

## **Cross-contamination of non-target feedingstuffs by nicarbazin authorised for use as a feed additive<sup>1</sup>**

### **Scientific opinion of the Panel on Contaminants in the Food Chain**

**Question N° EFSA-Q-2005-220K**

**Adopted on 9 April 2008**

#### **PANEL MEMBERS**

Jan Alexander, Guðjón Atli Auðunsson, Diane Benford, Andrew Cockburn, Jean-Pierre Cravedi, Eugenia Dogliotti, Alessandro Di Domenico, Maria Luisa Fernández-Cruz, Peter Fürst, Johanna Fink-Gremmels, Corrado Lodovico Galli, Philippe Grandjean, Jadwiga Gzyl, Gerhard Heinemeyer, Niklas Johansson, Antonio Mutti, Josef Schlatter, Rolaf van Leeuwen, Carlos Van Peteghem, Philippe Verger.

#### **SUMMARY**

Nicarbazin is a non-ionophoric synthetic complex composed of an equimolar amount of 4,4'-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP) that is authorised as a coccidiostat feed additive for use in chickens for fattening at a maximum concentration of 50 mg/kg in complete feed as a combination product with narasin (List of authorised additives in feedingstuffs (2004/C 50/01)). Despite the requirements set for feed business operators in Regulation (EC) No 183/2005, it is generally acknowledged that under practical conditions during the production of mixed feeds, a certain percentage of a feed batch remains in the

---

<sup>1</sup>For citation purposes: Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the European Commission on cross-contamination of non-target feedingstuffs by nicarbazin authorised for use as a feed additive, *The EFSA Journal* (2008) 690, 1-34.

production circuit and these residual amounts can contaminate the subsequent feed batches. This cross-contamination may result in the exposure of non-target animal species, and hence the potential health risks for non-target animal species as well as the potential residue deposition in foods derived from these non-target animal species have been evaluated.

Based on a limited range of tolerance studies performed on various non-target animal species the CONTAM Panel concluded that accidental ingestion of feed containing nicarbazin at the maximum authorised level for chickens (50 mg/kg feed), is unlikely to cause adverse effects in non-target animal species.

Cross-contamination of feed with nicarbazin at a level of 10% (5 mg/kg feed) of the maximum authorised level for target animal species, would result in an intake for non-target animal species that would correspond to a dose of 0.25 mg/kg b.w. per day. This dose is well below the no observed adverse effect level (NOEL) of 200 mg/kg b.w per day based on studies on chronic toxicity in dogs and developmental toxicity in rats. The Panel on Contaminants in the Food Chain (CONTAM Panel) concluded that adverse health effects in non-target animal species are unlikely to occur as a result of cross-contamination of feed up to a hypothetical level of 10% of the maximum authorised level of nicarbazin in feed for target animal species.

Consumer exposure was estimated using residue data from chicken eggs, liver and muscle and kinetic data from chickens for fattening at close to zero withdrawal time. The estimated exposure levels of nicarbazin resulting from eating chicken liver that were fed diet containing 10% carry over of nicarbazin (5 mg/kg) was 1.4 µg/kg b.w. per day. The contribution from eggs after a similar exposure to laying hens would be 1.8 µg DNC/kg b.w. per day. Food survey data show that nicarbazin concentrations of up to 7200 µg/kg have been detected in chicken liver, 900 µg/kg in chicken eggs and 110 µg/kg in chicken muscle. When using a conservative daily intake estimate: a person eating 100 g chicken eggs, 100 g chicken liver and 300 g chicken muscle would be exposed to 843 µg of nicarbazin (corresponding to 14 µg/kg b.w. per day for a 60 kg person).

The CONTAM Panel used this overall NOEL to evaluate the risk related to cross-contamination of feed materials produced for non-target animal species. The margin of exposure (MOE) was calculated to be approximately 10,000 based on the NOEL for DNC and the estimated intake. Therefore, the Panel concluded that there is no indication of an appreciable risk to consumers' health from the ingestion of nicarbazin residues in products from animals exposed to cross-contaminated feed up to a hypothetical level of 10% of the maximum authorised level.

**KEYWORDS:** nicarbazin, cross-contamination, carry-over, coccidiostat, anticoccidial, feed additive, occurrence, exposure, animal health, intoxication, human health.

**TABLE OF CONTENTS**

BACKGROUND AS PROVIDED BY THE REQUESTOR.....	4
1. Cross-contamination .....	4
2. Legal provisions as regards minimisation of cross-contamination .....	4
3. Legal provisions as regards the authorisation of coccidiostats (and histomonostats) for use as feed additive.....	5
4. Unavoidable cross-contamination (under practical conditions).....	6
5. Tolerances .....	6
TERMS OF REFERENCE AS PROVIDED BY THE REQUESTOR.....	7
ACKNOWLEDGEMENTS .....	8
GLOSSARY OF TERMS USED BY THE PANEL IN ITS OPINIONS ON COCCIDIOSTATS .....	8
ASSESSMENT .....	10
1. Introduction .....	10
1.1. Biological activity of nicarbazin.....	11
1.2. Previous evaluations of the toxicological properties and the safety of nicarbazin.....	12
1.3. Cross-contamination of feed batches.....	14
2. Methods of analysis for nicarbazin .....	17
2.1. Analysis of nicarbazin in premixes and animal feeds .....	17
2.2. Analysis of nicarbazin residue in animal products.....	18
3. Occurrence of nicarbazin.....	20
3.1 Occurrence of nicarbazin in feed materials for non-target animal species.....	20
3.2. Occurrence of nicarbazin residues in animal products derived from non-target animal species.....	20
4. Toxicity of nicarbazin .....	22
4.1. Mechanisms of toxicity .....	22
4.2. Toxicity of nicarbazin in target animal species.....	22
4.3 Toxicity of nicarbazin in non-target animal species.....	22
5. Kinetics and tissue disposition .....	24
5.1. Kinetics of nicarbazin in target animal species .....	24
5.2. Kinetics of nicarbazin in the non-target animal species.....	26
6. Risk characterization .....	28
6.1. Animal health risks in non-target animal species associated with the accidental consumption of feed materials designated for target animal species .....	28
6.2. Adverse health effect in non-target animal species as a consequence of cross-contamination of feed batches .....	28
6.3. Residues of nicarbazin in foods derived from non-target animal species exposed to feeds containing nicarbazin.....	29
6.4. Human health risk associated with residues in foods derived from non-target animal species following exposure of these animals to contaminated feed batches .....	29
CONCLUSIONS .....	30
RECOMMENDATIONS .....	31
REFERENCES .....	31
DOCUMENTS PROVIDED TO EFSA .....	34

## **BACKGROUND AS PROVIDED BY THE REQUESTOR**

### **1. Cross-contamination**

A feed manufacturing company produces a broad range of compound feedingstuffs. Therefore, in the same production line, different compound feedingstuffs have to be manufactured after each other. At the switch-over from one product to the subsequent one, it is unavoidable that traces of the first product remain in the production line and end up in the beginning of the production of the following product. The transfer from one production batch to the following batch is called “carry-over” or “cross-contamination”.

#### **Cross-contamination in purchased premixtures**

Purchased premixtures can contain traces of contamination of other substances due to cross-contamination during the production.

#### **Product-related cross-contamination**

The following properties of the feed additives and premixes also have an important influence on the cross-contamination behaviour:

- adhesive strength-adhesion to walls
- particle size and density (carrier, substance)
- electrostatic properties.

The cross-contamination decreases according to the product being less adhesive and electrostatic.

#### **Establishment related cross-contamination**

The design of the dosage, grinding and mixing equipment has an important influence on the level of cross-contamination. Also the transport and storage facilities and conditions are an important factor for cross-contamination

## **2. Legal provisions as regards minimisation of cross-contamination**

### **Directive No (EC) 95/69<sup>2</sup>**

Council Directive No (EC) 95/69 of 22 December 1995, laying down the conditions and arrangements for approving and registering certain establishments and operating in the animal feed sector, provides in Article 2 and 3, that establishments manufacturing coccidiostats, manufacturing premixtures prepared from coccidiostats, or manufacturing compound feedingstuffs containing premixtures prepared from coccidiostats have to receive approval for

---

<sup>2</sup> OJ L 332, 30.12.1995, p. 15. As last amended by Regulation No (EC) 806/2003 of 14 April 2003 (OJ L 122, 16.5.2003, p. 1)

these activities. Also intermediaries putting these products into circulation must be approved. The approval is subject to compliance with the minimum conditions laid down in the Annex.

One of these conditions concerning the facilities and the equipment provides that *“the lay-out, design and operation of the facilities and equipment must be as such to minimize the risk of error and permit effective cleaning and maintenance in order to avoid contamination, cross-contamination and any adverse effects generally on the quality of the products.”*

### **Regulation No (EC) 183/2005<sup>3</sup>**

Regulation No (EC) 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene was applicable from 1 January 2006 onwards and has replaced Council Directive No (EC) 95/69.

Article 10 of Regulation No (EC) 183/2005 provides that feed business operators shall ensure that establishments under their control are approved by the competent authority in case these establishments are manufacturing and/or placing on the market coccidiostats and histomonostats, manufacturing and/or placing on the market premixtures prepared using coccidiostats and histomonostats, manufacturing for placing on the market or producing for the exclusive requirements of their holdings, compound feedingstuffs using coccidiostats and histomonostats or premixtures containing coccidiostats and histomonostats.

Annex II to Regulation No (EC) 183/2005 contains requirements for the feed businesses mentioned in previous paragraph. As regards facilities and requirements it is provided under point 2 of Annex II that *“The lay-out, design and construction and size of the facilities and equipment shall:*

*(a) permit adequate cleaning and/or disinfection;*

*(b) be such as to minimize the risk of error and to avoid contamination, cross-contamination and any adverse effects generally on the safety and quality of the products. Machinery coming into contact with feed shall be dried following any wet cleaning process.”*

### **3. Legal provisions as regards the authorisation of coccidiostats (and histomonostats) for use as feed additive**

Article 3 of Council Directive No (EC) 70/524 concerning additives in feedingstuffs<sup>4</sup> provides that no additive may be put into circulation unless a Community authorisation has been granted. This Community authorisation can only be granted if, taking into account the

---

<sup>3</sup> OJ L 35, 8.2.2005, p. 1

<sup>4</sup> OJ L 270, 14.12.1970, p.1

conditions of use, it does not adversely affect human or animal health or the environment, nor harm the consumer by impairing the characteristics of animal products.

Nicarbazin has been authorised for use as a feed additive in accordance with the provisions of Council Directive 70/524/EEC (see Table 1).

Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition is applicable since 19 October 2004 and repeals Directive 70/524/EEC with effect from that date.

Table 1. Species or category of animals for which the use of nicarbazin is authorised (target animal), and authorised maximum content in complete feed

<b>Coccidiostat (active substance)</b>	<b>Species or category of animals for which the use of coccidiostats is authorised (target animal)</b>	<b>Authorised maximum content of nicarbazin in complete feed</b>
Nicarbazin in combination with narasin	Chickens for fattening	50 mg/kg (Maxiban)

#### **4. Unavoidable cross-contamination (under practical conditions)**

Nicarbazin is authorised for use as a feed additive for the production of feedingstuffs for target animal species according to the conditions of authorisation. However the production of feed containing nicarbazin can result in cross-contamination to feedingstuffs for non-target animal species.

Of major importance is the application by the feed operator of good manufacturing practices to avoid to the largest extent possible, the cross-contamination of residues of the coccidiostat in subsequent batches of compound feedingstuffs. However, even if all prevention measures are applied, including the use of rinsing batches, the cross-contamination of residues is unavoidable under practical conditions.

#### **5. Tolerances**

Therefore, the possibility to set tolerances for these in practice unavoidable residues of coccidiostats in feedingstuffs for non-target species should be considered in the frame of Directive (EC) No 2002/32 of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed<sup>5</sup>.

<sup>5</sup> OJ L 140, 30.5.2002, p. 10. Directive as amended by Directive (EC) No 2005/6 (OJ L 24, 27.1.2005, p. 33)

Such tolerances in feedingstuffs for non-target animals could be set following the ALARA principle (As Low As Reasonably Achievable) taking into account good manufacturing practices. According to information received from professional organisations, levels of cross-contamination of 3-10% with a majority at 5% or lower can be achieved after implementing thorough actions to reduce cross-contamination.

Such tolerances in feedingstuffs for non-target animals should not have any pharmacological activity and should not threaten animal health and public health, as in some cases the tolerances for feedingstuffs for non-target animals could result in presence of residues in foodstuffs of animal origin.

### **TERMS OF REFERENCE AS PROVIDED BY THE REQUESTOR**

In accordance with Article 29 (1) (a) of Regulation (EC) No 178/2002 the Commission asks EFSA to provide an opinion of the risks involved for animal health and public health as the consequence of undesirable cross-contamination of nicarbazin authorised as feed additive into non-target feeds.

The assessment should take into account hypothetical carry-over rates of 2%, 5% and 10% from feed produced with the highest authorised dose of nicarbazin into the afterwards produced non-target compound feed (for non-target animal species).

The EFSA is requested to provide an opinion whereby

- the animal health risk for non-target species (food producing farm animals) will be assessed,
- the adverse effects as a consequence of cross-contamination of nicarbazin into feed for non-target animals,
- on the basis of the available information, an estimate of the level of residues present in food of animal origin from non-target species as the consequence of cross-contamination is performed,
- the possible risks for human health as the consequence of the presence of such residues in food of animal origin (eggs, milk, meat, edible offal) from non-target species are assessed.

## **ACKNOWLEDGEMENTS**

The European Food Safety Authority wishes to thank the members of the Working Group for the preparation of this opinion: Arturo Anadón, Alexander Feil, María Luisa Fernández-Cruz, Johanna Fink-Gremmels, Kimmo Peltonen, Derek Renshaw, Pascal Sanders and Pieter Wester.

## **GLOSSARY OF TERMS USED BY THE PANEL IN ITS OPINIONS ON COCCIDIOSTATS**

Considering the current EU legislation, the following terms will be applied in the Opinion:

**Coccidiosis:** Coccidiosis is a common protozoan infection in farm animals, affecting predominantly young animals. Under common farm conditions, herd health management cannot exclude coccidial infections in large poultry and rabbit units and the use of coccidiostatic agents (coccidiostats) remains necessary to control animal health and welfare, and to avoid substantial losses due to acute and often lethal coccidiosis.

**Coccidiostats:** Currently, in the EU 11 coccidiostatic substances are authorised for the prevention of coccidiosis in one or more animal species. Authorisation is given for a minimum and maximum level to be included as feed additive into the animal's diet, and may prescribe the animal species as well as the species categories (as for example chickens for fattening and chickens reared for laying) and in some cases withdrawal periods. Of the 40.65 million tonnes of feed produced annually for chickens for fattening, turkeys and rabbits, approximately 18.33 million tonnes is manufactured with the addition of a coccidiostat (IFAH, 2007, document provided to EFSA).

Various coccidiostats exert also a distinct antibacterial effect and are licensed in Third Countries (countries outside the EU) as growth promoting agents in fattening ruminants (lambs or cattle) and fattening pigs.

**Target animal species:** Animal species or animal category within a species for which the compound under consideration is authorised for use as a coccidiostat.

**Non-target animal species:** Any other animal species or category for which the compound is not authorised.

**Feed additive:** A substance, micro-organism or preparation, other than feed material and premixtures, which are intentionally added to feed at concentrations up to a defined maximum level (mg/kg feed). Currently, coccidiostats are authorised for use as feed additives according to the provisions of Council Directive 70/524/EEC and Council Regulation No (EC) 1831/2003 that repeals Directive 70/524/EEC (see also the background chapter). According to these provisions, authorisation and prerequisites for use of coccidiostats are defined for

individual products (brands) following review by Panel on Additives and Products or Substances Used in Animal Feed (FEEDAP) of data provided by the applicant.

**Premixture:** A mixture of feed additives with feed materials. Premixtures are not intended for direct consumption by animals, and are therefore not addressed in the Opinion.

**Cross-contamination:** Contamination of feeds that are produced after the production of a mixed feed containing additives with residual amounts of the previous feed batch.

**Levels of cross-contamination:** According to the mandate as described in the Terms of Reference, three levels of cross-contamination will be considered, i.e. 2%, 5% and 10% of the maximum concentration authorised for target animal species, respectively.

**Assessment of animal exposure and adverse health effects in animals:** Adverse health effects occurring in non-target animal species are described. A distinction is made between the likelihood of adverse health effects that are associated with an accidental consumption of feeds prepared for a target animal species by a non-target animal species, and the involuntary exposure of non-target animal species by residual amounts of coccidiostats occurring in feed as a consequence of cross-contamination.

**ADI values:** Acceptable daily intake (ADI) of a substance that can be consumed by a human over a lifetime without adverse health effects. As the CONTAM Panel did not have access to the complete safety (toxicological, pharmacological and microbiological) database available for the individual substances under consideration, the ADI value as derived by the FEEDAP Panel and where appropriate also the ADI(s) derived by other relevant scientific committees (e.g. the CVMP<sup>6</sup> or the JECFA<sup>7</sup>) is used for the risk characterization and assessment. The CONTAM Panel noted in some cases the divergence between ADI values derived by the FEEDAP Panel and the ADI values derived by the CVMP and/or JECFA. These differences were attributable to the application of different uncertainty factors, or the inclusion of new endpoints, such as antimicrobial activity (antimicrobial no-effect level) in the assessment. The CONTAM Panel decided to consider both values in the presentation of its risk assessment for non-target animal species.

**MRL values:** Maximum residue limits. The CVMP applied Regulation No (EC) 1055/2006<sup>8</sup> amending the Annexes I and III of Regulation No (EC) 2377/90<sup>9</sup> to propose maximum residue limits (MRLs) for a number of coccidiostats. However, none of the compounds under consideration are licensed at present as veterinary medicinal product. The FEEDAP has also

---

<sup>6</sup> The Committee for Medicinal Products for Veterinary Use of the European Medicines Agency

<sup>7</sup> The Joint FAO/WHO Expert Committee on Food Additives (JECFA) is an international expert scientific committee that is administered jointly by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO).

<sup>8</sup> OJ L 192, 13.7.2006, p. 3-5

<sup>9</sup> OJ L 224, 18.8.1990, p. 1-8

recommended MRLs for some coccidiostats, and the CONTAM Panel considered these recommendations in the evaluation process.

**Residues of coccidiostats in edible tissues, milk and eggs:** According to Directive No (EC) 96/23<sup>10</sup> Member States are obliged to monitor certain substances and residues thereof in animals and animal products. These data are collected by the Commission and a compilation of the results from 2004 and 2005 are used in the human exposure assessment.

**Equivalents:** Where kinetic studies have been conducted with the coccidiostat <sup>14</sup>C-radiolabelled, the concentration of total radioactive residue levels measured in the different tissues are expressed as µg parent coccidiostat equivalents/kg tissue, to indicate that these levels could be the parent compound and/or metabolites.

**Human dietary exposure:** The present assessment is confined to the evaluation of residues of coccidiostats in foodstuffs derived from non-target animals. Where appropriate, total exposure originating from different products including edible tissues, milk and eggs is estimated.

**Risk characterization:** The risk characterization is based on the ADI and MRL values from either the FEEDAP Panel, the CVMP or the JECFA as outlined above. These levels are compared with levels of residues found in tissues and/or products (for example eggs) of non-target animal species as far as these are available. Where appropriate uncertainties in the establishment of ADI values are discussed.

## ASSESSMENT

### 1. Introduction

Nicarbazin is a synthetic complex composed of an equimolar amount of 4,4'-dinitrocarbanilide (DNC: molecular weight = 300) and 2-hydroxy-4,6-dimethylpyrimidine (HDP: molecular weight = 112). DNC is also known as *N,N'*-bis(4-nitrophenyl)urea. Nicarbazin is a light yellow powder with the CAS number 330-95-0. The chemical structures of DNC and HDP are presented in Figure 1.

---

<sup>10</sup> OJ L 125, 23.5.1996, p. 10-32

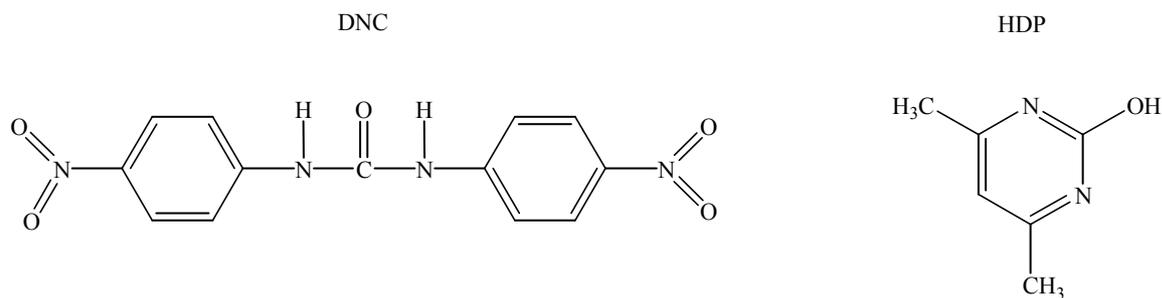


Figure 1. Chemical structure of 4,4'-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP).

DNC and HDP presents a water solubility of 0.02 and >10000 mg/L, respectively, and a log Kow of 3.6 and 0.94 at pH 5-9, respectively (EFSA, 2003).

As summarised in the background chapter, nicarbazin is authorised as a coccidiostat for use in chickens for fattening at a minimum-maximum level of 40-50 mg nicarbazin/kg feed as a component of the feed additive product Maxiban® which also contains narasin at a minimum-maximum level of 40-50 mg narasin/kg feed. The withdrawal period before slaughter is five days<sup>11</sup>.

## 1.1. Biological activity of nicarbazin

### Anticoccidial activity

DNC, but not HDP, has anticoccidial activity when used alone against *Eimeria*. The potency of DNC is increased ten fold when the molecule is complexed with HDP as nicarbazin, but no increase in anticoccidial activity is observed with a simple mixture of the two compounds. The complex is formed of hydrogen bonds between the amide carbonyls and amino hydrogens (Rogers *et al.*, 1983). Nicarbazin is at least partially broken down in the gut to DNC and HDP, which may be absorbed independently.

Nicarbazin acts primarily by inhibiting the further development of the 2<sup>nd</sup> generation and, to a lesser extent, 1<sup>st</sup> generation schizont stage of *Eimeria* spp. parasites. The anticoccidial effect of nicarbazin was shown to be mediated to a large extent by its systemic absorption (EFSA; 2003).

### Antibacterial activity

The antibacterial activity of nicarbazin and its individual components was investigated on 45 strains from 9 facultatively aero-anaerobic and micro-aerophilic bacterial species. All of the

<sup>11</sup> List of the authorised additives in feedingstuffs published in application of Article 9t (b) of Council Directive 70/524/EEC concerning additives in feedingstuffs. OJ C 50, 25.02.2004, p. 1-144.

minimum inhibitory concentrations (MIC) values determined *in vitro* for the 45 strains were > 256 mg L and it was concluded that neither the nicarbazin complex nor the individual compounds exert anti-bacterial activity (EFSA, 2003).

## **1.2. Previous evaluations of the toxicological properties and the safety of nicarbazin**

Toxicological assessment of nicarbazin in laboratory animals and consumer safety have been evaluated by the Scientific Committee for Animal Nutrition (SCAN) (EC, 1991, 1995), the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (FAO/WHO, 1999) and more recently by the FEEDAP Panel as part of its evaluation of the use of the formulation Koffogran® in feed for chicken for fattening (EFSA, 2003).

The acute oral toxicity of nicarbazin is low in rodents (LD<sub>50</sub> values >25 000 mg/kg b.w. in mice and >10,000 mg/kg b.w. in rats). The individual components of nicarbazin namely DNC and HDP, also showed a low acute oral toxicity (LD<sub>50</sub> values 4000 and >18,000 mg/kg b.w. for HDP and DNC respectively in mice) (FAO/WHO, 1999b). The 50<sup>th</sup> meeting of the JECFA had set an ADI of 0.4 mg/kg b.w. for nicarbazin by applying an uncertainty factor of 500 to the NOEL of 200 mg/kg b.w. per day for maternal toxicity (increased mortality, reduced bodyweight gain and feed intake) and foetotoxicity (delayed ossification and reduced foetal weights) in the rat developmental toxicity study. The large uncertainty factor was used “to account for limitations in the available data”. As nicarbazin had, at that time, a 40-year history of use in products fed to food producing animals and as its use was limited to use in chicken feed, the JECFA felt it was appropriate to set an ADI despite the absence of certain toxicological data that would normally be required (FAO/WHO, 1999).

Reports submitted to the FEEDAP Panel included repeat-dose oral short-term toxicity studies in rats, rabbits, cats, guinea-pigs, mice and dogs; 2-year oral toxicity studies in rats and dogs; mutagenicity studies (bacterial tests and a mouse bone marrow micronucleus test); two generation reproduction studies in rats; and a developmental toxicity study in rats (EFSA, 2003).

A 2-year chronic toxicity study was carried out in rats fed diets containing the DNC and HDP components at doses of 0, 50, 150 or 300 mg DNC and 0, 17, 50 or 100 mg HDP/kg b.w. per day. There was no treatment-related toxicity and the incidence of tumours was unaffected. The no observed effect level (NOEL) was judged to be the highest dose employed i.e. 300 and 100 mg/kg b.w. per day of DNC and HDP, respectively (EFSA, 2003).

A toxicity study was conducted in dogs given diets containing DNC and HDP for six days a week for two years. Intakes were 0, 60, 180, or 600 mg DNC/kg b.w. per day and 0, 20, 60, or 200 mg HDP/kg b.w. per day. Body weight gain, food intake, haematological and urine parameters were unaffected by treatment. No treatment related changes were seen in organ

weights and gross pathological appearance. There was an elevation in serum glutamic pyruvic transaminase (ALT), especially in dogs receiving the highest dose around twelve months into the study, but further investigations suggested no hepatic abnormalities. The NOEL was 180 mg DNC/kg b.w. per day and 60 mg HDP per kg b.w. per day calculated on the basis of administration on six days per week. For a weekly dosage, the NOELs were 154 mg DNC/kg b.w. per day and 51 mg HDP/kg b.w. per day resulting in a NOEL for nicarbazin (corresponding to a 3:1 mixture of DNC and HDP) of 200 mg/kg b.w. per day (EFSA, 2003).

In a two generation reproduction toxicity study, rats were treated with 0, 50, 150 or 300 mg DNC/kg b.w. per day and 0, 17, 50, or 100 mg HDP/kg b.w. per day continuously through the production of two litters per generation for three successive generations. There were isolated occurrences of slightly reduced litter size at birth or depressed body weight gain during lactation at the highest dose level. These effects were not observed in the majority of litters and showed no progression with the duration of the study (EFSA, 2003). The JECFA (1999) concluded that the results of this study showed that nicarbazin had no significant effects on reproduction in rats and proposed a NOEL of 400 mg/kg b.w. day, the highest dose tested (FAO/WHO, 1999). The FEEDAP Panel, (EFSA, 2003), used a more conservative approach and proposed the intermediate dose level as the NOEL i.e. 200 mg/kg b.w. per day of the 3:1 mixture (i.e. 150 mg DNC:50 mg HDP/kg b.w.).

Developmental toxicity was also studied in rats given nicarbazin at 0, 70, 200 or 600 mg/kg b.w. per day by gavage during gestation days 7-17. At 600 mg/kg b.w. per day some rats died (7/25), maternal food intake and body weights were reduced, foetal body weights were decreased and there was reduced ossification suggesting retarded foetal development. The NOEL for nicarbazin was considered to be 200 mg/kg b.w. per day for maternal and foetal toxicity. Teratogenic effects were not observed (FAO/WHO, 1999).

The FEEDAP Panel concluded that the dossier on toxicology was far from satisfactory since most of the studies were not performed to modern standards. In addition, nicarbazin gave equivocal results in a bacterial reverse mutation test, there were no *in vitro* mutagenicity tests in mammalian cells and, although a negative result *in vivo* was obtained in a micronucleus test in bone marrow erythrocytes, no other test in a second somatic tissue confirmed the absence of *in vivo* mutagenicity. Overall, the FEEDAP Panel considered it unlikely that nicarbazin is genotoxic (although new data from the sponsor were required) and that there were no hints to suggest that nicarbazin is a carcinogenic compound. Also, the available studies did not provide indications of a significant potential for target tissue toxicity, such as neurotoxicity, immunotoxicity or impaired fertility in mammals. As regards to developmental toxicity, even though no major effects on litter size or pup growth were observed in a two-generation reproduction toxicity study in rats, the toxicity data of the study was inadequately reported; thus, an unequivocal conclusion could not be drawn (EFSA, 2003). Therefore, the FEEDAP Panel did not establish an ADI for nicarbazin (as DNC or HDP) due to their concern regarding the quality of the toxicological database and concerns that more studies were

required to clarify the possible genotoxicity and developmental toxicity of the compound. As a result of these concerns, the CONTAM panel could not use an ADI for the risk characterization of nicarbazin as a feed contaminant.

There are currently no EU MRLs for nicarbazin, but there are Codex MRLs<sup>12</sup> for nicarbazin residues in chicken tissues: muscle, liver, kidney and skin/fat of 200 µg/kg. The marker residue for these MRLs is DNC.

### **1.3. Cross-contamination of feed batches**

Feed additives, such as coccidiostats, are marketed as premixtures, intended to be incorporated into mixed feeds during the mixing and production process. Cross-contamination refers to the fact that under the practical conditions in a commercial feed mill, residual amounts of feed materials remain in the production line (see also the background chapter) and may contaminate following feed batches. The degree of cross-contamination depends on the technical facilities and procedures, as well as on product characteristics.

#### **1.3.1. Factors influencing the rate of cross-contamination**

Feed additives, such as coccidiostats, are marketed as premixtures, intended to be incorporated into mixed feeds during the mixing and production process. Cross-contamination refers to the fact that under the practical conditions in a commercial feed mill, residual amounts of feed materials remain in the production line (see also the background chapter) and may contaminate subsequent feed batches. The degree of cross-contamination depends on the technical facilities and procedures, as well as on product characteristics.

#### **1.3.1. Factors influencing the rate of cross-contamination**

Several studies have shown that a completely contamination-free production of premixes and compound feeds in existing multi-product plants is impossible in practice (Strauch, 2003). Various process parameters and the physicochemical characteristics of the product act together to determine the residual amount remaining in the circuit and hence the rate of cross-contamination from one feed batch to the subsequent batches produced in the same production line (Kennedy *et al.*, 1996, 1998b; McEvoy *et al.*, 2003; Harner *et al.*, 1996). In a similar way, the purchased premix that is incorporated into the feed can itself contain traces of contamination of other substances, due to cross-contamination during the production of the premix.

---

<sup>12</sup> <http://www.codexalimentarius.net/mrls/>

The **technological equipment** in the feed mill can influence the amount of cross-contamination that may occur. The following sites in the circuit have been identified as being places where fractions of feeds can be retained, with the possible consequence of contamination of later batches:

- Areas of reduced flow in piping, material ledges, and non-plane surfaces (screw couplings, weld seams, moulded tanks) can lead to a sedimentation of feed materials.
- Oversized and long conveying systems, and non-continuous earthing of parts of the production plant.
- In silos or containers, differences in flow rate may cause segregation of the bulk material, which accumulates in dead zones with solidification of the bulk material.
- Conveyors which do not empty completely, such as screw conveyors and elevator boots.
- Wear of mixing equipment and conveying systems can cause a reduced flow in certain areas at which material can accumulate.
- Filter systems may accumulate residues, in particular with material featuring high dusting potential and strong aspiration flow.

The **physicochemical characteristics** of additives can contribute to cross-contamination in the following ways:

High dusting potential, low product moisture, adherence due to electrostatic charge, as well as environmental conditions (e.g. adhesions caused by surrounding moisture) contribute to cross-contamination. The more dispersed in air and the lower the density of the components, the more sensitively they react to current fields. Basically, particle sizes < 500 µm are dispersible in the air which facilitates the discharge of suitable, airborne components by aspiration air. An accumulation of feed material in filters and incomplete or inappropriate cleaning (see above) can lead to cross-contamination of these components into the next production batch. Also a high electrostatic loading potential as well as higher product moistures can cause adhesions inside production plants and can result in cross-contamination.

Finally, it should also be mentioned that activities in or outside the feed mill may contribute to undesired contamination of non-target animal feed, for instance, insufficient rinsing or no rinsing during product changes will result in a greater amount of cross-contamination. The beneficial effect of using rinsing batches can be reduced considerably if the residual material adhering to the equipment cannot be fully removed by the material flow of the rinsing batch (McEvoy *et al.*, 2003; Noser *et al.*, 2006; Dorn *et al.*, 1988). Further cross-contamination can occur at the feed plant during conveying (contaminated conveying equipment) and on-farm (e.g. during storage and transport to the feeding location).

### 1.3.2. Assessing cross-contamination in feed mills

In investigations involving the majority of German compound-feed plants (approximately 450), more than half of the examined production plants had a level of cross-contamination of less than 4% (Strauch, 2002). A survey of Belgian compound-feed production companies showed similar values for pelleted products (OVOCOM, 2004, document provided to EFSA). Similar results were achieved with mashed (not pelleted) feeds (approx. 69% containing less than 5% cross-contamination).

The investigations described above, refer to a general technical control of the mixing facilities used by commercial feed mills. Comparable investigations on the behaviour of coccidiostats during compound-feed production have not been carried out. As yet, analytical controls of the produced feeds for the presence of coccidiostats were only conducted in cases for which residual amounts of the coccidiostatic agents were found in food obtained from accidentally exposed animals. Systematic investigations of the behaviour of coccidiostats at compound-feed production companies have been carried out for lasalocid, narasin, nicarbazin and monensin (Kennedy *et al.*, 1996, 1998a,b; McEvoy *et al.*, 2003; Noser *et al.*, 2006). These authors concluded that:

- Cross-contamination can be reduced significantly by suitable measures.
- Contamination by coccidiostats was detected in several rinsing batches.

### 1.4. Specific data for nicarbazin-based feed additives products

Nicarbazin is strongly electrostatic. This is an important factor in cross-contamination of feedingstuff (McEvoy *et al.*, 2003).

Information on particle size and dusting potential was not available.

Studies from Dorn *et al.* (1988) have demonstrated cross-contamination of nicarbazin in different mixing plants. In one case, in the fourth batch after medication, 8.2% of the original nicarbazin concentration was found.

Some mechanisms of nicarbazin contamination in a feed mill were investigated by McEvoy *et al.* (2003). Three sequential 3-tonne cleaning batches of nicarbazin-free feed were produced directly after a batch of nicarbazin-containing feed (125 mg/kg). Sampling of the cleaning batches took place at two points before pelleting and at one point post-pelleting. The study was repeated on two further occasions. Pre-pelleting, the highest nicarbazin concentrations (3.4-0.26 mg/kg) were observed in the first tonne milled after the nicarbazin-containing ration. Thereafter, concentrations steadily declined in successive batches. Post-pelleting samples contained much higher concentrations of nicarbazin. After 8 tonnes had passed through, the concentrations (7.2-1.29 mg/kg) were between 10 and 20 times greater than the

corresponding concentrations detected post-mixing. The practice of returning post-press sieved material to the pre-press bins was identified as the cause of the problem. Re-routing of sieved material along with better segregation of nicarbazin-containing and nicarbazin-free feedingstuffs by separated mixing lines, the installation of a new elevator with reduced dead space (3 instead of 15-20 kg) markedly reduced the incidence of feed contamination with this compound.

## 2. Methods of analysis for nicarbazin

### 2.1. Analysis of nicarbazin in premixes and animal feeds

The nicarbazin content of feed can be analysed by measuring UV absorbance at 430 nm, following extraction with N,N-dimethylformamide and alumina clean-up (AOAC Method 956.11) (Johnston *et al.*, 2001).

Yakkundi *et al.* (2001) described a method for determining DNC and HDC in feeds using high performance liquid chromatography (HPLC) with ultra violet (UV) detection. The same article cited a method based on LC-tandem mass spectrometry (MS/MS) that had been used for measuring DNC in feed (Cannavan *et al.*, 1999).

Feed samples were analysed after extraction with an organic solvent using LC-MS/MS. Detection was performed on a triple quadrupole mass spectrometer in the multiple reaction monitoring (MRM) mode and electrospray ionisation. The regression analysis demonstrated a mean slope ( $\pm$  std) 0.88, ( $\pm$  14.21), a  $R^2$ -value of 0.987 ( $n = 11$ ) at the concentration range 0-20  $\mu\text{g}/\text{kg}$  feed. The decision limit ( $CC\alpha$ )<sup>13</sup> and the detection capability ( $CC\beta$ ) was 5.8 and 8.6  $\mu\text{g}/\text{kg}$  feed respectively (Mortier *et al.*, 2005a).

A reversed-phase liquid chromatography method for nicarbazin in broiler feeds was validated. The extraction solvent was an acetonitrile-methanol mixture. The 4,4'-dinitrocarbanilide moiety of nicarbazin was detected at a wavelength of 350 nm. Recovery was >87%. At 20 mg/kg, the repeatability was 0.7% and the within-laboratory reproducibility was 2.7%. The limit of determination was <20 mg/kg. In an inter-laboratory study, 4 positive feed samples for chickens for fattening, 1 blank pig feed, and 1 premixture sample for chickens for fattening were analysed by 19 laboratories. The relative standard deviation for repeatability

---

<sup>13</sup>Definitions of limit of detection (LOD), limit of quantification (LOQ), decision limit ( $CC\alpha$ ) and detection capability ( $CC\beta$ ): Commission decision 2002/657 of 12 August 2002 implementing Directive No (EC) 96/23 concerning the performance of analytical methods and the interpretation of results (OJ L 221, 17.08.2002, p. 8-36) define the performance of analytical methods used for residue control and the interpretation of results.  $CC\alpha$  means the limit at and above which it can be concluded with an error probability of  $\alpha$  that a sample is non-compliant.  $CC\beta$  means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of  $\beta$ .  $CC\alpha$  is equivalent to the LOD defined by IUPAC guidance (IUPAC, 1995). The LOQ (ISO, 1997) is defined by the relative standard deviation of the estimated quantity. Generally, it corresponds to the lowest concentration tested with a relative standard deviation below the performance value needed, such as the performance for repeatability defined by Decision No (EC) 2002/657 (OJ L 221, 17.8.2002, p. 8-36).

(RSDr) of the feedingstuffs (20-240 mg/kg) varied between 2.6 and 10.2%. Recoveries were 91-108%. The limit of detection (LOD) and the limit of quantification (LOQ) was 0.5 mg/kg and 1.0 mg/kg, respectively (De Jong *et al.*, 2004).

Nicarbazin in feeds was extracted with acetonitrile-water and extracts were analysed using UV detection at 340 nm. For medicated feeds, the method uses a standard linear range between 5 and 100 µg/mL. For lower levels, a linear range of 50 to 150 ng/mL can be used. The method has a LOD of 0.25 mg/kg and a LOQ of 0.5 mg/kg in a 40 g feed sample. Recovery was 99.1%, with a range of 95.2 to 101.8%. For a dose range of 27 to 113.5 mg/kg, the precision of the method based on one analyst, one day, and 2 weightings ranged from 2.8% (113.5 mg/kg) to 4.7% (27 mg/kg) (Krabel *et al.*, 2000).

The ground feed samples containing nicarbazin were extracted using aqueous dimethylformamide after moistening with water. Co-extracted feed constituents were removed with a solid-phase extraction alumina-basic column and the eluates were analysed using UV absorption of 4,4'-dinitrocarbanilide portion of nicarbazin at 265 and 345 nm. The mean recovery from nicarbazin spiked samples was 95% with a relative standard deviation of 4% in a concentration range of 2-150 mg/kg. The limits of detection of nicarbazin in feed (S/N= 3) was estimated to be 1 mg/kg, and the lowest levels tested in feeds by this procedure were 2 mg/kg (Dusi *et al.*, 2000).

## **2.2. Analysis of nicarbazin residue in animal products**

According to the list of methods used by the National Reference Laboratories (NRL) for residue control, edited by the Community Reference Laboratory (CRL) (Bohm *et al.*, 2005), nicarbazin residues methods are reported for muscle and liver tissues by 4 and 2, respectively, and for eggs by 4 out of the 20 NRLs within the EU. The Member States used different methods such as liquid chromatography coupled with diode array detector (DAD), liquid chromatography–mass spectrometry (LC-MS) or liquid chromatography – tandem mass spectrometry (LC-MS/MS) for screening and confirmatory purposes.

No EU maximum residue limit (MRL) or minimum required performance level (MRPL) has been established for nicarbazin in eggs or animal tissues.

### **2.2.1. Screening methods**

An ELISA method for screening of DNC in eggs was developed (Daeseleire *et al.*, 2005). The CC $\alpha$  and CC $\beta$  were 0.5 µg/kg and below 3 µg/kg.

Dubois *et al.* (2004) described a multi-residue qualitative method based on LC-MS/MS for determining nine coccidiostats in muscle and eggs. The method uses extraction in acetonitrile

followed by a clean-up on SPE. Two mass transitions ( $m/z$  301.0→137.0 and  $m/z$  301.0→107.0) were monitored. For DNC residue in muscle, extraction recovery was 60% and  $CC\alpha$  was 0.4  $\mu\text{g}/\text{kg}$ . This qualitative method is also applicable to eggs.

### **2.2.2. Quantitative and confirmatory methods**

JECFA (FAO/WHO, 1999) reviewed nicarbazin residue methods that measured the DNC component. Early methods relied on colourimetry and polarography, more recent methods used HPLC with UV detection and HPLC mass spectrometry, achieving LOQs of approximately 0.1 mg/kg.

When chickens are given nicarbazin in the feed, the HDP fraction is absorbed and excreted more rapidly than the DNC fraction. Most residue analyses for nicarbazin are therefore based on methods for the DNC molecule.

Yakkundi *et al.* (2001) cited a method based on LC-MS/MS that had been used for measuring DNC in feed (Cannavan *et al.*, 1999), and which the authors used for the confirmation of DNC in chicken liver and eggs. Samples were homogenised with acetonitrile. After centrifugation, the supernatant was evaporated and residues reconstituted in hexane. This was extracted with methanol-water before analysis. Identification of DNC was performed with two mass transitions ( $m/z$  301→137 and 301→107). The performance of the method were determined around the JECFA MRL (200  $\mu\text{g}/\text{kg}$ ) for muscle. The quantification limits were 100 and 10  $\mu\text{g}/\text{kg}$  for DNC in chicken liver and eggs, respectively.

DNC residues in plasma and eggs extracted with acetonitrile and N,N-dimethylformamide, after minimal clean-up, were analysed by HPLC with UV detection at 347nm (Johnston *et al.*, 2001). A very similar method was later used on eggs and egg-shells, where the LOD was quoted as 75  $\mu\text{g}/\text{kg}$  for fortified chicken egg samples (Stahl *et al.*, 2003).

A multi-residue LC-MS/MS method was developed to quantify nicarbazin (DNC) residue in sheep and chicken liver and eggs (Matabudul *et al.*, 2002). After mixing sample with anhydrous sodium sulphate, residues were extracted with acetonitrile. Extracts were purified on SPE cartridge before analysis. Two DNC mass transitions ( $m/z$  301→137 and 301→107) were monitored. Recoveries in eggs, sheep and chicken liver ranged around 90%. The LOD was 1  $\mu\text{g}/\text{kg}$  for all matrices.

A method based on LC-MS/MS with negative electrospray was developed for analysis of DNC residue in eggs (Daeseleire *et al.*, 2005). After liquid-liquid extraction with acetonitrile, the extract was injected for liquid chromatography. Two ion mass transitions ( $m/z$  301.1→137.1 and 301.1→107.1) were monitored. The concentration range was 0-75  $\mu\text{g}/\text{kg}$ . For eggs, the mean slope was  $27.06 \pm 5.57$  ( $r^2 = 0.993$ ). The  $CC\alpha$  and  $CC\beta$  were 1 and 1.2

µg/kg respectively. Recoveries ranged from 97.1 ±5.4% at 50 µg/kg to 101.2 ±6.7% at 5 µg/kg.

A multi-residue method based on LC-MS/MS was described by Mortier *et al.* (2003). After acetonitrile extraction, extract was concentrated and filtrated before analysis. Three mass transitions ( $m/z$  301.4→136.8, 301.4→107.0 and 301.4→45.8) were monitored. DNC recovery was 61% and CC $\alpha$  was 2.5 µg/kg.

### **3. Occurrence of nicarbazin**

#### **3.1 Occurrence of nicarbazin in feed materials for non-target animal species**

Data on cross-contamination of feed are very scarce. The Czech Republic analysed 254 samples in 2006 of different feed commodities. One sample of pre-mixture for pigs contained 43.5 mg/kg of nicarbazin with a LOD of 10 mg/kg.

Information from the Rapid Alert System for Food and Feed (RASFF)<sup>14</sup> that was collected between April 2002 and April 2006 showed no incidents of nicarbazin in feed for non-target animal species (data provided by the European Commission).

#### **3.2. Occurrence of nicarbazin residues in animal products derived from non-target animal species**

Residues of nicarbazin in non-target animal tissues and eggs can arise from cross-contamination but also if a non-target animal is given feed formulated for target animal species, intentionally or accidentally. Several controlled studies and surveys of egg samples have demonstrated that nicarbazin can accumulate in eggs. However, cross-contamination at the feed mill is found to be the main reason for the presence of residues in eggs.

Eggs, muscle and liver from different animal species are analysed for residues of coccidiostats by the Member States according to requirements in Directive No (EC) 96/23<sup>15</sup>. However, the results from the Member States were very different in terms of LODs and the definition of compliant and non-compliant. The levels at which a result is defined as non-compliant are not harmonised within the Member States, but several countries use 10 µg nicarbazin/kg tissue as their non-compliant limits. For nicarbazin, combined results of 2004 and 2005 show that out of a total of 10077 samples 133 out of 4311 samples of poultry, 23 out of 3314 samples of eggs, one out of 340 samples of rabbit and one out of 168 samples of farmed game were found to be non-compliant. The LODs were between 1 and 100 µg/kg.

---

<sup>14</sup>For more information on the RASFF system: [http://ec.europa.eu/food/food/rapidalert/index\\_en.htm](http://ec.europa.eu/food/food/rapidalert/index_en.htm)

<sup>15</sup>Directive No (EC) 96/23 of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products. OL L 125, 23.5.1996, p 10-32.

Belgium has provided individual data of 958 samples of foods that were analysed in 2005 and 2006. Six poultry samples contained residues of nicarbazin that were greater than the Belgian defined non-compliant limit of 10 µg/kg. Twelve samples of poultry, two of eggs and one of rabbit contained a concentration of nicarbazin that was greater than the LOD of 5 µg/kg but less than and the Belgian non-compliant limit of 10 µg/kg (data provided to EFSA).

In targeted food surveys performed in the United Kingdom (UK-VMD, 1995-2004), nicarbazin (DNC) was looked for in chicken eggs (2178 samples), quail eggs (97), egg-based baby food (108), chicken liver (1796), turkey liver (223), duck liver (41), deer liver (20), pâté of chicken and/or pig liver (120), raw chicken meat (514), chicken-based baby food (100), raw turkey meat (41), breaded turkey products (22) and rabbit (92). Some of the samples were found to contain nicarbazin (DNC) at concentrations greater than the respective reporting limits:

- 224 samples of chicken liver (12.5%) had more than 100 µg/kg of nicarbazin (DNC), with the highest residue level detected being 7200 µg/kg;
- 123 samples of chicken eggs (5.6%) had more than 10 µg/kg of nicarbazin (measured as DNC), with the highest residue level detected being 900 µg/kg;
- 22 samples of quail eggs (22.7%) had more than 25 µg/kg of nicarbazin (measured as DNC), with the highest residue levels detected being 540 µg/kg;
- 4 samples of raw chicken (0.8%) had more than 25 µg/kg of nicarbazin (measured as DNC), with the highest residue levels detected being 110 µg/kg;
- 3 samples of liver pâté (2.5%) had more than 40 µg/kg of nicarbazin (measured as DNC), with the highest residue level detected being 70 µg/kg.

In a pan European surveillance study in eggs (Mortier *et al.*, 2005a), 4.1 % of the tested samples were found to contain measurable amount of DNC with the highest level measured of 10 µg/kg.

A very recent survey in poultry produced in Ireland during the period 2002-2006 revealed the presence of DNC in less than 2% of poultry eggs with a highest level of 122 µg/kg. In livers from chickens for fattening approximately 7% of the tested samples contained DNC at levels exceeding 200 µg/kg (Danaher *et al.*, 2008).

## 4. Toxicity of nicarbazin

### 4.1. Mechanisms of toxicity

Data on the mechanism of toxicity of nicarbazin has not been identified.

### 4.2. Toxicity of nicarbazin in target animal species

In chickens, the acute oral toxicity of nicarbazin was low, with an LD<sub>50</sub> of 2400 mg/kg b.w. (data provided by the industry). The primary toxic effects of high oral doses of nicarbazin are decreased body weight gain and reduced feed conversion efficiency (Chapman, 1994). 50-200 mg nicarbazin/kg feed fed to chickens from 1 day to 10-11 weeks of age allowed good growth and feed conversion. However, at 400 mg nicarbazin/kg feed, there was decreased body weight gain and lower feed efficiency. Nicarbazin fed to chickens at 600 mg per kg feed for 12 weeks produced no signs of toxicity or abnormal behaviour and did not increase mortality, but the treatment decreased body weight gain and lowered feed efficiency. Increased mortality occurred at doses of 1500 and 2000 mg nicarbazin/kg feed. Other toxic effects at 800 mg/kg feed included anaemia and fatty change and haemosiderin deposits in the liver.

A study was designed to investigate the tolerance of the target species at the incorporation rates of 125, 250 and 375 mg nicarbazin/kg complete feed for 29 consecutive days followed by a 9-day recovery period. No treatment-related clinical signs of toxicity were observed in any group during the treatment period. A reduction in body weight gain occurred in all the treated groups as compared to control. Feed consumption was only affected in the 375 mg/kg group. Treatment-related reductions in kidney, liver and spleen weight were observed at 375 mg nicarbazin/kg feed. Autopsies revealed no gross pathology attributable to treatment with nicarbazin.

### 4.3 Toxicity of nicarbazin in non-target animal species

#### 4.3.1. Laying hens

Nicarbazin has adverse effects on the hatchability and the quality of the eggs. At 125 mg/kg of feed, nicarbazin caused a reduction in egg production of 65% and completely inhibited the hatchability of fertilised eggs (Booth and McDonald, 1982). In another study, hatchability was impaired at 50 mg/kg feed, with a NOEL of 25 mg/kg feed (Hughes *et al.*, 1991). At high dietary concentrations of nicarbazin (1600 mg/kg feed), testis development was retarded (Chapman, 1994).

Nicarbazin was fed at 0, 20, 50, 100 and 125 mg/kg feed to broiler breeders (Jones *et al.*, 1990a) and no depression in egg production (with the exception of 125 mg/kg), egg weight or fertility from feeding these levels were observed. However, a linear decrease in hatchability was observed together with an increase in DNC amount in egg yolks and egg-shell pigmentation as the level of nicarbazin increased. At 125 mg/kg nicarbazin reduced egg production, and produced a more severe decrease in hatchability and a complete bleaching of

brown-shell eggs by the third day of treatment. However, after seven days of nicarbazin withdrawal from the feed, pigmentation returned to pre-treatment level.

In another study, nicarbazin was fed to White Leghorns layers at 0, 20, 50, 100 and 125 mg/kg feed for ten days and hatchability and egg shell pigmentation was decreased at dietary concentrations of 50 and 100 mg/kg feed, and but fertility, egg production and egg weight fertility were not affected (Jones *et al.*, 1990b). At 125 mg/kg reduction of egg production, egg weight, egg yolk mottling were observed but egg quality as measured by Haugh Units was unaffected by the treatment (Jones *et al.*, 1990b).

#### **4.3.2. Ducks**

The dietary toxicity of nicarbazin was determined in 10-day old mallard ducks. Mallards were exposed to diets that contained concentrations of 0, 62, 200, 560, 1800 or 5000 mg nicarbazin per kg of feed for 5-days during an 8-day study. No mortality occurred at any treatment level and there were no signs of toxicity. The LD<sub>50</sub> was greater than 5000 mg/kg feed. Mean body weight gains of groups given 200 mg/kg or greater were significantly ( $P \leq 0.05$ ) less than the control group value during the treatment phase. Mean body weight gain values for all treatment groups were equal to or greater than the control group value during the basal diet test phase. Food consumption was substantially reduced for the 1800 and 5000 mg/kg feed groups during the treatment phase. No adverse effects on mortality, body weight gain and food consumption were observed at a concentration of 62 mg/kg feed (data provided by the industry).

#### **4.3.3. Quail**

Adult bobwhite quail (*Colinus virginianus*) were given single oral doses of nicarbazin of 0, 125, 250, 500, 1000, or 2000 mg/kg b.w. and observed for 14 days. The effects of nicarbazin were evaluated using the parameters of mortality, signs of toxicity, mean body weight fluctuations, and food consumption. One bird in the 125 mg/kg b.w. died from a cage related injury. No other mortality occurred. There were no clinical signs of toxicity during the study. Mean body weight and food consumption values for birds at all treatment levels were considered equivalent to the value of the control birds throughout the 14-day observation period. No signs of toxicity were observed at a concentration of 2000 mg/kg b.w., the highest dose tested (data provided by the industry).

The dietary toxicity of nicarbazin was determined in 13-day old bobwhite quail. The birds were exposed to diets that contained concentrations of 0, 62, 200, 560, 1800 or 5000 mg nicarbazin/kg feed for 5-days during an 8-day study. No mortality occurred at any treatment level and there were no signs of toxicity. The LD<sub>50</sub> was greater than 5000 mg/kg feed. Mean body weight gain for the groups that received dietary levels  $\leq 560$  mg/kg feed were equivalent to the control group during the treatment phase. Mean body weight gain values for groups that

received the 1800 and 5000 mg/kg feed were significantly ( $P \leq 0.05$ ) less than the control group value during same period. During the basal diet phase the mean body weight gain for the 5000 mg/kg feed group was significantly less than the control group value. Mean body weight gain values for all other treatment groups were equal to or greater than the control group value during the basal diet test phase. Food consumption was substantially reduced for the 5000 mg/kg feed groups during the treatment phase. Food consumption for groups that received dietary levels  $\leq 1800$  mg/kg feed was considered equivalent to the control group during the treatment phase. During the basal diet test phase all food consumption values for treated birds were considered equivalent to the control group value. Based on mortality, an analysis of body weight and food consumption measurements, and signs of toxicity were observed at a concentration of 560 mg/kg feed (data provided by industry)

#### **4.3.4. Fish**

Juvenile bluegills (*Lepomis macrochirus*) were exposed to nominal nicarbazin concentrations of 0 and 100 mg/L for 96 hours. No physical signs of toxicity were observed (data provided by the industry).

Both HDP and DNC were tested for acute toxicity to guppy (*Poecilia reticulata*) and rainbow trout (*Onchorhynchus mykiss*). HDP was tested at two concentrations (guppy: 5600 and 1000 mg/L, trout: 2300 and 5600 mg/L). DNC was tested at a saturated solution in the aqueous medium (0.02 mg/L). The 96-hour  $LC_{50}$  for HDP was  $10^4$  mg/L and  $>5600$  mg/L for the guppy and rainbow trout respectively. For DNC the 96-hour  $LC_{50}$  was  $>0.02$  for both guppy and rainbow trout (data provided by the industry).

### **5. Kinetics and tissue disposition**

#### **5.1. Kinetics of nicarbazin in target animal species**

The FEEDAP Panel has reported kinetic studies on nicarbazin (EFSA, 2003). The comparative fates of nicarbazin [ $^{14}C$ ]-labelled either on the DNC or the HDP moiety of the molecular complex has been followed in chickens surgically prepared to recover urine and faeces separately and, administered orally for 7 days a feed supplemented with 125 mg/kg of each of these compounds. The highest radioactivity in plasma and tissues was reached after 2 and 4 days for DNC and HDP, respectively. Five different studies were performed to measure the excretion pattern of the metabolites using colostomised animals treated for three consecutive days (125 mg/kg with both DNC and HDP labelled using  $^{14}C$ ) followed by three days withdrawal. On the average 91% of the radioactivity derived from  $^{14}C$ -DNC labelled nicarbazin was recovered in the faeces over the 6-day experiments. The plasma clearance and liver residue levels measured (as total radioactivity) gave an indirect indication that DNC is absorbed at a relatively high extent. Moreover, the fact that the bulk of the radioactivity is eliminated in the excreta beyond 24 hours while the intestinal transit time in these animals has been established to 4 hours only, suggests enterohepatic circulation of DNC. For  $^{14}C$ -HDP

labelled nicarbazin, 90% of the radioactivity was recovered in the urine. Total radioactivity kinetics in tissues indicates that DNC related residues are much higher and persistent, especially in the liver, than the HDP ones which become undetectable after five days withdrawal. DNC residues are still measurable at a significant level after 21 days in the liver and muscle (63 and 74 µg/kg wet tissue average values of five animals, respectively). No data were obtained concerning the skin and fat. No tissue depletion studies measuring both the total and individual residues resulting from the two moieties of the nicarbazin molecule have been carried out (EFSA, 2003).

In another study (EFSA, 2003), the metabolic fate of DNC was studied in chickens fed a 50 mg <sup>14</sup>C-DNC labelled nicarbazin/kg diet for 5 consecutive days then slaughtered at zero withdrawal time. The highest radioactivity contents were found in the liver, followed by the kidneys, fat, skin and muscle. DNC was by far (40%) the major compound identified in the excreta, followed by one major (M-1; 25%), two minor (M-2 and M-3) and polar metabolites (35%). The major biliary metabolites were M-1 and M-3. DNC was the major compound found in the liver (79%), but represented only 6% in the kidney. Metabolite M-3 amounted 10 and 13% of the radioactivity measured in the liver and kidney while M-1 represented 2% and trace amounts, respectively. Traces of metabolite M-2 were found in the liver, but not in the kidneys, while polar metabolites represented 68% of the kidney radioactivity. The metabolite M-3 that was isolated from the bile was identified as the acetylamino derivative of DNC. The metabolite M-1, that was also isolated from the bile, was shown to correspond to the di-acetylamino derivative of the two nitro groups. Metabolite M-2, isolated from the excreta, was identified as the N,N'-1,4-phenylenebis (acetamide).

A study was carried out using <sup>14</sup>C-DNC labelled nicarbazin administered to chickens at a dose of 50 mg/kg diet at the same time as an ionophoric anticoccidial and for six consecutive days (EFSA, 2003). Total residual radioactivity and DNC contents were measured in different tissues after 1, 3, 5, and 7 days withdrawal. Liver appeared as the target tissue. Both total radioactivity and DNC concentrations expressed as nicarbazin equivalents followed parallel log-linear depletion curves in the liver, kidney, skin/fat and muscle. The ratios DNC/total residues indicate that DNC constitutes most of the residues found in the skin and fat (>0.77), but also a considerable fraction of those found in the muscle (0.64-0.69), independently of the withdrawal time. Until one day withdrawal the ratio was 0.61 in the liver but it declined to 0.42 and 0.45 after 3 and 5 days respectively. The ratio was much lower in the kidney (0.24 after one day and 0.13 after three days).

Three further experimental studies (EFSA, 2003) were carried out. They were designed to evaluate the depletion of DNC in chicken tissues in conditions similar to those encountered in practice, *i.e.* animals kept on floor pens administered all along the growing period and until commercial weight slaughter 125 mg non-labelled nicarbazin/kg feed with the application of different supplemented feed withdrawal periods. The results of the first study indicated a log-linear decrease of DNC in the liver, muscle and skin/fat over the limited withdrawal period

explored (3 days). The results of the two other studies concerning the target tissue (liver) and the marker residue (DNC) indicated a log-linear decrease of DNC from zero withdrawal until the 7th day of withdrawal. In the other study, an increase of DNC concentration was noted. The problem of birds that ingest the droppings containing the excreted (non-absorbed) nicarbazin has been raised by different authors and it was concluded that under field conditions and use of 125 mg nicarbazin/kg feed, residue concentrations in the liver, but not in the muscle, could exceed 200 µg/kg (EFSA, 2003).

Yoder *et al.* (2005) tested the absorption in chickens gavaged with 8.4 mg of non-labelled nicarbazin per kg b.w. (equivalent to 125 mg/kg feed) for 8 days. Peak plasma DNC levels were 2.87 µg/mL in chickens. It took 6 days to obtain peak DNC levels in chickens. The elimination half-life of DNC in plasma was 1.43 days in chickens. Chickens eliminated 99% of plasma DNC by 8 days post-treatment.

The JECFA (FAO/WHO, 1999) reported results of residue studies in chickens. In one study, chickens were fed diets containing 125 mg/kg of non-labelled nicarbazin for 49 days. The concentrations of DNC in muscle, liver and skin/fat were measured at 24, 36, 48, 60 and 72 hours withdrawal. At 36 hours after withdrawal, the residue levels were highest in liver (2720-7090 µg/kg) followed by skin/fat (680-1060 µg/kg) and muscle (370-880 µg/kg).

In another study (FAO/WHO, 1999), chickens were fed 125 mg non-labelled nicarbazin/kg in the diet from 3 days of age until 44 days of age. At one day withdrawal, the concentrations of DNC in liver ranged from 14 to 21 mg/kg. From these results and by linear extrapolation, a concentration of 0.17, 0.42 and 0.84 mg DNC/kg liver would be anticipated if the chickens had been given feed cross-contaminated at a level of 2, 5 and 10 %, respectively.

## **5.2. Kinetics of nicarbazin in the non-target animal species**

### **5.2.1. Laying hens**

The kinetics of nicarbazin and distribution of residues to body tissues are expected to be the same in laying hens as in chickens for fattening. Several controlled studies and surveys of egg samples have demonstrated that nicarbazin can accumulate in eggs:

An experiment has been designed to establish the relationship between nicarbazin-contaminated feed and nicarbazin residues in eggs (Cannavan *et al.*, 2000). The concentrations of both the DNC and DHP components of the drug in eggs were proportional to feed levels. This relationship for DHP and DNC are respectively:

$$\text{feed nicarbazin (mg/kg)} = 0.230 \times \text{whole egg residue DHP (}\mu\text{g/kg)} + 0.737$$

$$\text{feed nicarbazin (mg/kg)} = 0.0195 \times \text{whole egg residue DNC (}\mu\text{g/kg)} + 0.05$$

Experimentally, a feed concentration of 12.1 mg/kg of nicarbazin (that consisted of 8.6 mg/kg

DNC and 3.5 mg/kg HDP) gave rise to mean maximum whole egg concentrations of 631 µg/kg DNC and 51.8 µg/kg HDP. These results are very close to those expected from the equations. After withdrawal of the experimental diet, DNC was undetectable in eggs after 12 days and HDP after 3 days. DNC was contained almost entirely in the yolk of the egg, whereas HDP was distributed between albumen and yolk in a ratio of approximately 3:1 (Cannavan *et al.*, 2000). For a cross-contamination of 10% of the maximum authorised level (50 mg/kg feed) and using the equations, DNC and HDP levels of 254 and 18.5 µg/kg would be anticipated, respectively in eggs.

Mortier *et al.* (2005b) investigated the effect of nicarbazin cross-contamination at the feed mill at two dietary concentrations 40 mg/kg and 2 mg/kg. Eggs were sampled during treatment (14 days) and for a period of 30 days after withdrawal of the treated feed. Residues were determined by ELISA and LC-MS/MS. For the highest concentration group from day 11 onward, a plateau DNC concentration of 6500 µg/kg egg was observed. This plateau was observed until day 18 of the experiment. Residues can be found for more than 23 days after nicarbazin-free feed was given. For the lowest concentration group the plateau DNC concentration (from day 10 to day 18) was 300 µg/kg egg which points to the risk of cross-contamination. Eggs free of residues are obtained after 15 days withdrawal. From the highest concentration and by linear extrapolation, a concentration of 160, 410 and 810 µg DNC/kg egg would be anticipated if the laying hens had been given nicarbazin at a level of 2, 5 and 10 % of the highest level authorised for target animal species (50 mg/kg), respectively. In a study from Japan (Oishi and Oda, 1989) laying hens were given nicarbazin-containing feed (1.0, 0.5, 0.1 and 0.05 mg/kg) for 10 days, and then control feed for ten days. A comparatively high concentration (226 µg/kg) of nicarbazin (DNC) was found in the eggs in the case of nicarbazin 1.0 mg/kg containing feed. The concentration ratio (nicarbazin in whole egg/nicarbazin in feed x 100) of nicarbazin (DNC) was found to be 22.6% on average, and nicarbazin in the eggs disappeared within approximately ten days. Nicarbazin was barely detectable in the eggs from birds given 0.1 and 0.05 mg/kg nicarbazin-containing feed. From the highest concentration and by linear extrapolation, a concentration of 230, 560 and 1100 µg DNC/kg egg would be anticipated if the laying hens had been given feed cross-contaminated at a level of 2, 5 and 10 % of the maximum level authorised for target animal species, respectively.

### **5.2.2. Ducks**

Mallard ducks (*Anas platyrhynchos*) were fed nicarbazin fortified feed at a concentration of 250 mg/kg during 14 days (Stahl and Johnston, 2002). Samples of excreta were collected on days 7 and 15 (1 day of withdrawal). The concentrations of DNC found in the excreta were 94.4 µg/g on day 7 and 0.30 µg/g DNC on day 15. The low level observed is consistent with reports of rapid excretion of nicarbazin by poultry (FAO/WHO, 1999).

Yoder *et al.* (2005) tested the absorption in mallard ducks given oral gavage doses of 8.4 mg of nicarbazin per kg (equivalent to 125 mg/kg feed) for 8 days. Peak plasma DNC levels were

2.39 µg/mL. It took 8 days to reach this peak. The half-life of DNC in plasma was 0.72 days. Mallards cleared 100% of plasma DNC by 4 days post-treatment.

### **5.2.3. Geese**

Yoder *et al.* (2005) tested the absorption in Canada geese (*Branta canadensis*) given oral gavage doses of 8.4 mg nicarbazin/kg for 8 days. Peak plasma DNC levels were 1.53 µg/mL in Canada geese. It took 8 days to reach this peak. The half-life of DNC in plasma was 1.26 days. Canada geese cleared 100% of plasma DNC by 8 days post-treatment.

Other studies have also examined the absorption of nicarbazin and egg concentrations in Canada geese (Johnston *et al.*, 2001). A peak plasma DNC level of 3.2 µg/mL was measured in chickens free fed 100 mg/kg nicarbazin treated feed. This plasma level correlated with a peak egg DNC level of 5980 µg/kg.

## **6. Risk characterization**

### **6.1. Animal health risks in non-target animal species associated with the accidental consumption of feed materials designated for target animal species**

The limited tolerance studies with non-target animal species provide no evidence that accidental ingestion of feed containing nicarbazin at the maximum authorised level for target animal species of 50 mg/kg feed presents a health risk for non-target animal species. However, the lowest effect level was found in laying hens at a concentration of 25 mg/kg in feed where impairment of egg shell pigmentation was observed. A NOEL for this effect on shell pigmentation was not identified. No case reports describing intoxications in other animal species were available.

### **6.2. Adverse health effect in non-target animal species as a consequence of cross-contamination of feed batches**

Cross-contamination of feed for non-target animal species at a level of up to 10% (equal to 5 mg/kg feed) of the maximum authorised level of nicarbazin would correspond to a dose of 0.25 mg/kg b.w. per day, based on an average feed intake of 50 g/kg b.w. per day. This dose would represent less than 1% of the overall NOEL of 200 mg/kg b.w. per day (150 from DNC and 50 from HDP) from studies in laboratory animals (based on maternal- and foetotoxicity in rats). The Panel concluded that adverse health effects are unlikely to occur in non-target animal species as a result of cross-contamination of feed up to a level of 10% of the maximum authorised level for target animal species.

### **6.3. Residues of nicarbazin in foods derived from non-target animal species exposed to feeds containing nicarbazin**

Liver residues of DNC were measured in chickens that had been given feed containing 125 mg/kg of nicarbazin (see section 5.2.1.). From these results and by linear extrapolation, a concentration of 170, 420 and 840 µg DNC/kg liver would be anticipated if the chickens had been given feed cross-contaminated at a level of 2, 5 and 10 % of the maximum authorised level for target animal species, respectively.

Different studies showed a linear correlation between nicarbazin feed intake and nicarbazin residues in eggs (see chapter 5.2.1.). From these studies and taking the highest levels calculated by linear extrapolation, a concentration of 230, 560 and 1100 µg DNC/kg eggs would be anticipated if the laying hens had been given feed cross-contaminated at a level of 2, 5 and 10 % of the maximum authorised level for target animal species, respectively.

Data on nicarbazin residues in animal-derived food products are available from the monitoring system according to Directive EC 96/23, from extensive surveillance studies conducted in the UK over several years and from market studies as described in Chapter 3.2. Highest residue levels have been found in liver with a maximum concentration of 7200 µg/kg in chicken liver. The highest levels found in chicken muscle, quail eggs and chicken eggs were 110, 540 and 900 µg/kg DNC, respectively.

### **6.4. Human health risk associated with residues in foods derived from non-target animal species following exposure of these animals to contaminated feed batches**

The FEEDAP Panel did not establish an ADI for nicarbazin (as DNC or HDP) in their review of data as provided by the applicant in the course of the premarketing approval procedure of the product koffogran. The reasons were that the toxicological dossier was far from satisfactory according to the present scientific standards, the reporting of the teratogenicity study was inadequately, studies on prokaryotes gave equivocal results, and the lack of studies on eukaryotic cells *in vitro* and only one *in vivo* study. The JECFA established an ADI of 0.4 mg/kg b.w. for nicarbazin based on the NOEL of 200 mg/kg b.w. and applying an uncertainty factor of 500 in consideration of the quality of studies evaluated. The CONTAM Panel used this overall NOEL to evaluate the risk related to cross-contamination of feed materials produced for non-target animal species.

The values for daily human food consumption relevant for calculation of human exposure to nicarbazin from cross-contaminated feed are 100 g of liver, 300 g of muscle and 100 g of eggs<sup>16</sup>.

---

<sup>16</sup> Values for daily human food consumption, as defined in Directive No (EC) 2001/79 are for birds: 300 g muscle, 100 g liver, 10 g kidney (50 g for mammals), 90 g skin/fat in natural proportions (50 g for mammals)

From kinetic data, the estimated levels of ncarbazin (DNC) as a result of eating chicken livers that were fed a diet containing 10% of the maximum level of ncarbazin authorised for chickens for fattening (5 mg/kg) was 84 µg/person or 1.4 µg DNC/kg b.w. per day. The contribution from eggs after a similar exposure to laying hens would be 1.8 µg DNC/kg b.w. per day.

Food survey data show that ncarbazin (DNC) concentrations of up to 7200 µg/kg have been detected in chicken liver, 900 µg/kg in chicken eggs and 110 µg/kg in chicken muscle. Using a conservative approach, a person eating standard portions of 100g chicken eggs, 100 g chicken liver and 300 g chicken muscle would be exposed to 843 µg of DNC (corresponding to 14 µg DNC/kg b.w. per day for a 60 kg person).

Using the NOEL for DNC of 150 mg/kg b.w. and taking a conservative approach using the highest measured concentrations in food as given above, which estimated an exposure of 14 µg DNC/kg b.w. per day for a 60 kg person, the margin of exposure (MOE) would be approximately 10,000. Therefore the CONTAM Panel concluded that there is no indication of an appreciable risk to consumers' health from the ingestion of ncarbazin residues in products from animals exposed to feed cross-contaminated with ncarbazin up to a hypothetical level of 10% of the maximum level authorised for target animal species. Comparing this calculated exposure level of 14 µg DNC/kg b.w. per day to the overall ADI established for ncarbazin, this exposure corresponds to approximately 3.5%.

## CONCLUSIONS

- The limited tolerance studies with non-target animal species did not provide evidence that accidental ingestion of feed containing ncarbazin at 50 mg/kg feed at the maximum authorised level for target animal species, may present a health risk for non-target animal species. The only report of adverse effect below this level was found at a dose of 25 mg/kg in laying hens for which impairment of egg shell pigmentation was observed. No case reports describing intoxications in other animal species were available.
- Animals exposed to cross-contaminated feed, containing diclazuril in a concentration of up to 10% of the maximum authorised level, are unlikely to experience adverse health effects as these concentrations would lead to an exposure well below the overall NOEL of 200 mg/kg b.w. per day from a 2-year rat study.
- No occurrence data or kinetic studies were available to estimate the human exposure to ncarbazin residues from milk.

---

and 100 g eggs (and 1500 g milk). Values for mammals are given in parenthesis when they differ from bird values

- The level of human exposure that was estimated to result from consumption of foods derived from non-target animal species exposed to 10% cross-contaminated diet was estimated from survey data reporting liver, muscle and egg residues in chickens for fattening. Human exposure to nicarbazin residues (dinitrocarbanilide, DNC), resulting from the ingestion of such contaminated diet would be approximately 843 µg/person per day (corresponding to 14 µg/kg b.w. per day).
- Using the NOEL for DNC of 150 mg/kg b.w. and taking a conservative approach using the highest measured concentrations in food as given above, which estimated an exposure of 14 µg DNC/kg b.w. per day for a 60 kg person, the margin of exposure (MOE) would be approximately 10,000.
- The CONTAM Panel concluded that there is no indication of an appreciable risk to consumers' health from the ingestion of nicarbazin residues in products from animals exposed to feed cross-contaminated with nicarbazin up to a hypothetical level of 10% of the maximum level authorised for target animal species.

## **RECOMMENDATION**

- Sensitive analytical methods are available for the detection of nicarbazin in animal products. These methods should be validated also for feed at concentrations below the maximum authorised level, to assess their applicability in the control of cross-contamination of feed batches during the production process.

## **REFERENCES**

- Bohm, D., Hahn, K. and Gowik, P. 2005. List of the screening and confirmatory analytical test methods used in the NRLs of the European Union for the substance groups A5, B2a, B2b, B2e and in the German routine field laboratories for all substance groups of Annex I, Council Directive 96/23/EC. BVL, Berlin.
- Booth, N.H. and McDonald, L.E. 1982. *Veterinary Pharmacology and Therapeutics*. Fifth Edition, The Iowa State University Press, Ames
- Cannavan, A., Ball, G. and Kennedy, D.G. 1999. Determination of nicarbazin in feeds using liquid chromatography-electrospray mass spectrometry. *Analyst*, 124: 1431.
- Cannavan, A., Ball, G. and Kennedy, D.G. 2000. Nicarbazin contamination in feeds as a cause of residues in eggs. *Food Addit. Contam.* 17: 829-836.
- Chapman, H.D. 1994. A review of the biological activity of the anticoccidial drug nicarbazin and its application for the control of coccidiosis in poultry. *Poultry Sci. Res.* 5: 231-234.
- Daeseleire, E., Mortier, L., Delahaut, P., Huet, A.-C. and Huyhebaert, G. 2005. Integrated approach for the control on residues of coccidiostats in eggs. *SPSD2*, 2005.

- Danaher, M., Campbell, K., O'Keeffe, M., Capurro, E., Kennedy, G. and Elliott, C.T. (2008) Survey of the anticoccidial feed additive nicarbazin (as dinitrocarbanilide residues) in poultry and eggs. *Food Addit Contam.* 25:32-40.
- De Jong, J., Tomassen, M., Driessen, J., Keukens, H., Hans-Artur, P. and Brambilla, G. 2004. Liquid chromatographic method for nicarbazin in broiler feeds and premixtures: development, validation, and interlaboratory study. *J. AOAC Int.* 87 (6): 1269-1277.
- Dorn, V.P., Neudegger, J. and Knöppler, H.O. 1988. Zur Problematik der Wirkstoffverschleppung bei der Mischfuttermittelherstellung am Beispiel Nicarbazin. *Tierärztliche Umschau* 43: 524-529.
- Dubois, M., Pierret, G., and Delahaut, P. 2004. Efficient and sensitive detection of residues of nine coccidiostats in egg and muscle by liquid chromatography-electrospray tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 813(1-2): 181-9.
- Dusi, G., Faggionato, E., Gamba, V. and Baguiera, A. 2000. Determination of nicarbazin and clodolol in poultry feeds by liquid chromatography. *J. Chromatogr. A* 882: 79-84.
- EC (European Commission), 1991. Complementary report of the Scientific Committee for Animal Nutrition on Question 52 by the Commission on Narasin + Nicarbazin in feedingstuffs for chickens (Opinion expressed on 7 July 1995). [http://ec.europa.eu/food/fs/sc/oldcomm6/antibiotics/32\\_en.pdf](http://ec.europa.eu/food/fs/sc/oldcomm6/antibiotics/32_en.pdf)
- EC (European Commission), 1995. Report of the Scientific Committee for Animal Nutrition on the use of Narasin + Nicarbazin in feedingstuffs for chickens. (Provisional opinion : 10 July 1991). [http://ec.europa.eu/food/fs/sc/oldcomm6/antibiotics/31\\_en.pdf](http://ec.europa.eu/food/fs/sc/oldcomm6/antibiotics/31_en.pdf)
- EFSA (European Food Safety Authority), 2003. Opinion of the Scientific Panel on Additives and products or Substances Used in Animal Feed on the request from the Commission on the efficacy and safety of the coccidiostat Koffogran", *The EFSA Journal*, 16: 1-40. Adopted on 3 December 2003. [http://www.efsa.eu.int/science/feedap/feedap\\_opinions/185/opinion\\_feedap05\\_ej16\\_koffogran\\_en1.pdf](http://www.efsa.eu.int/science/feedap/feedap_opinions/185/opinion_feedap05_ej16_koffogran_en1.pdf)
- FAO/WHO (Food and Agriculture Organisation/World Health Organization), 1999a. Evaluation of certain veterinary drug residues in food. Fiftieth report of the Joint FAO/WHO Expert Committee on Food Additives, WHO Technical Report Series 888. [http://whqlibdoc.who.int/trs/WHO\\_TRS\\_888.pdf](http://whqlibdoc.who.int/trs/WHO_TRS_888.pdf)
- Harner, J.P., Herrman, T., Falk, J. and Behnke, K. 1996. Avoiding drug carry-over during feed processing and delivery. Kansas State University, MF-2055. <http://www.oznet.ksu.edu/library/grsci2/mf2055.pdf>
- Hughes, B.L., Jones, J.E., Toler, J.E., Solis, J., and Castaldo, D.J. (1991) Effects of exposing broiler breeders to nicarbazin contaminated feed. *Poult Sci.*70:476-82.
- ISO (International Standards Organization), 1997. Capability of detection — Part 1: Terms and definitions, Part 2: Methodology in the linear calibration case Part 2: Methodology in the linear calibration case. ISO 11843-1: 1997. [http://www.iso.org/iso/iso\\_catalogue/catalogue\\_tc/catalogue\\_detail.htm?csnumber=1096](http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=1096)
- IUPAC (International Union of Pure and Applied Chemistry), 1995. Protocol for the design, conduct and interpretation of method-performance studies, *Pure Applied Chem.* 67: 331.

- Jones, J.E., Solis, J., Hughes, B.L., Castaldo, D.J. and Toler, J.E. 1990. Reproduction responses of broiler-breeders to anticoccidial agents. *Poult. Sci.* 69(1): 27-36.
- Johnston, J.J., Britton, W.M., MacDonald, A., Primus, T.M., Goodal, M.J., Yoder, C.A., Miller, L.A. and Fagerstone, K.A. 2001. Quantification of plasma and egg 4,4'-dinitrocarbanilide (DNC) residues for the efficient development of a nicarbazin based contraceptive for pest waterfowl. *Pest Management Science*, 58: 197-202.
- Jones, J.E., Solis, J., Hughes, B.L., Castaldo, D.J. and Toler, J.E. 1990a. Production and egg-quality responses of White Leghorn layers to anticoccidial agents. *Poultry Sci.* 69: 378-387.
- Jones, J.E., Solis, J., Hughes, B.L., Castaldo, D.J. and Toler, J.E. 1990b. Reproduction responses of broiler breeders to anticoccidial agents. *Poultry Sci.* 69: 27-36.
- Kennedy, D.G., Blanchflower, W.J., Hughes, P.J. and McCaughey, W.J. 1996. The incidence and cause of lasalocid residues in eggs in Northern Ireland. *Food Addit. Contam.* 13(7): 787-794.
- Kennedy D.G., Hughes P.J. and Blanchflower, W.J. 1998a. Ionophore residues in eggs in Northern Ireland: incidence and cause, *Food Addit. Contam.* 15(5): 535-541.
- Kennedy, D.G., Smyth, W., Hewitt, S. and McEvoy, J. 1998b. Monensin carry-over into unmedicated broiler feeds. *Analyst* 123: 2529-2533.
- Krabel, B.J., Dickson, D.A., Zimmermann, A.G. and Coleman, M.R. 2000. Liquid chromatographic determination of nicarbazin in feeds. *J. AOAC Int.* 83 (5): 1027-1038.
- Matabudul, D.K., Lumley, I.D. and Points, J.S. 2002. The determination of 5 anticoccidial drugs (nicarbazin, lasalocid, monensin, salinomycin and narasin) in animal livers and eggs by liquid chromatography linked with tandem mass spectrometry (LC-MS-MS). *Analyst* 127(6): 760-8.
- McEvoy, J.D., Smyth, W.G. and Kennedy, D.G. 2003. Contamination of animal feedingstuff with nicarbazin: investigations in a feed mill. *Food Addit. Contam.* 20: 136-40.
- Mortier, L., Huet, A.C., Charlier, C., Daeselaire, E., Delahaut, P. and Van Peteghem, C. 2005a. Incidence of residues of nine anticoccidials in eggs. *Food Addit. Contam.* 22: 1120-1125.
- Mortier, L., Huet, A.C., Daeseleire, E., Huyghebaert, G., Fodey, T., Elliott, C., Delehaut, P. and Van Peteghem, C. 2005b. Deposition and depletion of five anticoccidials in eggs. *J. Agric. Food Chem.* 53: 7142-7149.
- Noser, J., Wenk, P., Sutter, A. and Schneider, P. 2006. Kokzidiostatika in Eiern, Geflügelfleisch und Futtermitteln: Eine Stichproben-Kontrolle über den Nordwestschweizer Markt. *Mitt Lebensm Hyg* 97: 107-120.
- Oishi, Y. and Oda, T. 1989. Residues and disappearance of nicarbazin in eggs when it is fed in a minute dosage to egg laying hens. *Shokuhin Eiseigaku Zasshi (Journal of Food Hygienics)* 30(6): 542-547.
- Rogers, E.F., Brown, R.D., Brown, J.E., Kazazis, D.M., Leanza, W.J., Nichols, J.R., Ostlind, D.A. and Rodino, T.M.. 1983. Nicarbazin complex yields dinitrocarbanilide as ultrafine crystals with improved anticoccidial activity. *Science* 222: 630-632.
- Stahl, R.S. and Johnston, J.J. 2002. High-performance liquid chromatography-based determination of nicarbazin excretion in waterfowl. *Journal of Chromatography B* 775: 103-108.

- Stahl, R.S., VerCauteren, K.C., Kohler, D. and Johnston, J.J. 2003. 4.4'-Dinitrocarbanalide (DNC) concentrations in egg shells as a predictor of nicarbazin consumption and DNC dose in goose eggs. *Pest Management Science* 59: 1052-1056.
- Strauch, W. 2002. Causes and control of carry-over and cross-contamination. *Kraftfutter/Feed Magazine* 85 (4): 151-159 (Part 1). *Kraftfutter/Feed Magazine* 85 (6): 239-249 (Part 2).
- Strauch, W. 2003. Is contamination-free feed production realistic? *Feed Tech* 7 (7): 23-25.
- UK-VMD (United Kingdom-Veterinary Medicines Directorate), 1995-2004. The Veterinary Medicines Directorate Annual Reports on Surveillance for Veterinary Residues for 1995-2004. Published by the Veterinary Medicines Directorate, New Haw, Surrey, England. <http://www.vmd.gov.uk/Publications/AnnReps/AnnReps.htm>
- Yakkundi, S., Cannavan, A., Elliott, C.T., Lovgren, T. and Kennedy, D.G. 2001. Development and validation of a method for the confirmation of nicarbazin in chicken liver and eggs using LC-electrospray MS-MS according to the revised EU criteria for veterinary drug residue analysis. *Analyst* 126(11): 1985-9.
- Yoder, C.A., Miller, C.A. and Bynum, K.S. 2005. Comparison of nicarbazin absorption in chickens, mallards, and Canada geese. *Poult. Sci.* 84: 1491-1494.

## **DOCUMENTS PROVIDED TO EFSA**

- Elanco-Eli Lilly, 2006. Safety of nicarbazin.
- IFAH (International Federation for Animal Health), 2007. IFAH-Europe report to the European Commission on The Future of Coccidiostats and Histomonostats. Final tripartite, 16 July 2007.
- OVOCOM, 2004. Carry-over of coccidiostats authorized as feed additives and medicinal substances in medicated feed. Letter from the European NGOs for Agriculture (COPA-COGECA), Feed manufacturers (FEFAC) and Animal Health (IFAH) to Dr. W. Penning (Head of Unit Animal Nutrition, EU Commission, DG SANCO), dated 25 March 2004.

## **Occurrence data**

- Belgium. AFSCA, The Food Agency.
- Czech Republic. Central Institute for Supervising and Testing in Agriculture.
- European Commission, DG SANCO.