful and would lead to a better chance of binding robenidine.
- *preliminary conclusions and recommendations :* No titer was obtained with immunizations with robenidine. Titers were obtained with immunizations with diclazuril but not allowing displacement. New immunogens must be designed and produced to try new immunizations.

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

- *execution period :* January 2002 – December 2003
- *current status :* in progress
- *partners responsible :* CER
- *objective :* Rabbits were injected subcutaneous with the immunogens every month. During the immunization, titer is checked in order to determine the ideal period to proceed to the final bleeding.
- *results :*

  a) Diclazuril

  Rabbits M 118 to M 122 and M 113 to M 117 were injected with immunogen diclazuril-CMO - BTG and diclazuril -CMO - HSA respectively. All diclazuril antibodies were characterized with regards to titer using conjugate diclazuril-CMO - HRP (1/20.000). No titer was obtained

neither for rabbits M 113 to M 117, nor for M 118 and M119. But the bleedings results of M 120, M 121 and M 122 showed a production of specific antibodies (*table 3)*. Several conditions were tested to observe a displacement by a standard curve but all failed (all experiments were also tested with an incubation of two hours).

b) Robenidine

Five rabbits were injected subcutaneous with robenidine-succ. - HSA, rabbits M 143 to M 147 and five others were immunized with robenidine-succ. - BTG, rabbits M 148 to M 152. The resulting antibodies have been characterized by enzyme immunoassay using robenidine-succ. - HRP (1/5.000). The two immunogens produced failed to render a detectable immune response.

- *preliminary conclusions and recommendations :* No titer was obtained with immunizations with robenidine. Antibodies against diclazuril were obtained but were unable to lead to satisfactory displacement. We will test antibodies after immunizations with new immunogens.

task A.5 : Development of enzyme linked immuno sorbent assays (nicarbazin, dimetridazole, halofuginone, diclazuril and robenidine)

- *execution period :* January 2002 – June 2004
- *current status :* in progress
- *partners responsible :* CER
- *objective :* The best conjugate and the best antibodies were selected and their dilution rate was evaluated. ELISA tests were examined according several parameters to be optimal.
- *results :*

a) ELISA for nicarbazin

Immunogens / conjugates and antibodies

Nicarbazin is an equimolar mixture of dinitrocarbanilide (DNC) and hydroxy-dimethyl pyrimidine (HDP) *(figure 4)*. Chickens excrete DNC more slowly than HDP and so it is the marker residue for detection.

The structure of DNC allows the direct conjugation through hydrazone derivatisation; however, this approach has been tried previously and shown to be unsuccessful **(1, 2)**. The immunogen used to immunize rabbits PC 21 and PC 23 was made of nitrosuccinanilic acid - cHSA (NSA). N-succinyl-L-alanyl-L-alanyl-L-alanine 4-nitroanilide - cHSA (SAN) was used to immunize rabbits M 184 to M 188. Nitrosuccinanilic acid and SAN share a common substructure with DNC and allowing an optimal mimicking of the target analyte *(figure 4)*.

Two conjugates were tested, nitrosuccinanilic acid - HRP (Nic-C-HRP) and N-succinyl-L-alanyl-L-alanyl-L- alanine 4-nitroanilide - HRP (Nic-A-HRP). The conjugates were synthesized using the same method but HRP replaced the carrier protein.

Results *(table 4)*

PC 23 antibodies were better than PC 21 antibodies because they exhibited a higher titer. The analysis of the standard curve given by the nicarbazin PC 23 antibody features a good sensitivity under a direct competitive assay configuration. Studies of the cross-reactivity profile of PC 23 confirmed 100 % reactivity to nicarbazin and insignificant reactivity to halofuginone and metronidazole.

Three different incubation times with the analyte of interest (one hour, two hours and overnight) were compared for antibody PC23 as well as three different temperatures (4 °C, room temperature and 37 °C). Temperature seemed to have no influence on the improvement of the displacement: 4 °C was kept for practical reasons. An incubation of two hours was sufficient to create an acceptable displacement.

Rabbit M 186 did not produce antibodies but the others gave good titer when antibodies were evaluated in conjunction with Nic-C-HRP. However when antibodies obtained from animals M 184 to M 188 were evaluated in conjunction with the conjugate Nic-A-HRP (indirect competitive assay), they did not lead to any displacement.

The conclusion drawn was that these antibodies work best in a heterologous assay format, which is in agreement with previous work reported by Connolly **(1)**.

Such anti-sera displayed a response with a displacement under an indirect competitive assay and antibody M 187 was found to be the most sensitive. Studies on the cross-reactivity profile of M 187 confirmed 100 % reactivity with nicarbazin and insignificant reactivity with halofuginone and metronidazole *(figure 5)*. In order to compare the direct and indirect formats of the ELISA, antibodies M 187 were also immobilized onto the surface of the wells: the direct competitive assay format gave a better sensitivity. According to these results, M 187 was slightly more sensitive in comparison to PC 23 used under the same conditions of format and incubation.

b) ELISA for halofuginone

Immunogens / conjugates and antibodies

Rabbits M 98 to M 102 were immunized with halofuginone-hemisuccinate - HSA whereas rabbits PC 38, PC 40 and PC 42 were injected with the same immunogen that have undergone a purification by thin layer chromatography. More precisely, the halofuginone immunogen was produced by protecting the hydroxyl group on the drug, enabling succinoylation to occur at -NH group. These derivatives were conjugated to HSA via the carbodiimide reaction.

The halofuginone-hemisuccinate - HRP was synthesized adopting the same strategy as for the immunogen.

Results *(table 5)*

Antibodies PC 40 presented a titer that was considered too low to envisage the development of an ELISA procedure. Antibodies PC 38 were the least sensitive while PC 42 gave interesting results. Different incubation conditions were tested with PC 42: different incubation times (one hour, two hours and overnight) and different temperatures (4 °C, room temperature and 37

°C) were compared. The optimal condition was found for a two hours incubation at 4 °C. The cross-reactivity profile was also examined for antibody PC 42. No cross reactivity was found with standards of nicarbazin and metronidazole. However standards of halofuginone led to a displacement curve with 50 % inhibition concentration equal to 0,135 ng/ml.

In the group of rabbits M 98 to M 102, all rabbits produced a specific response except rabbit M101. The four treated animals led to anti-sera giving a displacement under an indirect competitive format. Antibody M 98 was the most sensitive. Studies on the cross-reactivity profile of M 98 confirmed 100 % reactivity to halofuginone and insignificant reactivity to nicarbazin and metronidazole *(figure 6)*. The comparison of the assay configuration between the direct and indirect format was carried out with antibodies M 98 leading to better sensitivity under the direct format (antibodies directly immobilized onto the surface of the wells). According to these results, M 98 antibodies were slightly more sensitive than PC 42 antibodies used under the same conditions of format and incubation.

c) ELISA for nitroimidazoles

The nitroimidazole compounds : dimetridazole (DMZ) and ronidazole (RNZ) are metabolised to hydroxydimetridazole (DMZOH) while metronidazole (MNZ) is metabolised to hydroxymetronidazole (MNZOH) and ipronidazole (IPZ) becomes hydroxyipronidazole (IPZOH). Dimetridazole has been the most frequently used compound of the group of nitroimidazoles to treat coccidiosis. DMZ, RNZ and their common metabolite are suspected carcinogens and mutagens. Moreover, DMZ is metabolised rapidly and DMZOH is present at higher concentrations in tissues and eggs emphasizing the need for a method that detects not only DMZ but also the others nitroimidazoles **(3)**. 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 broad-spectrum of recognition.

Immunogens / conjugates and antibodies

DMZOH and MNZ were selected as haptens as they could provide sites for conjugation at two different positions of the general nitroimidazole structure *(figure 7)*. The nitroimidazoles are relatively small in size therefore a jeffamine spacer was used to extend the distance between the drug and the carrier protein. The extra length of the spacer reduces the effect of steric hindrance on conjugation and makes the hapten more distinct from the rest of the immunogen allowing the immune system of the host animal to elicit a specific response to this part of the molecule. The synthesis of such immunogens was carried out using disuccinimidyl carbonate (DSC). Animals immunized with a MNZ preparation yielded an antibody whereas none of the animals treated with DMZOH immunogens displayed a response **(4)**. Rabbits PC 100 to PC 104 and M 93 to M 97 were injected with MNZ-DSC-jeffamine - BTG and rabbits PC 107 to PC 109 were injected with MNZ-DSC-jeffamine - HSA.

For the preparation of enzyme labelled drugs, MNZ and DMZOH were conjugated to the horseradish peroxidase : either MNZ-DSC - HRP or DMZOH-pmpi - HRP (P-maleimidophenyl

isocyanate). The jeffamine spacer was excluded in the preparation of these two labels. Another conjugate was also synthesized, DMZOH-DSC-jeffamine - HRP.

Results *(table 6)*

Only the results obtained with the conjugate DMZOH-pmpi-HRP or DMZOH-DSC-jeffamine - HRP are presented in this table because the results examined with the conjugate MNZ-DSC - HRP were not satisfactory. We observed again that the heterologous format (different compounds were used to produce the antibody and peroxidase conjugate) was the best to adopt **(4)**.

No titer was obtained for antibodies PC 102, PC 103, PC 104, PC 106, PC 108 and PC 109. Antibody PC 105 was eliminated because when used in a direct format, no titer was obtained. Despite a satisfactory titer obtained for antibodies PC 100 and PC 101, no displacement (or a slight displacement) was obtained. In the group of rabbits M 93 to M 97, only M 96 produced antibodies but was not able to create a displacement (no titer was obtained with conjugate DMZOH-pmpi-HRP).

Only PC 107 antibodies showed both a high titer and an acceptable displacement with a direct assay. Studies on the cross-reactivity profile of PC 107 with standard halofuginone and standard nicarbazin showed no cross-reactivity *(figure 8)*. To improve the sensitivity of PC 107, indirect competitive assay was examined but the displacement was too weak to lead to an ID 50. The comparison of ID 50 values and cross-reactivity profiles of the metronidazole PC 107 with a range of nitroimidazoles is presented in *table 7*.

PC 107 antibody displayed a cross reactivity profile that was able to bind a range of nitroimidazoles but the antibody recognized with difficulties the metabolites IPZOH and MNZOH. The use of ipronidazole (even prior to the EC ban) is very limited. Moreover, the presence or absence of a single hydroxyl group was observed to influence the antibody binding. On the other hand, the conjugation of the hapten to the carrier protein is a critical step and greatly influences the quality of the resulting antibodies. 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 **(5)**.

d) Multiresidues ELISA

This part of the report is a discussion on the feasibility of multiresidue ELISA aimed at reducing the number of tests by sample and consequently leading to a considerable gain of time. The ideal system of detection for the five coccidiostats is to immobilize the five specific antibodies onto the surface onto one well. The sample is to be added before the addition of mixed conjugates (five specific conjugates for each coccidiostat).

This scheme has been tested with coating into one well a solution containing PC 23 (nicarbazin - 1/20.000) + PC 42 (halofuginone - 1/15.000) + PC 107 (metronidazole - 1/10.000). The results, for an incubation of two hours at 4 °C, are presented in *table 8*. The analysis of these results indicates that the system using three antibodies per well in

conjunction with three mixed conjugates is not promising. Indeed, zero standard values (B0) were too high (O.D. higher than 2) and NSB values are also higher than those obtained when conjugates are used individually (additive NSB effect). Moreover, displacements in direct competitive assay have completely disappeared. Because of these problems, it seemed difficult to forecast the scheme for five antibodies per well with five mixed conjugates. On the other hand, three antibodies immobilized onto one well with conjugate added separately gave more encouraging results: displacement remains and similar sensitivity to single antibody configuration was obtained but a problem remains : B0 values were significantly lower.

- *preliminary conclusions and recommendations :* We have successfully developed three ELISAs allowing the detection of halofuginone, nicarbazin and nitroimidazoles. *Table 9* summarizes the conditions established for these three coccidiostats ELISAs and their respective sensitivity. Two ELISA configurations were used in this project. The direct competitive assays where specific antibodies are coated onto the surface of wells seemed to be more sensitive. Experiments to reach a multi-residue ELISA were initiated but must be further developed. It was however demonstrated that the use of five antibodies per well in conjunction with a mixture of five conjugates was impossible since no displacement occurred.

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

- *execution period :* November 2002 – June 2004
- *current status :* NOT YET STARTED

task A.7 : Development of immunoaffinity columns

- *execution period :* January 2003 – June 2004
- *current status :* NOT YET STARTED

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

task B.1 : literature study

- *execution period* : November 2001 – December 2003
- *current status* : in progress
- *partners 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 is updated on a regular base.
- *preliminary conclusions and recommendations* : 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

- *execution period* : December 2001
- *current status* : completed
- *partners 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, will be examined.
- *results* : An internal standard should react in the same way as the compound during analysis. Therefore, a deuterated analogue is very suitable as internal standard. But when those are not available, a compound with a similar molecular structure and belonging to the same compound group can also be used as internal standard. Only for two compounds a deuterated analogue was available. Dimetridazole-d_3 could be bought at RIVM (Bilthoven, The Netherlands). For nicarbazine not a deuterated nicarbazine but a deuterated dinitrocarbanilide could be found. Nicarbazine is the generic name of the equimolar complex of 4,4'-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP). When chickens are given nicarbazine in the feed, the HDP moiety is absorbed and excreted more rapidly than the DNC moiety and consequently most residue analyses for nicarbazin are based on methods of the DNC moiety. Therefore, we started by focussing only on the DNC compound. Dinitrocarbanilide-d_8 was gathered from the Veterinary Science Division of the Queen's University of Belfast. Since we couldn't acquire a deuterated analogue of the other compounds, a compound with similar structure was searched for. For diclazuril, two different internal standards from Janssen Animal Health were received. For halofuginone, which is a quinazolinone, a compound belonging to the same product group was bought at Sigma-Aldrich. For robenidine that belongs to the guinanidine derivates, a compound with analogue structure was bought at Sigma-Aldrich. In *figure 9* the molecular structures of the compounds and their internal standards are given. For each compound a suitable solvent had to be found. Then a stock solution of each standard of 1mg/ml was made and stored at 4°C. In *table 10* an overview is given of the standard, internal standards and their solvent.

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

task B.3 : optimisation of the mass spectrometric conditions

- *execution period* : January 2002 – June 2002
- *current status* : completed
- *partners 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 optimisation of the mass spectrometric conditions was done by tuning standard solutions that are brought directly into the mass spectrometer by a syringe pump. Starting from the stock solutions, a dilution of 1ng/µl was made. Different solvents were tested and for all compounds the best tuning results were obtained with the solution in acetonitrile-water (50/50, v/v) containing 0.1% formic acid. Tuning experiments were performed in electrospray as well as in APCI (atmospheric pressure chemical ionisation) both in positive and negative mode. The best results were obtained in negative electrospray mode for diclazuril and its two internal standards and for the DNC compound and his deuterated analogue and in positive electrospray mode for dimetridazole, dimetridazole-d3, halofuginone and its internal standard and robenidine and its internal standard. Only for de HDP compound of nicarbazin, tuning results were best in the APCI ionisation mode. One of the objectives of this task was to find out whether it is possible to develop a multi-residue method i.e. all compounds can be determined in one run. Given that all compounds but HDP gave the best results in the electrospray ionisation mode and that we only have to focus on the DNC moiety of nicarbazin makes that possible. For each compound the $(M+H)^+$ or $(M-H)^-$ ion was determined and dissociation of this precursor ion with argon was induced. This is called Collision Induced Dissociation (CID). Cone voltage and collision energy were tuned to optimise the transition of the precursor ion into the most abundant product ions. Only for diclazuril, only one product ion could be generated. According to Commission Decision 2002/657/EC, a minimum of 4 identification points is required for forbidden substances. For LC-MS-MS, in which the transition of 1 precursor ion into two product ions is followed, 4 identification points are earned. This implies that we can fulfil this requirement for dimetridazole, halofuginone, dinitrocarbanilide and robenidine but not for diclazuril. This problem was solved by looking at the molecular composition of diclazuril. Chlorine has two natural isotopes (^{35}Cl and ^{37}Cl) and the diclazuril molecule contains three chlorine atoms. The ions at m/z 411, 409, 407 and 405 in the negative MS mode for diclazuril represents the chlorine isotopes, i.e. the $^{37}Cl_3$, $^{35}Cl^{37}Cl_2$, $^{35}Cl_2{}^{37}Cl$ and $^{35}Cl_3$ versions of diclazuril as a deprotonated molecule (nominal mass = 407.64), respectively. Product ions in the MS-MS-mode of m/z 411, 409, 407 and 405 are 340, 338, 336 and 334, respectively. According to the

natural occurrence of the different isotopes, diclazuril contains 1.4% $^{37}Cl_3$, 13.3% $^{35}Cl^{37}Cl_2$, 41.7% $^{35}Cl_2{}^{37}Cl$ and 43.5% $^{35}Cl_3$. In the MS spectrum those 4 fractions could be seen *(figure 10)*. Since the ions at m/z 407 and 405 have about the same abundance, both transitions 407<336 and 405<334 can be used. In this way 4 identification points can be earned for diclazuril as well.

The MS parameters where almost all precursor ions are converted to product ions are shown in *table 11*.

Also the specificity of the MS response was tested. Standard solutions of 1ng/µl of the individual compounds were analysed on the presence of the other compounds. No interference between the 5 compounds could be detected. *Figure 11* shows the injection of the diclazuril standard : none of the other compounds generated a signal.

- *preliminary conclusions and recommendations* : The mass spectrometric conditions were optimised for al 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. The possibility of an multi-residue method is still open since all compounds can be detected in the electrospray ionisation mode.

task B.4 : optimisation of the chromatography

- *execution period* : July 2002 – September 2002
- *current status* : completed
- *partners 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. This is done by the use of standard solutions.
- *results* : To begin with, the liquid chromatographic conditions that are used in the laboratory for other analyses were tested *(table 12)*. These conditions resulted in a chromatogram that is shown in *figure 12*. Complete chromatographic separation between dimetridazole and halofuginone was not accomplished but this is not necessary due to the very specific detection with multiple reaction monitoring. Also a detailed literature study on chromatographic conditions was performed. On the base of that literature research and own experience, 17 different chromatographic conditions were tested. All these tests were performed on the same C_{18} column and precolumn. A list of all the tested conditions is given in *table 13* . A lot of the tested conditions were suitable for one or sometimes two compounds. But since it was the aim to develop a multi-residue method, only these conditions were taken in further consideration that gave good results for all the compounds. The method described by Croubels et al. (Rapid Commun. Mass Spectrom. 2002; 16: 1463-1469) seemed to be the most appropriate for further optimisation. The solvent gradient, the concentration of NH_4OAc, the injection volume and the

column temperature had to be further optimised. In *figure 13a* the chromatogram is shown that was obtained when the gradient described in the article was applied. Since peak shape and separation are not ideal, the gradient applied was further optimised. *Figure 13b* shows the chromatogram that is acquired when the optimised gradient is applied. The optimisation resulted in bigger peaks, a better separation and a better peak shape. Since methanol is less harmful than acetonitrile to work with, a chromatogram was recorded with methanol in stead of acetonitrile as eluent B. Peaks were smaller and for robenidine a bad peak cheap could be observed so no further experiments were executed with methanol.

Also the concentration of ammoniumacetate that was added to both eluents to improve the ionisation was optimised. Since the testing of different chromatographic conditions is very time consuming, it was decided to test the influence of the ammoniumacetate concentration by tuning standard solutions. This was done for dimetridazole and dinitrocarbanilide. For dimetridazole 0.01M NH_4OAc and for dinitrocarbanilide 0.005M NH_4OAc seemed to be the optimum concentration. To check whether these results could be transferred to the influence of the ammoniumacetate concentration on the chromatographic results, a chromatogram was recorded with 0.01M NH_4OAc in the mobile phase and one with 0.1M NH_4OAc in the mobile phase. A concentration of 0.01M NH_4OAc gave the best results for all compounds. Also the injection volume has to be optimised. This will be done when the clean-up of the egg matrix is fully optimised since it can be expected that the matrix will play an important role : when a bigger volume is injected it is to be expected that not only the signal will increase but also the noise. The column temperature has to be optimised as well. This will be done when we have a new LC-MS equipment that is expected in the spring of 2003 available.

- *preliminary conclusions and recommendations* : At the moment there are 2 different liquid chromatographic conditions that are suitable for a multi-residue analysis of the 5 coccidiostats. Since the matrix can play an important roll when egg samples are analysed, a definitive choice between both methods will be made in a further stage of the project.

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

- *execution period* : July 2002 – June 2003
- *current status* : in progress
- *partners 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* : As with the liquid chromatographic conditions, we started by testing a clean-up procedure that was already used in the laboratory for the determination of nitroimidazoles in

milk. After mixing the egg with an ultra-turrax, 10 g homogenized egg was weighed in a centrifuge tube. At this stage, internal standard was added and samples were spiked if necessary by adding an appropriate amount of standard in water solution. The sample was vortex mixed and allowed to stand for 10 min. Then 10 ml of acetonitrile was added and the sample was vortex mixed for 1 min and placed in an ultrasonic bath for 5 min. The sample then was centrifuged during 10 min at 3000 rpm. The supernatant was transferred into a graduated tube and was concentrated to a volume of 4 ml under nitrogen in a water bath at 60°C. After filtration through a 0.22 µm filter, 40 µl of the remaining extract was injected into the LC-MS system. Since the Belgian government asked to analyse already egg samples for a monitoring program, these clean-up procedure was fully validated according to Commission Decision 2002/657/EC. Results of this validation study are presented in the publication "Simultaneous detection of five coccidiostats in eggs by liquid-chromatography – tandem mass spectrometry" (Analytica Chimica Acta, accepted for publication).

Besides this 'ACN-extraction', a number of other sample preparation methods were already tested : a trichloric acid-precipitation followed by solid phase extraction (SPE) with a C_{18} column, an extraction with acetonitrile and hexane and an extraction with acetonitrile followed by SPE with on cation exchange column. Further testing and optimisation is still in progress. Also the use of immunoaffinity columns will be fully investigated.

- *preliminary conclusions and recommendations* : We already developed a clean-up procedure that is fully validated and that can be used for routine analysis of egg samples. But based on literature, a number of other procedures will be tested and if necessary optimised. Results will be compared to the validated method to decide which one is to be preferred for further use.

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

Although this task hasn't been started yet, we found out that 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.

- *execution period* : July 2003 – September 2004
- *current status* : NOT YET STARTED

4.4. TASK D : APPLICABILITY OF THE METHODS FOR INCURRED SAMPLES

- *execution period* : January 2004 – March 2005
- *current status* : NOT YET STARTED

4.5. TASK E : MONITORING OF COMMERCIALLY AVAILABLE EGGS

- *execution period* : January 2005 – May 2005
- *current status* : NOT YET STARTED

5. Future prospects and planning

At CER the following points will be considered : (a) the development of enzyme linked immunosorbent assays for diclazuril and robenidine needs to be carried out. For this task, new immunogens must be synthesized and then injected to rabbits to obtain polyclonal antibodies which will be tested with new conjugates, (b) the test involving all antibodies into one well for practical reasons must be improved, (c) the clean-up of egg matrices especially for ELISA procedures leading to a rapid and simple method with a high recovery will be optimised, (d) immunoaffinity columns must be developed so a specific and selective purification of samples before the LC-MS detection can be obtained. First the different gels will be tested separately and then they will be mixed to lead to a multiresidue immunoaffinity column and (e) the knowledge concerning the immunological tests for the detection of coccidiostats must be actualised.

In July 2003, the validation of the methods according to Commission Decision 2002/657/EC will be started.

According to the time schedule, DVK will go on with task B.4. When we have decided on the clean-up procedure, the method will be validated according to Commission Decision 2002/657/EC. In the spring of 2003, a new LC-MS instrument will arrive at DVK. With this new machine, more sensitivity can be expected. In the mean time, monitoring of egg samples is performed on demand by FAVV with the validated method.

6. Annexes

6.1. References

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6.2. Publications

- *Simultaneous detection of five coccidiostats in eggs by liquid-chromatography – tandem mass spectrometry*
  Leen Mortier, Els Daeseleire and Philippe Delahaut
  Analytica Chimica Acta, accepted for publication

- *Simultaneous detection of five coccidiostats in eggs by liquid-chromatography – tandem mass spectrometry*
  Leen Mortier, Els Daeseleire and Philippe Delahaut
  4th International symposium on hormone and veterinary drug residue analysis (June 4-7, 2002, Antwerp, Belgium), abstract book, p3

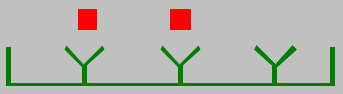
6.3. Detailed results

6.3.1) figures

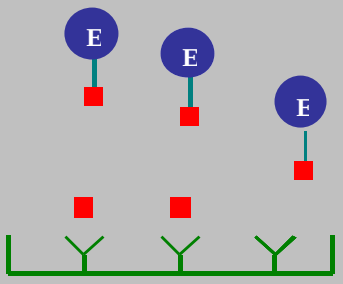
figure 1: schematic representation of the competitive ELISA used in our studies

1. Adsorb specific antibodies produced by hyper-immunization of rabbit to a solid phase.

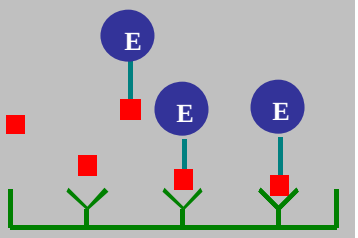
2.a) Add sample or standard.



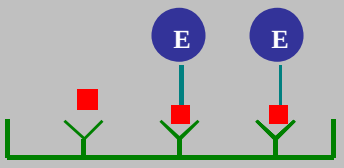
b) Add enzyme-labelled antigen.



3. Two hours incubation at 4 °C.



4. Remove the excess of enzyme.



5. Add TMB/H_2O_2 – 30 min.
6. Stop the reaction with H_2SO_4.
7. Read at 450 nm.

figure 2 : schematic presentation of MRM principle

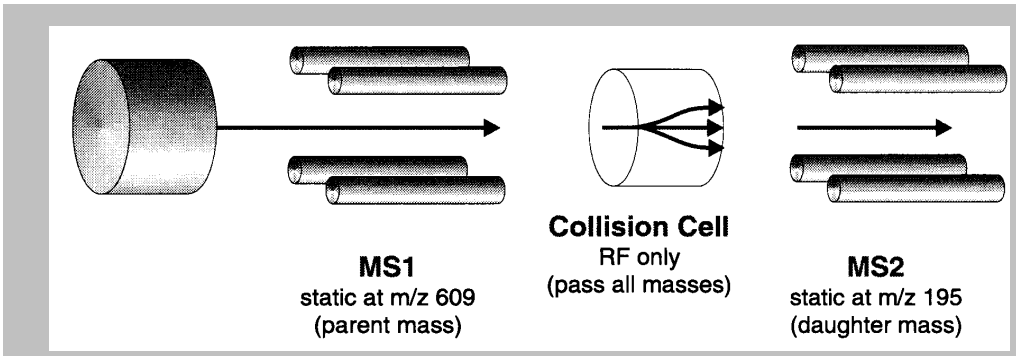


figure 3 : chemical structures of robenidine and 1-(2,6-dichlororbenzildeneamino)-guanidine

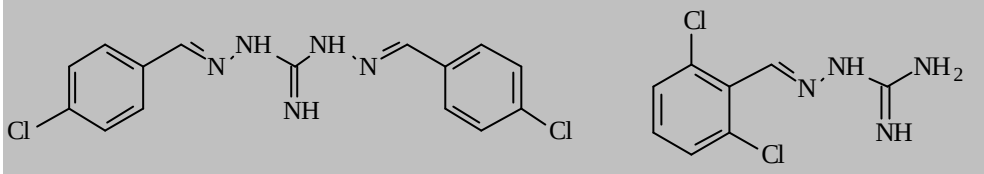


figure 4 : chemical structures of nicarbazin (DNC + HDP) and two mimics of DNC, SAN and NSA

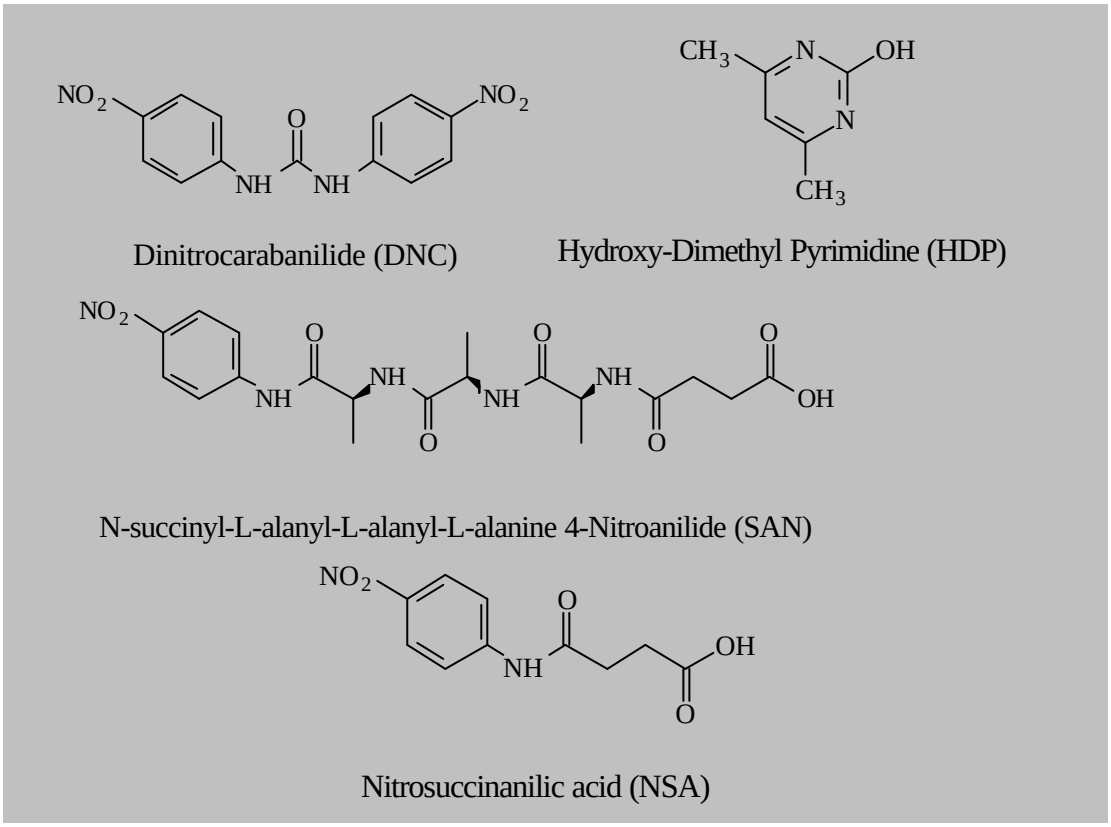


figure 5 : standard curve analysis of the nicarbazin M 187 polyclonal and cross reactivity studies with these antibodies (indirect competitive assay)

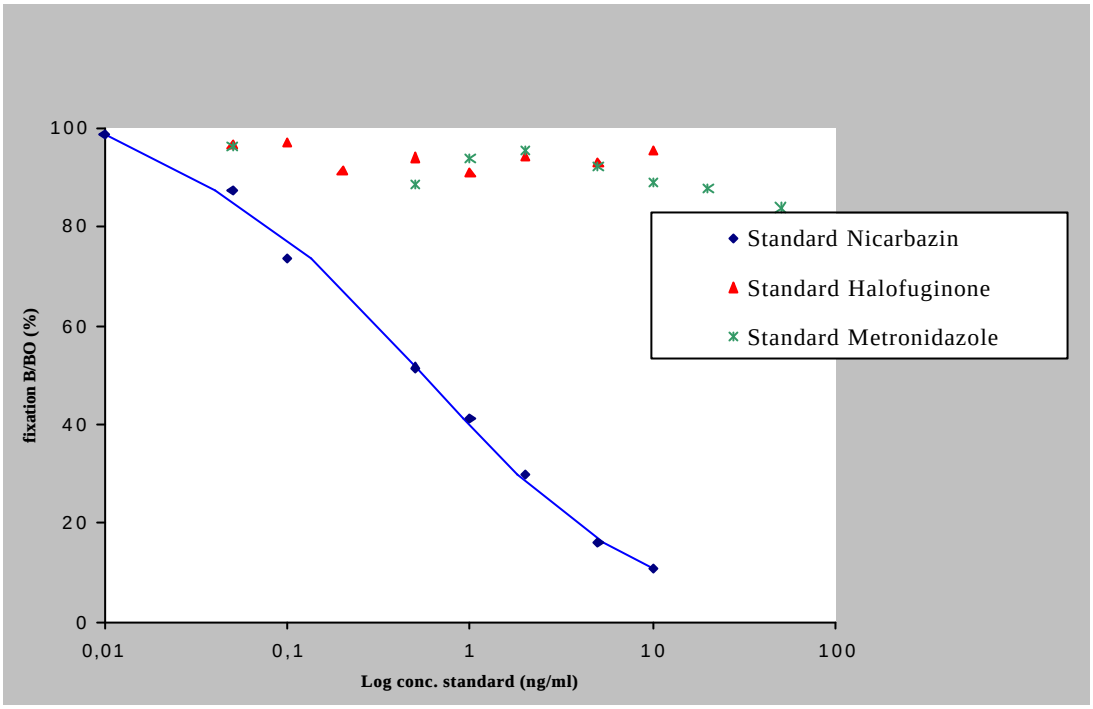


figure 6 : standard curve analysis of the halofuginone M 98 polyclonal and cross reactivity studies with these antibodies (indirect competitive assay)

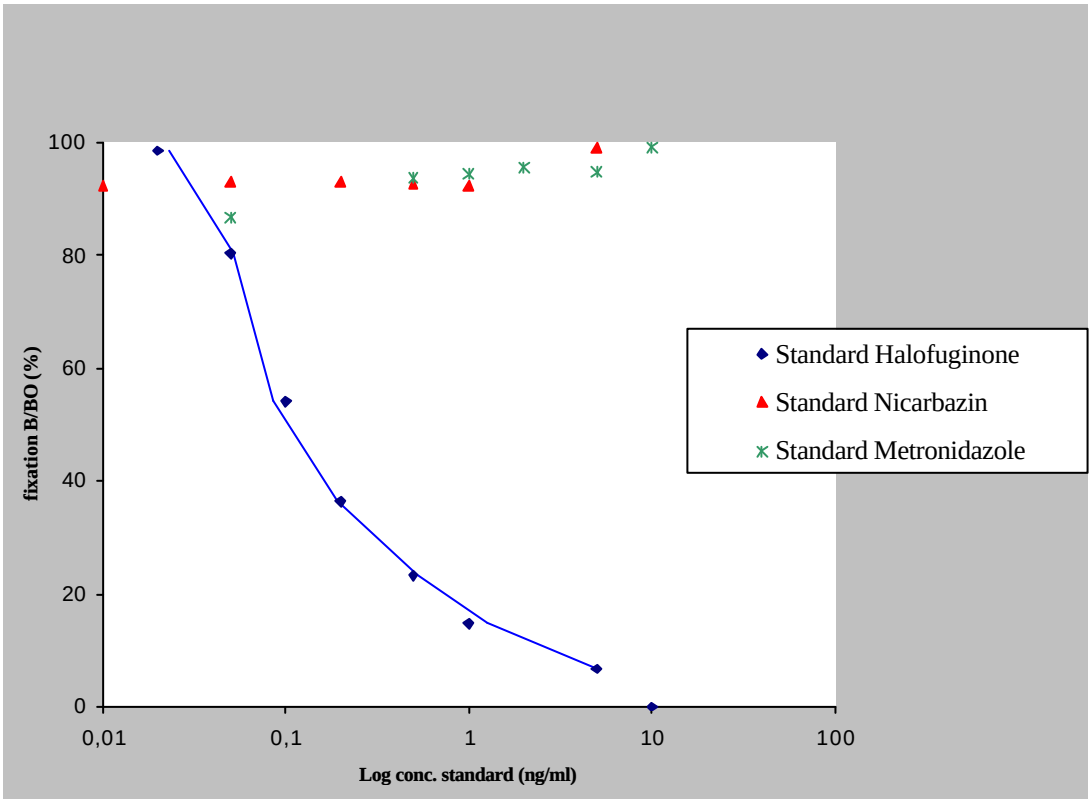


figure 7 : chemical structures of metronidazole and hydroxydimetridazole which are susceptible to serve as hapten for the synthesis of immunogens

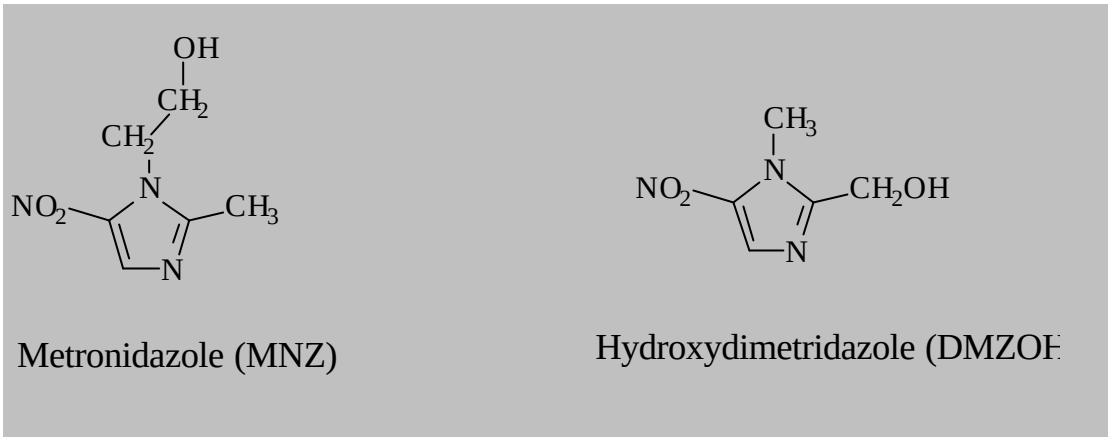


figure 8 : standard curve analysis of the metronidazole PC 107 polyclonal and cross reactivity studies with these antibodies

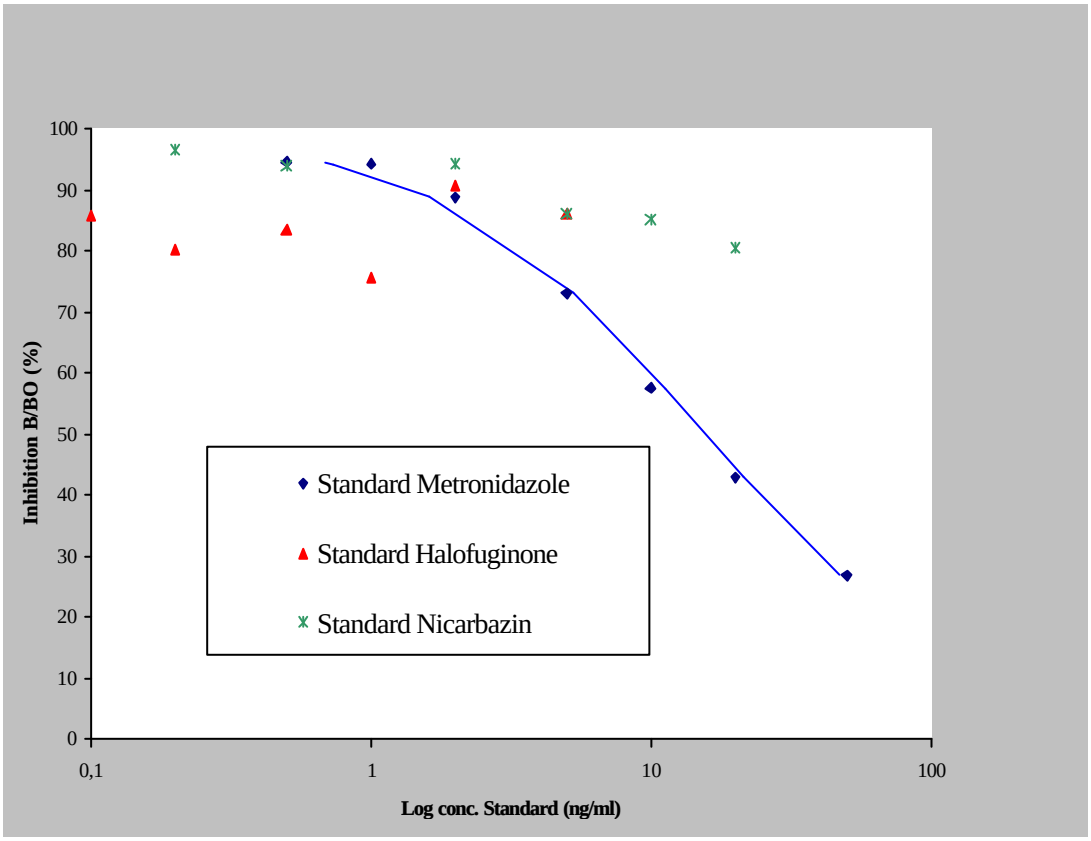


figure 9 : molecular structures of the compounds and internal standards

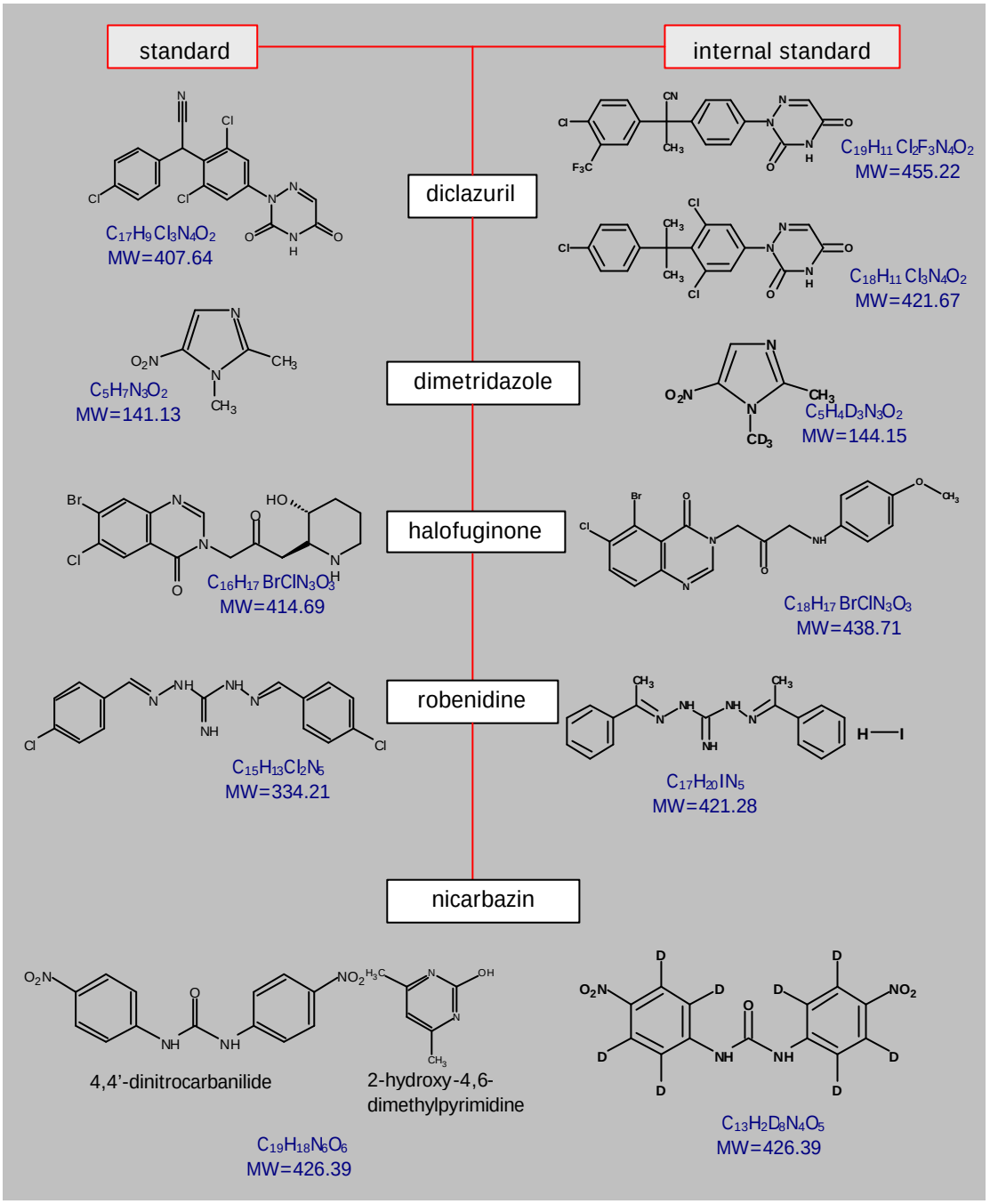
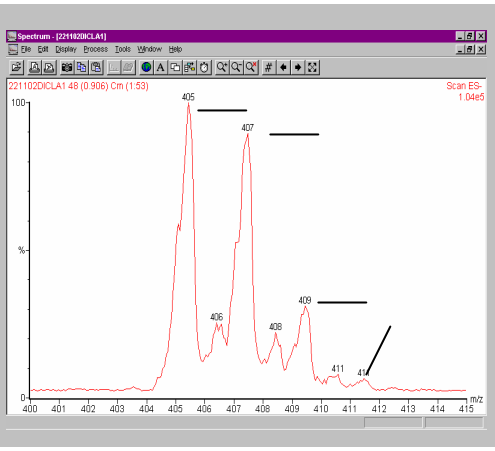
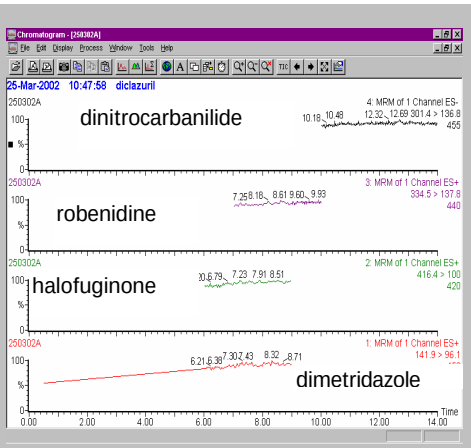


figure 10 : MS-spectrum of diclazuril



ions at m/z 411, 409, 407 and 405 in the negative mode for diclazuril

figure 11 : specificity of the MS – response



when a diclazuril standard is injected, no signal is obtained for the other compounds

figure 12 : chromatogram acquired with 'ACN-extraction' and 'laboratory' LC method

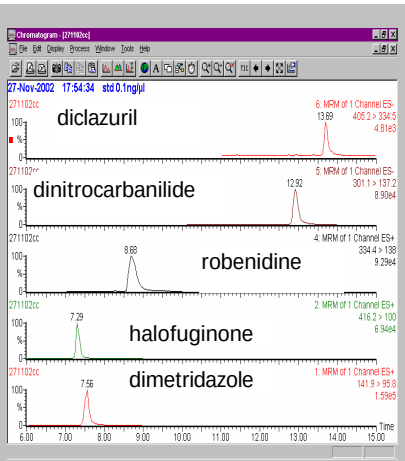


figure 13 : chromatograms acquired with LC conditions from Croubels et al.

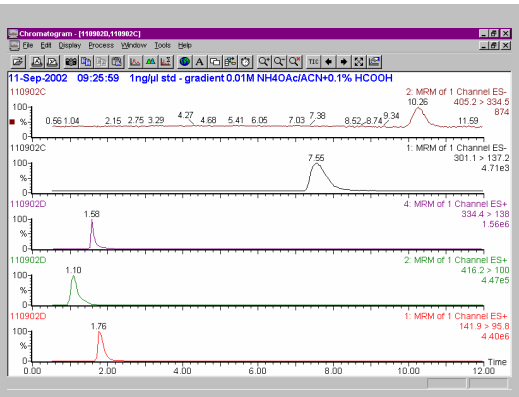


figure 13a : gradient from article

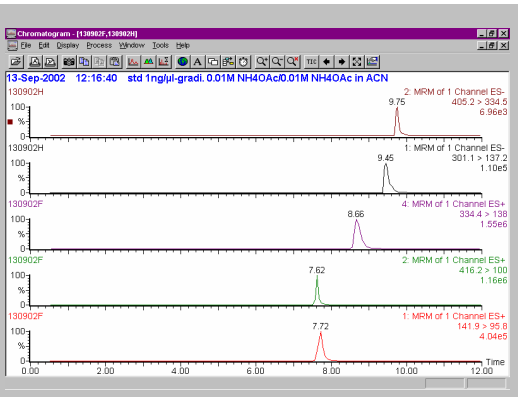


figure 13b : optimised gradient

6.3.2) tables

table 1 : task schedule

task	partner responsible		year 1 : 15 Nov. 2001 – 14 Nov. 2002	year 2 : 15 Nov. 2002 – 14 Nov. 2003	year 3 : 15 Nov.2003 – 14 Nov. 2004	year 4 : 15 Nov. 2004 – 14 May 2005
A : Development immunological screening methods	CER	A.1	November 2001 – December 2003			
		A.2	November 2001 – June 2002			
		A.3	January 2002 – December 2002			
		A.4	January 2002 – December 2003			
		A.5	January 2002 – June 2004			
		A.6		November 2002 – June 2004		
		A.7		January 2003 – June 2004		
B : Development LC-MS confirmation methods	DVK	B.1	Nov. 2001 – December 2003			
		B.2	December 2001			
		B.3	January 2002 – June 2002			
		B.4	January 2002 – September 2002			
		B.5	July 2002 – June 2003			
C : Validation	CER	C.1		July 2003 – September 2004		
	DVK	C.2		July 2003 – September 2004		
D : Applicability	DVV + DVK	D.1			January 2004 – September 2004	
	DVK + CER	D.2			September 2004 – March 2005	
E : Monitoring	DVK + CER	E.1				January 2005 – May 2005
	CER	E.2				January 2005 – May 2005
	DVK	E.3				January 2005 – May 2005

table 2 : overview of standards

COCCIDIOSTATS	STANDARDS	ORIGIN
Halofuginone	Halofuginone	Roussel-UCLAF
Nicarbazin	Nicarbazin	Sigma-Aldrich
	Dinitrocarbanilide	Veterinary Sc.Division, Belfast (UK)
Nitroimidazoles	Dimetridazole	Sigma-Aldrich
	Hydroxydimetridazole	Veterinary Sc.Division, Belfast (UK)
	Ipronidazole	B.g.V.V., Berlin (D)
	Hydroxyipronidazole	B.g.V.V., Berlin (D)
	Ronidazole	Veterinary Sc.Division, Belfast (UK)
	Metronidazole	Veterinary Sc.Division, Belfast (UK)
	Hydroxymetronidazole	B.g.V.V., Berlin (D)
Diclazuril	Diclazuril (R064433)	Janssen Research
Robenidine	Robenidine	Alpharma Animal Health Division

table 3 : summary of results obtained with different antibodies raised against diclazuril

ANTIBODIES	DILUTION RATE ANTIBODIES	CONJUGATE	DILUTION RATE CONJUGATE	ID 50 (NG/ML)	FORMAT	INCUBATION
M 120	1/8.000	diclazuril-CMO - HRP	1/40.000		Indirect	overnight 4°C
	1/16.000	diclazuril-CMO - HRP	1/20.000		Direct	overnight 4°C
M 121	1/16.000	diclazuril-CMO - HRP	1/40.000		Indirect	overnight 4°C
	1/16.000	diclazuril-CMO - HRP	1/40.000		Direct	overnight 4°C
M 122	1/16.000	diclazuril-CMO - HRP	1/40.000		Indirect	overnight 4°C
	1/8.000	diclazuril-CMO - HRP	1/40.000		Direct	overnight 4°C

table 4 : summary of results obtained with different antibodies raised against nicarbazin

ANTIBODIES	DILUTION RATE ANTIBODIES	CONJUGATE	DILUTION RATE CONJUGATE	ID 50 (NG/ML)	FORMAT	INCUBATION
PC 21	1/4.000	Nic-C-HRP	1/8.000		Indirect	2 hours 4°C
PC 23	1/16.000	Nic-C-HRP	1/8.000		Indirect	2 hours 4°C
	1/20.000	Nic-C-HRP	1/9.000	0,39	Direct	2 hours 4°C
M 184	1/20.000	Nic-C-HRP	1/4.000	0,87	Indirect	2 hours 4°C
M 185	1/30.000	Nic-C-HRP	1/3.000	2,8	Indirect	2 hours 4°C
M 186	_	Nic-C-HRP	1/4.000		Indirect	2 hours 4°C
M 187	1/5.000	Nic-C-HRP	1/5.000	0,52	Indirect	2 hours 4°C
	1/16.000	Nic-C-HRP	1/8.000	0,28	Direct	2 hours 4°C
M 188	1/10.000	Nic-C-HRP	1/3.000	6,05	Indirect	2 hours 4°C

table 5 : summary of results obtained with different antibodies raised against halofuginone

ANTIBODIES	DILUTION RATE ANTIBODIES	CONJUGATE	DILUTION RATE CONJUGATE	ID 50 (NG/ML)	FORMAT	INCUBATION
PC 38	1/30.000	Halofuginone succ. -HRP	1/3.750	1,99	Direct	overnight 4°C
PC 40	1/500	Halofuginone succ. -HRP	1/3.000		Direct	2 hours 4°C
PC 42	1/15.000	Halofuginone succ. -HRP	1/20.000	0,135	Direct	2 hours 4°C
M 98	1/32.000	Halofuginone succ. -HRP	1/32.000	0,21	Indirect	2 hours 4°C
	1/16.000	Halofuginone succ. -HRP	1/60.000	0,11	Direct	2 hours 4°C
M 99	1/32.000	Halofuginone succ. -HRP	1/20.000	0,724	Indirect	2 hours 4°C
M 100	1/20.000	Halofuginone succ. -HRP	1/20.000	0,483	Indirect	2 hours 4°C
M 101	_	Halofuginone succ. -HRP	1/4.000		Indirect	2 hours 4°C
M 102	1/32.000	Halofuginone succ. -HRP	1/32.000	0,441	Indirect	2 hours 4°C

table 6 : summary of results obtained with different antibodies raised against nitroimidazole

ANTIBODIES	DILUTION RATE ANTIBODIES	CONJUGATE	DILUTION RATE CONJUGATE	ID 50 (NG/ML)	FORMAT	INCUBATION
PC 100	1/32.000 1/10.000	DMZOH-pmpi-HRP DMZOH-pmpi-HRP	1/16.000 1/16.000		Indirect Direct	overnight 4°C 2 hours 4°C
PC 101	1/16.000 1/10.000	DMZOH-pmpi-HRP DMZOH-pmpi-HRP	1/8.000 1/8.000		Indirect Direct	overnight 4°C 2 hours 4°C
PC 102	_	DMZOH-pmpi-HRP	1/1.000 to 1/128.000		Indirect	overnight 4°C
PC 103	_	DMZOH-pmpi-HRP	1/1.000 to 1/128.000		Indirect	overnight 4°C
PC 104	_	DMZOH-pmpi-HRP	1/1.000 to 1/128.000		Indirect	overnight 4°C
PC 105	1/8.000 _	DMZOH-pmpi-HRP DMZOH-pmpi-HRP	1/8.000 1/1.000 to 1/128.000		Indirect Direct	overnight 4°C overnight 4°C
PC 106	_	DMZOH-pmpi-HRP	1/1.000 to 1/128.000		Indirect	overnight 4°C
PC 107	1/10.000 1/8.000	DMZOH-pmpi-HRP DMZOH-pmpi-HRP	1/65.000 1/24.000	14,95 (MNZ)	Direct Indirect	2 hours 4°C 2 hours 4°C
PC 108	_	DMZOH-pmpi-HRP	1/1.000 to 1/128.000		Indirect	overnight 4°C
PC 109	_	DMZOH-pmpi-HRP	1/1.000 to 1/128.000		Indirect	overnight 4°C
M 96	1/8.000 1/12.000	DMZOH-DSC-JEFF - HRP DMZOH-DSC-JEFF - HRP	1/6.000 1/6.000		Direct Indirect	2 hours / ON 4°C overnight 4°C

table 7 : comparison of ID 50 values and cross-reactivity profiles of the metronidazole PC 107 with a range of nitroimidazoles

	MNZ	MNZOH	RNZ	DMZ	DMZOH	IPZ	IPZOH
% cross-reactivity	100	10,2	55,3	400,7	81,8	61,8	4,7
ID 50 (ng/ml)	14	137,5	25,3	3,5	17,1	22,7	297

table 8 : summary of results obtained with the coating of three antibodies in the same well, using conjugates either individually or mixed

STANDARDS	CONJUGATES	ID 50 FOR NICARBAZIN	ID 50 FOR HALOFUGINONE	ID 50 FOR MNZ
Nicarbazin	Nic-C - HRP 1/9.000	0.23 ng/ml	no displacement	no displacement
	Mix	no displacement - Bo / NSB too high	no displacement - Bo / NSB too high	no displacement - Bo / NSB too high
Halofuginone	Halof.-suc.-HRP 1/20.000	no displacement	0.1 ng/ml	no displacement
	Mix	no displacement - Bo / NSB too high	no displacement - Bo / NSB too high	no displacement - Bo / NSB too high
Metronidazole	DMZOH-pmpi-HRP 1/65.000	no displacement	no displacement	14 ng/m
	Mix	no displacement - Bo / NSB too high	no displacement - Bo / NSB too high	no displacement - Bo / NSB too high

table 9 : summary of immunoassay developed for Halofuginone, Nicarbazin and Nitroimidazole with their best conditions

ELISA	ANTIBODIES	DILUTION RATE ANTIBODIES	CONJUGATE	DILUTION RATE CONJUGATE	ID 50 (NG/ML)	FORMAT
Nicarbazin	PC 23	1/20.000	Nic-C - HRP	1/9.000	0,39	Direct
	M 187	1/16.000	Nic-C - HRP	1/8.000	0,28	Direct
Halofuginone	PC 42	1/15.000	Halof. succ. - HRP	1/20.000	0,135	Direct
	M 98	1/16.000	Halof. succ. - HRP	1/60.000	0,11	Direct
Nitroimidazole	PC 107	1/10.000	DMZOH-pmpi-HRP	1/65.000	14,95 (MNZ)	Direct

table 10 : overview of standards and internal standards

COMPOUND	STANDARD		INTERNAL STANDARD		SOLVENT
	compound	supplier	compound	supplier	
diclazuril	diclazuril	gift from Janssen Animal Health, Belgium	R0062646 ($C_{18}H_{11}Cl_3N_4O_2$)	gift from Janssen Animal Health, Belgium	dimethylformamide (DMF)
			R062370 ($C_{19}H_{11}Cl_2F_3N_4O_2$)	gift from Janssen Animal Health, Belgium	
dimetridazole	dimetridazole	bought at Sigma-Aldrich, Belgium	dimetridazole-d3	bought at RIVM, Bilthoven, the Netherlands	acetonitrile/water : 50/50
halofuginone	halofuginone hydrobromide	gift from Intervet, Belgium	R74717-3 ($C_{18}H_{17}BrClN_3O_3$)	bought at Sigma-Aldrich, Belgium	water
nicarbazine / dinitro-carbanilide	dinitro-carbanilide	bought at Sigma-Aldrich, Belgium	dinitrocarbanilide-d8	gift from Veterinary Science Division, Queen's University, Belfast, Ireland	dimethylsulfoxide (DMSO)
robenidine	robenidine hydrochloride	gift from Alpharma, USA	S3122-8 ($C_{17}H_{20}IN_5$)	bought at Sigma-Aldrich, Belgium	ethanol

table 11 : overview of the MS parameters

COMPOUND		IONISATION MODE	M/Z PRECURSOR ION	CONE VOLTAGE (V)	M/Z PRODUCT IONS	COLLISION ENERGY (EV)
diclazuril	standard	ES -	405.4 407.5	30	334.3* 335.9	17 17
	I.S. 1	ES -	453.3	28	381.9 355.3*	15 25
	I.S. 2	ES -	419.3	25	320.9*	25
dimetridazole	standard	ES +	141.9	25	96.1* 81.1 53.9	15 27 40
	I.S.	ES +	145.0	25	98.9* 58.7 115.2	17 15 15
halofuginone	standard	ES +	416.4	23	100.0* 137.9 119.8	22 20 17
	I.S.	ES +	424.3	20	317.4*	15
dinitrocarbanilide	standard	ES -	301.4	22	136.8* 107.0 45.8	10 40 70
	I.S.	ES -	309.3	10	140.9*	20
robenidine	standard	ES +	334.5	25	137.8* 129.0 110.9	25 40 45
	I.S.	ES +	294.3	25	77.0 95.0 117.8*	40 35 20

* : most abundant production

table 12 : chromatographic conditions

	'LABORATORY' METHOD	METHOD CROUBELS ET AL.	OPTIMISED METHOD CROUBELS ET AL.
column	Waters C18, 5µm, 150mmx2.1mm i.d.		
precolumn	C18		
eluent A	H_2O + 0.1% HCOOH	H_2O + 0.1M NH_4OAc	H_2O + 0.01M NH_4OAc
eluent B	ACN + 0.1% HCOOH	ACN + 0.1M NH_4OAc	ACN + 0.01M NH_4OAc
column temperature	room temperature		
flow rate	0.25ml.min^{-1}		
injection volume	40µl		
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	0'-7' : 50/50 A/B 7.5'-12' : 10/90 A/B 12.5'-17' : 50/50 A/B	0'-0.5' : 100/0 A/B 1'-3' : 50/50 A/B 3.2'-9.5' : 0/100 A/B 7.5'-12' : 10/90 A/B 10'-15' : 100/0 A/B

table 13 : list of the tested liquid chromatographic conditions

1. H_2O/MEOH 25/75, isocratic	10. H_2O(+0.1M NH_4OAC)/ ACN(+0.1%HCOOH), gradient
2. H_2O/MeOH 35/65, isocratic	11. H_2O(+0.1M NH_4OAc)/ ACN(+0.1M NH_4OAc), gradient
3. H_2O/MeOH 50/50, isocratic	12. H_2O/MeOH (+0.05M NH_4OAc)75/25, isocratic
4. H_2O/MeOH 30/70, isocratic	13. H_2O/ACN (+0.05M NH_4OAc)25/75, isocratic
5. H_2O/MeOH 25/75, gradient	14. H_2O/MeOH (+0.05M NH_4OAc)25/75, isocratic
6. H_2O/ACN (+0.1% HCOOH) 50/50, isocratic	15. H_2O(+0.5% NH_4OAc)/ACN/MeOH, gradient
7. H_2O/ACN (+0.1% HCOOH) 30/70, isocratic	16. ACN/H_2O (+0.25M NH_4OAc)/H2O 5/3/12, isocratic
8. H_2O/ACN (+0.05M NH_4OAc) 25/75, isocratic	17. H_2O(pH4.3)/ACN(+0.1M NH_4OAc) 30/70, isocratic
9. H_2O(+0.1M NH_4OAc)/MeOH 28/72, isocratic	