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Safety and efficacy of STENOROL® (halofuginone hydrobromide) as a feed additive for chickens for fattening and turkeys

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Abstract

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the coccidiostat STENOROL® containing halofuginone hydrobromide (halofuginone HBr) as active substance. The FEEDAP Panel was not able to conclude on the safety of STENOROL® for chickens and turkeys for fattening at the highest proposed use level. No incompatibilities or interactions with feedingstuffs, carriers, other approved additives or medicinal drugs are expected. Halofuginone HBr does not have antimicrobial activity at the highest dose proposed; it is not expected to exert adverse effects on chicken gut microbiota or select for resistance and cross-resistance with other antimicrobials. The Panel cannot conclude on the genotoxic potential of halofuginone HBr since an appropriate *in vivo* follow-up to exclude the mutagenic effect of the compound was not available. Therefore, the FEEDAP Panel cannot conclude on the safety of halofuginone HBr for the consumer. The additive is toxic by inhalation, dermal and ocular routes and is very irritant to both the eye and the skin. It is considered also a skin sensitiser. Inhalation exposure is considered a risk to persons handling the additive. Since the lack of genotoxic potential of halofuginone HBr has not been adequately demonstrated, it should be considered as an additional potential concern to users handling the additive. Due to limitations in some of the ecotoxicological studies, no conclusions can be drawn on the safety of the additive for the environment. The FEEDAP Panel is not in the position to conclude on the efficacy of STENOROL® in chickens for fattening and in turkeys for fattening.

Keywords: coccidiostats, halofuginone hydrobromide, chickens for fattening, turkeys, safety and efficacy

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Summary

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the coccidiostat STENOROL® containing halofuginone hydrobromide (halofuginone HBr) as active substance.

The FEEDAP Panel was not able to conclude on the safety of STENOROL® for chickens and turkeys for fattening at the highest proposed use level of 3 mg halofuginone HBr/kg complete feed. No incompatibilities or interactions with feedingstuffs, carriers, other approved additives or medicinal drugs are expected. Halofuginone HBr does not have antimicrobial activity at the highest dose proposed; it is not expected to exert adverse effects on chicken gut microbiota (including shedding of enteropathogens) or select for resistance and cross-resistance with other antimicrobials.

The Panel cannot conclude on the genotoxic potential of halofuginone HBr since an appropriate *in vivo* follow-up to exclude the mutagenic effect of the compound was not available. Therefore, the FEEDAP Panel cannot conclude on the safety of halofuginone HBr for the consumer.

Halofuginone HBr is toxic by inhalation, dermal and ocular routes and is very irritant to both the eye and the skin. It is considered also a skin sensitiser. The same conclusions are applied to STENOROL®. Inhalation exposure is considered a risk to persons handling the additive. Since the lack of genotoxic potential of halofuginone HBr has not been adequately demonstrated, it should be considered as an additional potential concern to users handling the additive.

The fate and behaviour in the environment was evaluated for halofuginone, which is the substance expected to be excreted and, therefore, to reach the environment. Predicted environmental concentrations (PECs) have been calculated for halofuginone in the different environmental compartments. No concern for groundwater is expected. Due to the major limitations in some of the ecotoxicological studies, the FEEDAP Panel cannot establish predicted no effect concentrations (PNECs) for earthworm and for aquatic organisms. Consequently, no conclusions can be drawn on the safety of the additive for the environment. These conclusions apply to chickens for fattening and turkeys.

The FEEDAP Panel is not in the position to conclude on the coccidiostatic efficacy of STENOROL® in chickens for fattening and turkeys for fattening due to the insufficient number of studies with positive results.

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 10(2) of that Regulation also specifies that for existing products within the meaning of Article 10(1), an application shall be submitted in accordance with Article 7, at the latest one year before the expiry date of the authorisation given pursuant to Directive 70/524/EEC for additives with a limited authorisation period, and within a maximum of seven years after the entry into force of this Regulation for additives authorised without a time limit or pursuant to Directive 82/471/EEC.

The European Commission received a request from Huvepharma NV[2] for re-evaluation of the product STENOROL® (halofuginone hydrobromide), when used as a feed additive for chickens for fattening and turkeys (category: coccidiostats and histomonostats).

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 10(2) (re-evaluation of an authorised feed additive). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 15 November 2012.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the product STENOROL® (halofuginone hydrobromide), when used under the proposed conditions of use (see Section 3.1.5).

1.2. Additional information

Halofuginone hydrobromide is included in the European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003 and is authorised for use in chickens for fattening and turkeys (maximum age 12 weeks) at a dose range of 2–3 mg/kg complete feedingstuffs with a withdrawal time of 5 days.[3]

The European Food Safety Authority (EFSA) issued an opinion on the coccidiostat STENOROL® containing 0.6% halofuginone hydrobromide for chickens for fattening and turkeys (EFSA, 2003).

For the use of halofuginone lactate in the prevention of diseases due to *Cryptosporidium parvum* in non-ruminant calves, maximum residue limits (MRLs) are set for halofuginone (marker residue) in the muscle (10 μg/kg), fat (25 μg/kg), liver (30 μg/kg) and kidney (30 μg/kg).[4] Products containing halofuginone lactate are not authorised for use in lactating animals producing milk for human consumption.

The Committee for Medicinal Products for Veterinary Use (CVMP) of the European Medicine Agency (EMA) published two assessment reports on maximum residue limits for halofuginone (EMA-CVMP, 1998, 2000).

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[5] in support of the authorisation request for the use of STENOROL® (halofuginone hydrobromide) as a feed additive.

[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. OJ L 268, 18.10.2003, p. 29.
[2] Huvepharma NV, Uitbreidingstraat 80, 2600, Antwerp, Belgium.
[3] Commission Directive 91/248/EEC of 12 April 1991 amending the Annexes to Council Directive 70/524/EEC concerning additives in feedingstuffs.
[4] Commission Regulation (EC) No 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin.
[5] FEED dossier reference: FAD-2010-0293.

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, peer-reviewed scientific papers and other scientific reports to deliver the present output.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the STENOROL® (halofuginone hydrobromide) in animal feed and the marker residue in tissues. The Executive Summary of the EURL report can be found in Annex A.[6]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of STENOROL® (halofuginone hydrobromide) is in line with the principles laid down in Regulation (EC) No 429/2008[7] and the relevant guidance documents: Guidance for the preparation of dossiers for coccidiostats and histomonostats (EFSA FEEDAP Panel, 2011a), Technical guidance: Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011b), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008a), Guidance for the preparation of dossiers for the re-evaluation of certain additives already authorised under Directive 70/524/EEC (EFSA, 2008b), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012a), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012b), Technical Guidance: Microbial Studies (EFSA, 2008c).

3. Assessment

The present opinion assesses the safety and efficacy of the STENOROL® containing halofuginone hydrobromide (halofuginone HBr) as active substance when used as a coccidiostat feed additive in chickens for fattening and turkeys.

3.1. Characterisation

3.1.1. Characterisation of the additive

The additive STENOROL® contains 0.6% halofuginone HBr, 1% povidone (polyvinylpyrrolidone), 2% castor oil (macrogol glycerol ricinoleate) and 96.4% corn cobs. Analysis of five batches of the additive indicated active substance consistency, mean halofuginone HBr content was 0.62% (0.59–0.63).[8,9] This was in line with the proposed specifications of 0.57–0.63%.

Cadmium, lead, mercury, arsenic levels (all below 0.1 mg/kg in one batch and < 0.01, 0.05, < 0.04 and < 0.005 mg/kg in two other batches) did not raise concerns.[10,11] Aflatoxins (B1, B2, G1 and G2, each below 0.5 μg/kg in one batches and below 1 μg/kg in other two batches) also did not raise concerns.[10,11] The analysis of three batches of the additive indicated the absence of *Salmonella* in 25 g samples.[12]

The additive is presented in form of yellowish to brown granules. A study of three batches (sieve analysis) showed that of the majority of the particles (81.1%) were between 250 and 500 μm and only 0.4% of the particles passed through the sieve with 100 μm mesh size.[13] Bulk and tapped density of the product were in the range of 337–449 and 372–507 kg/m^3, respectively.[13] The dusting potential of the additive (Stauber-Heubach method) was measured in one batch.[14] The average value of four measurements showed a dusting potential of 0.03 g/m^3. Some chemically related impurities might be also present in the additive; those are described below.

[6] The full report is available on the EURL website: https://ec.europa.eu/jrc/en/eurl/feed-additives/evaluation-reports/fad-2010-0293?search&form-return

[7] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

[8] Technical dossier/Section II/Reference 3.

[9] Technical dossier/Supplementary information/March 2015/Reference 1.

[10] Technical dossier/Section II/Reference 27.

[11] Technical dossier/Supplementary information/March 2015/Reference 2 and 3.

[12] Technical dossier/Section II/Reference 4.

[13] Technical dossier/Section II/Reference 5.

[14] Technical dossier/Section II/Reference 6.

3.1.2. Characterisation of the active substance

Halofuginone HBr, a compound of the quinazolinone derivatives group, is obtained via chemical synthesis as a racemic mixture (*trans*-(±)-7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperidyl)-2-oxopropyl]-4(3H)-quinazolinone hydrobromide, $C_{16}H_{17}BrClN_3$·HBr, molecular weight 495.60 g/mol, Chemical Abstracts Service No 64924-67-0). Its structural formula is given in Figure 1.

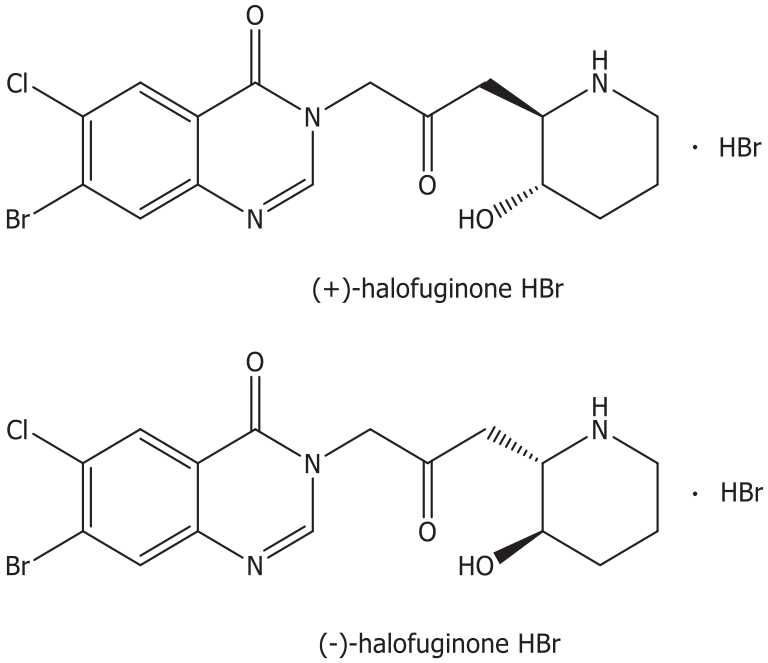

Figure 1: Structural formula of the two *trans*-enantiomers of halofuginone HBr

Chemically related impurities in the active substance were identified as the *cis*-isomer of halofuginone HBr, cebrazolone, methoxy cebegine and melylcebegine (see Appendix A). The specification limit in the active substance for each individual impurity is 0.5% and for the total impurities is 1.0%. In three batches, all impurities were below these limits with values of *cis*-isomer = 0.11%, cebrazolone ≤ 0.02%, methoxy cebegine ≤ 0.34%; melylcebegine was not detected. The total impurities were ≤ 0.43%.[15] Three additional certificates of analysis were provided in which the *cis*-isomer was ≤ 0.5%, any other impurities were 0.3% and the total content of impurities other than the *cis*-isomer was ≤ 1%.[16] Finally, the applicant provided analytical data on the *cis*-isomer in the final additive: in four batches, it was 0.2% while in one batch, it amounted to 0.7%.[8,9] The specification for the *cis*-isomer in the additive is ≤ 2.0%. This probably takes into account that the content of the *cis*-isomer increases over time; this was also noted in the stability tests (see below Section 3.1.4.1).

3.1.3. Manufacturing processes

Halofuginone HBr is produced via a chemical synthesis. [redacted] As the synthesis is not stereoselective, halofuginone HBr is obtained as a racemic mixture. The active substance is micronised. The final additive is manufactured by spray-drying. The micronised active substance is dissolved in the spraying solution which contains isopropyl alcohol, purified water, sulfuric acid, povidone (polyvinylpyrrolidone) and castor oil.

[15] Technical dossier/Section II/2.1.4.
[16] Technical dossier/Supplementary information/August 2016/Reference 1.

3.1.4. Stability and homogeneity

3.1.4.1. Shelf-life of the additive

Three batches of STENOROL® were stored at 25°C/60% relative humidity (RH) for 24 months, at 30°C/75% RH for 12 months and at 40°C/75% RH for 6 months in small size multiple layer bag with internal polyethylene layer. The content of halofuginone HBr and that of the *cis*-isomer were monitored. At the end of the test periods, recoveries of the active substance were in the range of 96.7–98.1, 97.8–98.3% and 91.1–91.8% at the three conditions, respectively.[17] The content of the *cis*-isomer at time zero was 0.2% in two batches while in one batch, it amounted to 0.7%. At the end of the storage period, the following *cis*-isomer content, measured at the three conditions, amounted to 1.43–1.68, 1.51–2.00 and 3.15–3.40%, respectively. The FEEDAP Panel noted that upon storage the *cis*-isomer content increased. Under the storage conditions of 25°C/60% RH (24 months) and 30°C/75% RH (12 months), it remained under the specification limit proposed by the applicant (2.0%) while under accelerated conditions (40 °C/75% RH) it increased up to a level of 3.4%.

3.1.4.2. Stability of the additive used in premixtures and feedingstuffs

Three batches of STENOROL® were incorporated in a vitamin/mineral premixture (with 150 g choline chloride/kg) for chickens for fattening containing 300 mg halofuginone HBr/kg premixture.[18] The samples were stored at 25°C/60% RH and at 40°C/75% RH for 6 months in polyethylene bags. At the end of the test period, the loss in terms of halofuginone HBr content was in the range of 7.4–9.5% and 20.1–22.6% at the two conditions, respectively.

Stability was also studied in a complete feed for chickens for fattening (2 and 3 mg halofuginone HBr/kg feed for each batch) using three batches of the additive.[19] The basal diet consisted on soybean groats, maize and wheat. Mash and pelleted samples (pelleting temperature 65–75°C) were stored at 25°C/60% RH and at 40°C/75% RH for 6 months in polyethylene bags. Concentrations of the active substance in the mash feeds (both inclusion levels) measured at the end of the storage periods showed recoveries in the range of 82–85 and 52–59% at the two conditions, respectively. Pelletisation resulted in a minimal loss of the active substance: recoveries were between 95.7% and 99.1%. Pelleted feed was also put on stability test under the same storage conditions. The results were similar to those obtained with mash feed; recoveries were in the range of 82–84% and 52–67% at 25°C/60% RH and at 40°C/75% RH, respectively.

3.1.4.3. Homogeneity

The same premixtures and complete feeds used for the stability studies were used to assess the capacity of halofuginone HBr from STENOROL® to homogeneously distribute. The coefficient of variation (CV) of the halofuginone HBr concentration in 10 samples was 3.10, 2.83 and 3.42% in the three premixtures prepared with three different batches of STENOROL®. CVs in mash feed were 9.7, 7.9 and 7.1% (inclusion level 2 mg/kg) and 7.6, 7.5 and 4.8% (inclusion level 3 mg/kg). CVs in pelleted feed were 1.8–9.9% at inclusion level of 2 mg/kg and 3.1–3.6% at inclusion level of 3 mg/kg.

3.1.5. Conditions of use

STENOROL® is intended to be used for the prevention of coccidiosis in chickens for fattening and in turkeys up to a maximum of 12 weeks of age at a concentration of 2–3 mg halofuginone HBr/kg complete feed. A withdrawal period of 5 days is foreseen by the current authorisation. The applicant asked the reduction of the withdrawal time to 4 days.

3.2. Safety

In addition to the studies assessed below under the specific chapters, the applicant performed a literature search covering the period 2000–2015[20] on the following aspects of safety: tolerance, microbial safety (including cross-resistance to frequently used antibiotics in human therapy),

[17] Technical dossier/Section II/Reference 13.
[18] Technical dossier/Section II/Reference 14.
[19] Technical dossier/Section II/Reference 15.
[20] Databases searched: PubMed (Technical dossier/Supplementary information March 2015/Reference 23), TOXNET (Technical dossier/Supplementary information March 2015/Reference 26), and Web of Science (Technical dossier/Supplementary information March 2015/Reference 28). Searched terms: halofuginone and broiler, chicken, turkeys, antibiotic, antimicrobial, resistance, cross resistance, toxicity, mutagenicity, genotoxicity, interaction, environment.

interaction with other drugs, toxicological data (including genotoxicity) and environmental safety. None of the papers retrieved by the applicant were considered relevant by the Panel for the current assessment.

3.2.1. Absorption, distribution, metabolism, excretion and residues

In its former assessment (EFSA, 2003), the FEEDAP Panel concluded that: (i) halofuginone was absorbed at a considerable extent in the chicken then metabolised extensively, (ii) liver was the target tissue in the chicken and in the turkey, (iii) unchanged halofuginone represented an important part of halofuginone residues in the liver. However, the following was noted: (i) halofuginone metabolites representing more than 10% total radioactivity isolated from chicken bile and excreta were not identified, (ii) no data on the metabolic fate of halofuginone in turkeys was available, (iii) no comparative metabolism study in chickens, turkeys and laboratory animals was performed and (iv) experimental conditions were not adequate (dose of halofuginone applied, analytical method with limited sensitivity). Moreover, major metabolites found in chicken bile and excreta were not present in the rat. Consequently, the safety of halofuginone for the consumer could not be established.

For the present application, the applicant submitted the following studies to cover the gaps previously identified: (i) metabolism and residue studies in chickens, turkeys and rats, (ii) an attempt to identify halofuginone metabolites in bile and excreta and (iii) an *in vitro* comparative metabolism study of halofuginone in hepatocytes of chickens, turkeys and rats. The *in vivo* and *in vitro* studies were performed with [quinazolinone-2-^{14}C] halofuginone hydrobromide. The labelling position used was justified in a former study (EFSA, 2003) showing the similarity of the metabolic fate of halofuginone ^{14}C-labelled either on the quinazolinone or the piperidine cycles of the molecule and the absence of cleavage of the molecule.

3.2.1.1. Metabolism and residue studies

Chickens for fattening

In the metabolism and residue study in chickens,[21] three groups (1, 2 and 3) of six birds each (three males and three females, 16 days old) were fed (from day 23) for 14 days a complete feed supplemented with a target dose of 3 mg ^{14}C-halofuginone HBr (analytically confirmed: 2.56–3.21 mg/kg feed). Another group (4) received orally a gelatine capsule containing the labelled compound at an equivalent daily dose split in two administrations of 3 ± 0.3 mg/kg feed (five animals) and 2.35 mg/kg feed (one animal). Birds from groups 1, 2 and 3 were slaughtered 0.25, 3 and 4 days after the last feed intake, birds from group 4 were slaughtered 6 days after the last gavage. Tissues (liver, kidney, muscle and skin/fat) were sampled individually. Total radioactivity and halofuginone residues in individual tissues were measured; halofuginone and metabolites were quantified using a validated high-performance liquid chromatography (HPLC) method with a limit of quantification (LOQ) of 0.001 mg/kg wet tissues. Bile was collected from the gallbladder of six birds per group, then pooled by sex (only samples from group 1 were analysed). Excreta from birds of group 4 were collected individually all along the experimental period. Blood was sampled individually from birds of groups 2 and 3 exposed via feed for 11, 12 and 13 days.

Blood radioactivity concentration plateaued after 11 days indicating that metabolic steady state was reached. Bile excretion of radioactivity was considerable (about 9 μg equivalent halofuginone/kg), halofuginone representing 65 and 68% of the whole radioactivity excreted in males and females, respectively, the rest being distributed between 11 metabolites (each < 6.4%). In the excreta, the radioactivity was distributed between halofuginone (83 and 68% in males and females, respectively) and two metabolites E1 (6.9 and 17.3%) and E2 (24.6 and 13.4%). Total radioactivity and halofuginone concentrations measured in tissues along the withdrawal period are reported in Tables 1 and 2, respectively.

[21] Technical dossier/Supplementary information March 2015/Reference 6.

Table 1: Total radioactivity (mg equivalent halofuginone/kg) in tissues of chickens administered 3 mg halofuginone HBr/kg feed for 14 days followed by a withdrawal period(1)

Withdrawal time (day)	Liver	Kidney	Muscle	Skin/fat
0.25	0.855 ± 0.219(1)	0.315 ± 0.073	0.036 ± 0.007	0.056 ± 0.016
3	0.092 ± 0.016	0.026 ± 0.013	0.003 ± 0.001	0.008 ± 0.002
4	0.056 ± 0.023	0.011 ± 0.004	0.002 ± 0.001	0.005 ± 0.001

(1): Average values ± standard deviation from six animals (three males and three females).

Table 2: Halofuginone (mg/kg) residues in tissues of chickens administered 3 mg halofuginone HBr/kg feed for 14 days followed by a withdrawal period(1)

Withdrawal time (day)	Liver	Kidney	Muscle(2)	Skin/fat
0.25	0.641 ± 0.151	0.259 ± 0.057	0.018 ± 0.003	0.039 ± 0.009
3	0.029 ± 0.008	0.013 ± 0.011	< LOQ(2)	0.003 ± 0.002
4	0.011 ± 0.005	0.004 ± 0.002	< LOQ	0.002 ± 0.001

(1): Average values ± standard deviation from six animals (three males and three females).
(2): LOQ = 0.001 mg/kg wet tissue.

Total radioactivity and halofuginone levels in tissues declined considerably along the withdrawal period (results after 6 days showed very low amounts of residues, therefore, not reported here). The ratios halofuginone vs. total residues are shown in Table 3. The FEEDAP Panel notes that these results are in the same range as those produced and assessed formerly (EFSA, 2003).

Table 3: Ratios halofuginone vs total residues in tissues of chickens for fattening at different withdrawal periods

Withdrawal time (day)	Liver	Kidney	Muscle	Skin/fat
0.25	0.75	0.82	0.50	0.70
3	0.32	0.50	–	0.36
4	0.20	0.36	–	0.40

In the study, attempts were made to separate and quantify halofuginone metabolites in tissues. The extractability of halofuginone and metabolites declined from 89% to 47% (after 0.25 and 3 days withdrawal, respectively) in the liver, 94–69% in the kidney and 94–73% in the skin/fat. Halofuginone appears as the marker residue in all tissues at the withdrawal times reported, and liver appears as the target tissue. After 0.25-day withdrawal, all metabolites were each below 7% the total radioactivity extracted from all tissues. After 3-day withdrawal, one metabolite (CL1) amounted to 12% in females and 11% in males in the liver; another metabolite (CK2) amounted to 20% in females and 11% in males in the kidney; and a third metabolite (CS1), in the skin/fat of male chicken only, was 7%. Due to the very low residual radioactivity in the muscle, no analysis was performed.

A marker residue study already assessed by the FEEDAP Panel in 2003 (EFSA, 2003) was provided. Chickens fed halofuginone supplemented feed for a 6-week period under the proposed conditions of use (3 mg halofuginone HBr/kg complete feed).[22] The FEEDAP Panel reassessed the study and noted that the results for the same withdrawal time are in line with the concentrations of halofuginone measured in the total residue study.

Turkeys for fattening

The metabolism and residue study in turkeys[23] followed the same protocol as the study performed in chickens for fattening with the following adjustments: the animals were 49- to 51-day-old at the beginning of the experiment; of the six birds per group initially involved, only two males and three females (group 1) and three males and two females (group 2) remained for tissue sampling. Feed was supplemented with 2.67–3.51 mg ^{14}C-halofuginone HBr/kg and gavage administration was equivalent to 2.37–2.76 mg/kg feed.

[22] Technical dossier/Section III/Reference 32.
[23] Technical dossier/Supplementary information March 2015/Reference 7.

Analysis was performed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Blood concentration of radioactivity plateaued after 12 days. Bile excretion of radioactivity was considerable (about 6 μg equivalent halofuginone/g), halofuginone representing 66% and 37% of the whole radioactivity excreted in males and females, respectively, with one major metabolite (B6) amounting to 17% and 12%, the rest being distributed between seven metabolites (each < 9.5%). In the excreta, the radioactivity was distributed between halofuginone (47% and 57% in the males and females, respectively) and two metabolites E3 (14%) and E4 (10% and 13%). Total radioactivity and halofuginone concentrations measured in tissues along the withdrawal period are reported in Tables 4 and 5, respectively.

Table 4: Total radioactivity (mg equivalent halofuginone/kg) in tissues of turkeys fed 3 mg halofuginone HBr/kg feed for 14 days followed by a withdrawal period

Withdrawal time (day)	Liver	Kidney	Muscle	Skin/fat
0.25(1)	0.666	0.213	0.033	0.070
3(2)	0.090	0.021	0.004	0.017
4(3)	0.039 ± 0.004	0.011 ± 0.002	0.002 ± 0.001	0.010 ± 0.001

(1): The highest values retained from five animals (two males and three females).
(2): The highest values retained from five animals (three males and two females).
(3): Average values ± standard deviation from six animals (three of each sex).

Table 5: Halofuginone (mg/kg) residues in tissues of turkeys administered 3 mg halofuginone HBr/kg feed for 14 days followed by a withdrawal period

Withdrawal time (day)	Liver	Kidney	Muscle(4)	Skin/fat
0.25(1)	0.596	0.179	0.018	0.048
3(2)	0.033	0.012	< LOQ	0.007
4(3)	0.010 ± 0.002	0.006 ± 0.002	< LOQ	0.004 ± 0.001

(1): The highest values retained from five animals (two males and three females).
(2): The highest values retained from five animals (three males and two females).
(3): Average values ± standard deviation from six animals (three of each sex).
(4): LOQ = 0.001 mg/kg wet tissue.

Total radioactivity and halofuginone levels declined considerably along the withdrawal period (results after 6 days showed very low amounts of residues, therefore, not reported here). The ratios halofuginone vs total residues are shown in Table 6. These results are in the same range as those produced and assessed formerly (EFSA, 2003).

Table 6: Ratios halofuginone vs. total residues in tissues of turkeys at different withdrawal periods

Withdrawal time (day)	Liver	Kidney	Muscle	Skin/fat
0.25	0.89	0.84	0.54	0.68
3	0.37	0.57	–	0.41
4	0.25	0.54	–	0.40

In the study, attempts were made to separate and quantify halofuginone metabolites in tissues. The extractability of halofuginone and metabolites declined by 40%, 26% and 42% in the liver, kidney and skin/fat, respectively, between 0.25-day and 4-day withdrawal. Halofuginone is considered the marker residue in all tissues at the withdrawal times reported and liver is the target tissue. Several compounds in the chromatographic analysis of all tissues are supposed to be metabolites of halofuginone, but their structure was not clarified.

A marker residue study, already assessed by the FEEDAP Panel in 2003 (EFSA, 2003) was provided. Turkeys were fed halofuginone supplemented feed for a 13-week period under the proposed conditions of use (3 mg halofuginone HBr/kg complete feed).[24] The FEEDAP Panel reassessed the study and noted that the results for the same withdrawal time are in line with the concentrations of halofuginone measured in the total residue study.

[24] Technical dossier/Section III/Reference 47.

Rat

^{14}C-halofuginone was orally administered to male and female rats (five each) at the dose of 0.2 mg/kg body weight (bw) for 7 days.[25] Faeces were collected daily and the urine in the last day of the experimental period; animals were slaughtered, blood and tissues were sampled. Biliary cannulation was performed in six males and six female rats administered the same dosage for 7 days and bile was collected during 3 days following surgery. Metabolite profiling was tentatively carried out on pooled sample (males and females separately) extracts, using radio-HPLC analysis. However, the separative power of the technique used was limited. Total radioactivity extracted was over 70% for liver and kidney and < 40% for faeces. Halofuginone was by far the main compound found in the urine, faeces and tissues; it was absent (males) or present at very limited extent (females) in the bile. An halofuginone-derived compound was possibly present (similar retention time in a single chromatographic system) in the liver (RL1), kidney (RK1), urine and faeces. In the study, no identification attempt of the metabolites was made.

3.2.1.2. Metabolic profiling and metabolites identification

Additional studies,[26,27] on the metabolic profiles and identification of halofuginone metabolites in the excreta (chicken and turkey), urine and faeces (rat), and tissues were performed using improved HPLC-radiochemical detection conditions, liquid chromatography with high resolution mass spectrometry (LC-HRMS, orbitrap) and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. The samples analysed (pooled by sex) were taken from the three studies described above.

Halofuginone was confirmed to be present in all tissues from chickens, turkeys and rats. One major metabolite corresponded (similar retention times in different chromatographic conditions) to CL1, TL1, TK2 and to one rat liver metabolite (RL1) already separated (see above). A reduction product (+2 mass units) of halofuginone, corresponding likely to the conversion of one of the ketone functions to the secondary alcohol, was reliably identified by LC-HRMS in chicken and turkey liver only. The limited amount of the metabolite detected in other tissues did not allow to draw conclusion on its identity.

Excreta analysis performed in the chicken allowed to separate one metabolite (CE4) amounting to 28% and 33% (males and females, respectively) of the whole radioactivity extracted, all other metabolites being each < 9%. In the turkey, one metabolite in the excreta (TE2) amounted to 19–18%. In the rat, halofuginone was 83–90% in the faeces and 36–42% in the urine; urinary metabolites were all < 9%. The retention times of CE4, TE2 and a minor metabolite (RU9) in rat urine were similar; the same was observed with the minor metabolites CE3 (chicken), TE1 (turkey) and RU6 (rat urine). The reduced metabolite of halofuginone was not detected in poultry excreta and rat urine. However, due to signal suppression related to matrix effects, no chemical structural elucidation of metabolites was not possible.

3.2.1.3. *In vitro* comparative metabolism in the rat, chicken and turkey

The aim of this study was to compare the metabolic profiles of [^{14}C]-halofuginone in rat, chicken and turkey hepatocytes, and to identify the major metabolites.[28] Rat (male Sprague-Dawley), chicken (female Lohman Brown) and turkey (female Norfolk Black) hepatocytes were prepared following a standard procedure. Isolated hepatocytes were tested for number of cells and viability (positive controls with ^{14}C-testosterone and [^{14}C]-7-ethoxycoumarin); the stability of the labelling (negative control) was tested also. Incubations were performed at a concentration of 10 μM halofuginone with 1 million cells/mL for 4 h.

Halofuginone reference standard and hepatocyte incubation samples were analysed by radio-chromatography (LC) and LC-MS/MS generating accurate mass full scans allowing the determination of the elemental composition of the separated compounds. Metabolites of [^{14}C]-halofuginone were observed to possess a characteristic isotope pattern due to the incorporation of the [^{14}C]-isotope and the presence of bromine (^{79}Br, ^{81}Br) and chlorine (^{35}Cl, ^{37}Cl) isotopes in the molecule. This unique isotope pattern in the full scan MS spectra was used during data processing to differentiate between potential metabolites of halofuginone, derived degradation products and unrelated endogenous material.

[25] Technical dossier/Supplementary information March 2015/Reference 8.
[26] Technical dossier/Supplementary information August 2016/Reference 5.
[27] Technical dossier/Supplementary information August 2016/Reference 6.
[28] Technical dossier/Supplementary information August 2016/Reference 7.

The extent of the test item metabolism was similar across the three species with 19.9%, 20.3% and 21.2%. Negative controls indicated an extended breakdown of halofuginone (12% in the absence of cells and 16% with heat inactivated cells). In the chicken and turkey, two metabolites (H2 and H3), each representing around 2% of the whole sample radioactivity, appeared to be formed in the presence of hepatocytes only. The proposed structures for H2 and H3 correspond to the stereo-selective reduction of the aliphatic ketone of the 3-oxopropyl part of halofuginone to the secondary alcohol, forming two diastereoisomers with different retention times. One of these metabolites would correspond to CL1 and TL1 seen in chicken and turkey liver *in vivo*, respectively (see above). Metabolites H2 and H3 were not detected in the rat at the detection limit of 1%. In the rat, a major metabolite was formed (about 7%); this compound was also detected at low levels (< 1%) in turkey and chicken hepatocyte incubations. This metabolite was identified as the glutathione conjugate resulting from the substitution of the bromine atom of halofuginone with the sulfur of the glutathione. It must be noted that the major part of the halofuginone depletion was due to a number of non-metabolic breakdown products, each representing less than 10% of the total radioactivity.

3.2.1.4. Conclusions on ADMER

Halofuginone was absorbed but excreted unchanged at a large extent in chicken and turkey excreta. A major metabolite common to chicken and turkey excreta was also present in rat faeces. Biliary excretion was substantial. Comparative *in vitro* metabolic fate in chicken, turkey and rat, indicated quantitatively similar biotransformation pathways but also a non-metabolic breakdown of the molecule. A major metabolite arising from the reduction of halofuginone was found to be common to the chicken and turkey, and likely the rat; this metabolite was shown to be present *in vivo* in chicken and turkey liver. A second metabolite (a glutathione-conjugate) was identified in rat liver incubations and shown to be present in chicken and turkey. Despite technical difficulties inherent to the chemical structure of halofuginone, the efforts made allow the FEEDAP Panel to conclude that the metabolic fate of this compound is very likely similar in the chicken, turkey and rat. In general, the residues of halofuginone in tissues and organs of chickens and turkeys are of the same magnitude and the ratios halofuginone vs. total residues were similar. Halofuginone is the marker residue and liver is the target tissue.

3.2.2. Safety for the target species

3.2.2.1. Tolerance studies in the target species

Chickens for fattening

A total of 224 1-day-old male and female chickens for fattening (Ross 308) was randomly allocated to four groups with four replicates per sex (five birds + two spare birds for the first week/replicate) each fed diets containing 0, 3.0 (1× maximum level), 4.5 (1.5×) and 6.0 (2×) mg halofuginone HBr/kg), respectively, for 42 days.[29] The basal diet consisted mainly of wheat, soya and maize, no quantitative composition by ingredient was provided. A starter diet (calculated 23.4% CP, 0.52% methionine, 13.3 MJ ME/kg) was provided for the first 14 days, followed by a grower diet (calculated 20.5% CP, 0.58% methionine, 13.6 MJ ME/kg) for the rest of the study. No indication of the physical feed form (mash or pellets) was given. The birds had ad libitum access to feed and water. The intended concentrations of halofuginone HBr in the starter and the grower diet were analytically confirmed at week 1 (for the starter) and 3 (for the grower). The experimental diets were freshly prepared each week, but data on the dietary halofuginone HBr content in weeks 2, 4, 5 and 6 were not submitted.

Health of chickens was monitored daily; body weight and feed intake were recorded at weekly intervals. At the end of the study, blood samples were taken from one bird per replicate (four males and four females per treatment) for haematology[30] and blood biochemistry[31] and one bird/replicate was killed and subjected to necropsy; organ weights were determined for heart, liver, kidneys and spleen. Histopathology was performed for duodenum, ileum, caeca, colon, liver, kidneys, spleen, heart

[29] Technical dossier/Supplementary information August 2016/Reference 2.

[30] Haematocrit, Haemoglobin concentration, Erythrocyte count, Mean corpuscular haemoglobin, Mean corpuscular haemoglobin concentration, Mean corpuscular volume, Total leucocyte count, Differential leucocyte count: Heterophils, Lymphocytes, Basophils, Eosinophils, Monocytes, Thrombocyte - Avian platelets.

[31] Alkaline phosphatase, Alanine aminotransferase, Aspartate aminotransferase, Lactate dehydrogenase, Creatine kinase, Total bilirubin, Bile acids, Uric acid, Creatinine, Glucose, Total cholesterol, Sodium, Potassium, Chloride, Calcium, Inorganic phosphorus, Magnesium, Total protein, Albumin.

and lungs. Two separate statistical analyses were provided; the first one based on separate data sets for male and female birds, and the second one on all birds.[32] Both analyses were based on analysis of variance (ANOVA) after verification of the normality of distributions and homogeneity of variances. If the conditions for applying an ANOVA were not proved, nonparametric procedures (i.e. Kruskal–Wallis one-way analysis of variance on ranks, Shirley's or Steel's test) were applied. Covariance analysis was applied to organ weights (using terminal organ weight as covariate), and the Fisher's exact test to clinical pathology. Group comparisons were done by the Tukey's test/Dunnett's method.

Mortality was low and limited to one bird per treatment (two birds died in the first 3 days, another two were euthanised for welfare reasons (twisted neck, broken wing)). Zootechnical performance data are summarised in Table 7.

Table 7: Zootechnical performance of chickens for fattening fed STENOROL® in a 6-week tolerance study

halofuginone HBr (mg/kg feed)	**0**		**3.0 (1×)**		**4.5 (1.5×)**		**6.0 (2.0×)**	
Sex	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Final body weight (g)	1,623	1,707	1,727	1,665	1,736	1,675	1,655	1,651
Feed intake (g/bird and day)	68	69	71	69	72	69	70	70
Feed to gain ratio	1.76	1.70	1.70	1.72	1.70	1.72	1.78	1.77

The FEEDAP Panel noted that the final body weights achieved during the study in all groups, including the untreated control group, were substantially lower than target values given in the Ross 308 Broiler Performance Objectives 2014[33] (males: 3,023 g; females: 2,595 g) and gender differences were smaller than expected. Also feed intake was considerably lower (performance objectives for males: 120 g/day; females: 105 g/day, respectively). In the control group at the end of the tolerance study (42 days of age), males reached only 56% and females only 65% of the commercially achievable body weight. The FEEDAP Panel considers that such a low performance would not allow to extend any conclusion to fast growing birds under standard European farming conditions; therefore, the study was not further considered.

Turkeys for fattening

The study followed the same experimental design as for chickens for fattening. A total of 224 1-day-old male and female turkeys for fattening (Hybrid Grade Maker) was randomly allocated to four groups with four replicates per sex (five birds + two spare birds for the first week/replicate) each fed soybean meal, wheat and barley-based diets for 57 days. A starter diet (calculated 28.0% CP, 0.69% methionine, 12.7 MJ ME/kg) was provided for the first 14 days, followed by a grower diet (calculated 26.4% CP, 0.61% methionine, 12.6 MJ ME/kg) for the rest of the study. No quantitative composition by ingredient was provided and no indication of the physical feed form (mash or pellets) was given. The intended concentrations of halofuginone HBr at weeks 1 (for the starter diet) and 3 (for the grower diet) were analytically confirmed. The experimental diets were freshly prepared each week, but data on the dietary halofuginone HBr content in weeks 2, 4, 5, 6, 7 and 8 were not submitted. The same protocol was applied as described for the study in chickens.

Mortality was low. Four birds (one each in the control and the 1.5× groups, two in the 2.0× group) were found dead within the first 6 days of the study. Three other birds were euthanised for welfare reasons (two males and one female in the 1.5× group). No treatment-related signs of toxicity were observed during the study. Zootechnical performance data are summarised in Table 8.

[32] Technical dossier/Clarification received via e-mail January 2020.

[33] The Performance Objectives indicate the performance achievable under good management and environmental conditions and when feeding nutrient levels described in the Ross 308 Broiler Nutrition Specifications.

Table 8: Zootechnical performance of turkeys for fattening fed STENOROL® in an 8-week tolerance study

halofuginone HBr (mg/kg feed)	0		3.0 (1×)		4.5 (1.5×)		6.0 (2.0×)	
Sex	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Final body weight (g)	3,843	3,135	3,749	2,958	3,319*	2,830	3,183*	2,985
Feed intake (g/bird and day)	158	116	153	119	164	121	137	110
Feed to gain ratio	2.13	1.96	2.02	2.15	2.54	2.46	2.27	1.97

*: Significant change compared to the respective control within sex ($p < 0.05$).

The final body weights achieved during the study were lower than target values given in the Hybrid Grade Maker Performance Goals 2014[34] (males: 4,610 g; females: 3,820 g) while feed intake was slightly higher (goal for males: 126 g/day; females: 109 g/day, respectively). Males reached in 56 days of the tolerance study only 83% and females only 82% of the commercially achievable body weight already in the control group. This lower growth rate coupled with higher feed intake indicates a poor feed utilisation due to insufficient management or feed quality. Analysis performed on males and females separately indicated differences to the control in final body weight in both overdose groups (1.5× and 2.0×) in males. However, analysis combining the data of both sexes showed that (i) there was no statistically significant interaction between gender and treatment; (ii) analysis of the mean values also indicated statistically significant reduced body weight in both overdose groups (mean values were 3,489, 3,353, 3,013 and 3,084 g in the four treatments). This effect is considered adverse.

There were no consistent treatment-related trends in haematological parameters and no statistically significant differences were seen. No treatment-related effects were seen in the blood biochemistry data. Analysis performed on males and females separately indicated statistically significant changes of the following parameters (Table 9): (i) a high alkaline phosphatase (ALP) value in males at the high dose group was noted owing to very high values of two birds (2,953 and 2,947 U/L), (ii) increased cholesterol concentration was seen in the same group, (iii) a significant increase of creatine kinase (CK) in females of the high dose group has been attributed to the anomalous value obtained in the control group. These differences were considered not toxicologically relevant. Combining male and female data the differences did not reach significance.

Table 9: Blood chemistry parameters showing statistically significant alterations in turkeys for fattening fed STENOROL® in an 8-week tolerance study

halofuginone HBr (mg/kg feed)	0		3.0 (1×)		4.5 (1.5×)		6.0 (2.0×)	
Sex	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
ALP (U/L)	1,930	2,041	2,169	1,979	2,151	1,883	2,457*	2,055
CK (U/L)	1,572	947	1,245	1,743	1,165	1,384	1,030	1,992*
Cholesterol (mmol/L)	3.57	3.44	3.52	3.61	3.44	3.60	4.09*	3.33

*: Significant change compared to the respective control within sex ($p < 0.05$).

No treatment-related findings were observed at necropsy and histopathology. No significant changes due to treatment were observed in absolute organ weights when the analysis was performed on males and females separately. Relative organ weights were calculated and analysed combining the data of both genders.[32] In this case, the 1.5× overdose group showed values significantly higher than the control, but without a macroscopic or microscopic correlate.

Considering the lower performance of the birds and the adverse effect seen at the 1.5× and 2.0× treatments on final body weight, the safety at the proposed use level cannot be established.

[34] The Performance Objectives indicate the performance achievable under good management and environmental conditions and when feeding nutrient levels described in the Hybrid Grade Maker Performance Goals 2014.

Conclusions on the tolerance studies

The FEEDAP Panel was not able to conclude on the safety of STENOROL® for chickens and turkeys for fattening at the highest proposed use level of 3 mg halofuginone HBr/kg complete feed.

3.2.2.2. Compatibility and interactions

For the current assessment, the applicant submitted two compatibility trials with therapeutic drugs and growth promoters (one in chickens[35] and one in turkeys[36]) that were already assessed in 2003. The FEEDAP Panel reassessed the data and confirmed that in both compatibility studies, no adverse effects of the combined use of halofuginone HBr with therapeutic drugs and growth promoters were observed.

Overall, in the absence of new information, the FEEDAP Panel maintains its former conclusion that no incompatibilities or interactions with feedingstuffs, carriers, other approved additives or medicinal drugs are expected.

3.2.2.3. Microbial studies

The applicant submitted an *in vitro* study designed to determine the minimum inhibitory concentration (MIC) of halofuginone HBr on 132 bacterial strains from culture collections already assessed by the FEEDAP Panel in 2003 (EFSA, 2003).[37] The study has been re-evaluated in this opinion and updated its previous conclusion. The strains tested included strain (numbers in parenthesis) of *Escherichia coli* (20), *Enterococcus faecalis* (20) and *E. faecium* (22), *Staphylococcus aureus* (10) and coagulase negative staphylococci (10), *Salmonella spp.* (20), *Clostridium perfringens* (20) and *Campylobacter spp*. (10), and also reference strains of *Bacteroides fragilis*, *Staphylococcus aureus*, *E. faecalis*, *E. coli* and *Pseudomonas aeruginosa*. The microdilution methodology was performed as described by the existing Guidance on the characterisation of micro-organisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018). The majority of the 132 tested strains were resistant at the highest concentration tested (MIC > 128 mg/L), without significant difference between Gram positive and Gram negative. The two most susceptible strains of the 31 showing an MIC below 128 mg/L, presented an MIC of 1 mg/L. This value is higher than the expected gut concentration of 0.75 mg/kg when halofuginone HBr is used at the highest dose of 3 mg/kg feed. The FEEDAP Panel notes that halofuginone HBr is not considered an antimicrobial compound and it is not included in the list of important antimicrobials, defined by the WHO (WHO, 2016). Based on the results of the study and the available scientific information, the FEEDAP Panel concludes that halofuginone HBr has no antimicrobial activity when used as a coccidiostat.

The FEEDAP Panel noted that since halofuginone HBr does not have antimicrobial activity at the highest dose proposed, it is not expected to exert adverse effects on chicken gut microbiota (including shedding of enteropathogens) or select for resistance and cross-resistance with other antimicrobials.

3.2.2.4. Conclusions on the safety for the target species

The FEEDAP Panel was not able to conclude on the safety of STENOROL® for chickens and turkeys for fattening at the highest proposed use level of 3 mg halofuginone HBr/kg complete feed.

No incompatibilities or interactions with feedingstuffs, carriers, other approved additives or medicinal drugs are expected.

Halofuginone HBr does not have antimicrobial activity at the highest dose proposed; it is not expected to exert adverse effects on chicken gut microbiota (including shedding of enteropathogens) or select for resistance and cross-resistance with other antimicrobials.

3.2.3. Toxicological studies

In 2003, the FEEDAP Panel assessed the toxicity of halofuginone HBr (EFSA, 2003). For the current application, the same data assessed in 2003 were submitted with the addition of some studies not available at the time of the previous assessment (genotoxicity) and a literature search covering the period 2000–2015.

The FEEDAP Panel noted that some of the studies reported were performed according to standards appropriate to the time, but in some cases, they were not in accordance either with good laboratory

[35] Technical dossier/Section III/Reference 16.
[36] Technical dossier/Section III/Reference 20.
[37] Technical dossier/Section III/Reference 21.

practice (GLP) or with previous and current OECD guidelines. However, the quality of the studies was considered sufficient for the assessment. An overview of the available studies and the main conclusions are given below.

3.2.3.1. Genotoxicity

The applicant submitted the same *in vitro* and *in vivo* genotoxicity studies assessed by the FEEDAP Panel in 2003 (EFSA, 2003) and newly performed rat bone marrow micronucleus test and an *in vivo* unscheduled DNA synthesis (UDS) in rat hepatocytes.

The FEEDAP Panel reassessed the studies already available in 2003[38] and agreed with its previous conclusions: the genotoxicity tests showed that halofuginone HBr is mutagenic in bacterial tests whereas the *in vitro* tests for gene mutations and clastogenicity in mammalian cells halofuginone HBr did not reveal any genotoxicity; the *in vivo* tests for genotoxicity in bone marrow gave negative results. On the basis of these results, the FEEDAP Panel expressed the need for a second *in vivo* study on a different somatic tissue in order to exclude the genotoxic hazard expressed *in vitro*.

In order to fulfil the requirements, the applicant submitted the following two new *in vivo* studies with halofuginone HBr.

In the micronucleus test,[39] compliant with OECD guideline 474 (revision 1997), 10 rats per sex per experimental group were treated with dosages up to 10 mg halofuginone HBr/kg bw as a single oral dose. Samples of bone marrow were taken 24 and 48 h after the administration and at least 2,000 polychromatic erythrocytes were analysed per animal. No increase was found in the frequency of micronucleated polychromatic erythrocytes, while a statistically significant reduction of the ratio polychromatic erythrocytes vs monochromatic erythrocytes (PCE/NCE ratio) was observed at all doses, demonstrating the exposure of the target cells to the test substance. The results of this study confirmed what was observed in the previous dataset.

In the unscheduled DNA synthesis (UDS) study performed according to OECD guideline 486,[40] three male rats per experimental group were treated orally with 10 (maximum tolerated dose) and 5 mg halofuginone HBr/kg bw. Two expression times were analysed: 2–4 h and 12–16 h after treatment. The group mean net nuclear grain count (NNG) values at the two doses tested of 10 and 5 mg/kg were less than zero in two independent experiments. Furthermore, no significant increase in the mean NNG count values of cells in repair (NNG $\geq$ 5) was observed and the mean percentage of cells in repair was comparable with the solvent control. Moreover, the frequency of cells in S-phase was not higher than in the control animals; therefore, the test item did not induce any proliferative effect in rat liver cells under the experimental conditions used.

The FEEDAP Panel notes that in its opinion clarifying some aspects related to genotoxicity assessment, the EFSA Scientific Committee (EFSA Scientific Committee, 2017) stated that: 'For re-assessment, in cases of already existing UDS data as a follow-up of a positive *in vitro* mutation test, there might be positive or negative results: Test results may be considered as adequate to assess genotoxic potential only in cases with positive results. If the outcome of the UDS is negative, the reliability and significance of results should be carefully evaluated in a WoE approach, taking into account all available information on mode of action (e.g. the type of DNA damage), metabolism, toxicokinetics etc., before deciding whether more sensitive tests such as TGR or *in vivo* comet assay would be needed to complete the assessment.'

In accordance with the above statement, the FEEDAP Panel carefully evaluated all the available information and noted that there is no evidence that the substance is inducing bulky DNA adducts and that the substance is only targeting the liver because it is inducing gene mutation *in vitro* in the presence and absence of metabolic activation. Based on these specific circumstances, the Panel concludes that there is no robust data supporting the negative outcome of the UDS assay.

Conclusions on genotoxicity

Halofuginone HBr did not induce chromosome damage *in vivo* as observed by the micronucleus test in two studies showing negative results in the presence of target tissue exposure. The test item induced significant increase of gene mutations in bacteria, while no gene mutations were observed in mammalian cells *in vitro*; the *in vivo* UDS study was considered not sufficiently informative. Since an appropriate *in vivo* follow-up to exclude the mutagenic effect of halofuginone HBr was not available,

[38] Technical dossier/Section III/Reference 61, 62, 63, 64, 65, 66, 67, 68.
[39] Technical dossier/Section III/Reference 69.
[40] Technical dossier/Section III/Reference 116.

the FEEDAP Panel cannot conclude on the genotoxicity of halofuginone HBr and considers that further testing is needed.

3.2.3.2. Toxicity studies

The applicant re-submitted a total of four sub-acute studies in mice,[41] two subchronic studies in rats[42] and two in dogs (13 weeks[43] and 26 weeks[44]). The FEEDAP Panel re-assessed the studies and summarised the main results as follow.

The lowest no observed adverse effect level (NOAEL) from the subacute mice studies was 0.07 mg halofuginone HBr/kg bw per day[45] based on adverse effects on haematology results (mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration) seen at a dietary level of 0.16 mg halofuginone HBr/kg bw per day.

The lowest NOAEL from the subchronic studies in rat was 0.2 mg halofuginone HBr/kg bw per day based on various effects (including vacuolation of hepatocytes in the livers) at 0.5 mg halofuginone HBr/kg bw per day[46]; the lowest NOAEL from the dog studies[44] was 0.075 mg/kg bw per day based on haematological effects (lower packed cell volume) at 0.175 mg/kg bw per day.

The applicant re-submitted one chronic toxicity/carcinogenicity study in mice[47] and one in rats.[48] In both studies, there were no treatment-related increases in the incidence of any type of tumour. The FEEDAP Panel re-assessed the studies and confirmed that the lowest NOAEL is from the study in rat: 0.11 mg/kg bw per day, based on haematological changes and alopecia in females given 0.23 mg halofuginone HBr/kg bw per day or more.

The applicant re-submitted one multi-generation study in mice[49] and results from the reproduction phase from the chronic toxicity/carcinogenicity study in mice.[47] In the multi-generation reproduction toxicity study in mice receiving 0.136 mg halofuginone HBr/kg bw per day and 0.068 mg halofuginone HBr/kg bw per day, the body weights of males were lower than control values in all generations for F0 and F1 generations, respectively. There was a small dose-related trend to decreased pup weight gain during lactation, but the weights were statistically significantly lower than controls only at 0.136 mg halofuginone HBr/kg bw per day. The NOAEL for the study was 0.034 mg/kg bw per day. A higher NOAEL of 0.15 mg/kg bw per day has been identified from the results from the reproduction phase from the chronic toxicity/carcinogenicity study in mice (highest dose tested in the study).

The applicant re-submitted two developmental toxicity studies in rats[50] and two in rabbits.[51] The lowest NOAEL was identified in rats to be 0.5 mg halofuginone HBr/kg bw per day, with maternal toxicity seen at 0.8 mg/kg bw per day or greater.[52] In the study in rabbits, dose-related reductions in food consumption and body weight gain were seen at 0.03 and 0.09 halofuginone HBr mg/kg bw per day. Increased mortality and morbidity were seen in the 0.09 mg/kg bw per day group, in which four animals aborted. None of the treatment levels caused any embryotoxicity, fetotoxicity or teratogenicity; the NOAEL for this study is 0.03 mg halofuginone HBr/kg bw per day.

The FEEDAP Panel noted that the applicant submitted also other toxicological and/or pharmacological studies.[53] These studies were performed using route of administration (e.g. intravenous) and doses (high and single administration) that are not considered relevant for the current assessment.

3.2.3.3. Conclusions on the toxicological studies

The Panel cannot conclude on the genotoxic potential of halofuginone HBr since an appropriate *in vivo* follow-up to exclude the mutagenic effect of the compound was not available. The evidence of slight maternal toxicity in a teratology study observed in rabbits at 0.03 mg halofuginone HBr/kg bw per day was described in the previous opinion (EFSA, 2003) but dismissed as insignificant as there was

[41] Technical dossier/Section III/Reference 74, 75, 76, 77.
[42] Technical dossier/Section III/Reference 72, 73.
[43] Technical dossier/Section III/Reference 78.
[44] Technical dossier/Section III/Reference 79.
[45] Technical dossier/Section III/Reference 76.
[46] Technical dossier/Section III/Reference 72.
[47] Technical dossier/Section III/Reference 80, 81.
[48] Technical dossier/Section III/Reference 82, 83.
[49] Technical dossier/Section III/Reference 85.
[50] Technical dossier/Section III/Reference 86, 87.
[51] Technical dossier/Section III/Reference 88, 89.
[52] Technical dossier/Section III/Reference 87.
[53] Technical dossier/Section III/Reference 91, 92, 93.

no effect on the offspring. The FEEDAP Panel considers that the lowest NOAEL should be considered as 0.03 mg halofuginone HBr/kg bw per day, based on reproductive effects and maternal toxicity seen in the rabbit teratology study.

3.2.4. Safety for the consumer

The toxicological package identified an NOAEL that could be the basis for setting a health-based guidance value (e.g. an acceptable daily intake (ADI)). Since the lack of genotoxic potential of halofuginone HBr has not been adequately demonstrated, the FEEDAP Panel is not in the position to establish an ADI on which to base the assessment of consumer safety. Therefore, the FEEDAP Panel cannot conclude on the safety of halofuginone HBr for the consumer.

3.2.5. Safety for the user

3.2.5.1. Effects on eyes and skin

Eye and skin irritation and skin sensitisation potential of halofuginone HBr and STENOROL® were already assessed in the FEEDAP opinion in 2003 (EFSA, 2003). The FEEDAP Panel concluded that 'halofuginone appears to be toxic by the cutaneous route and very irritant to both the eye and the skin.' Skin irritancy was not observed with the additive, but it was not tested for eye irritancy. Both halofuginone HBr and STENOROL® caused skin sensitisation; therefore, the Panel concluded that 'STENOROL® is likely to be a skin sensitising agent'. Finally, based on skin irritation studies, the Panel concluded that halofuginone HBr 'had the potential to cause systemic toxicity'. The FEEDAP Panel reassessed the former studies for the purpose of the current assessment.

In the absence of new data, the FEEDAP Panel reiterates its previous conclusions.

3.2.5.2. Effects on the respiratory system

The acute inhalation toxicity study submitted in the dossier was already assessed in 2003 (EFSA, 2003). In this study, the acute inhalation toxicity of halofuginone HBr dust was investigated in a 4-h whole body exposure study in Sprague-Dawley rats at dust concentrations of 3.5, 30, 48, 75, 95, 164 and 1,168 μg/L.[54] All animals died at doses of 75 μg/L and above and 2/10 died at a dose of 48 μg/L. The most severe signs of response in survivors were seen post-exposure and included dyspnoea, abnormal changes in the gastro-intestinal and urogenital tracts and muscular incoordination. An LC_{50} (4 h) of 53 μg/L was set. The FEEDAP Panel reassessed the study and confirmed the previous conclusions i.e. halofuginone HBr showed marked acute inhalation toxicity to the rat.

In the absence of new data, the former conclusion is reiterated: respirable dust of halofuginone HBr can cause respiratory and systemic toxicity in the user.

3.2.5.3. Inhalation exposure

In the former opinion, no data was available to perform an exposure assessment. In the present application, the dusting potential of STENOROL® (Stauber-Heubach method) was provided (0.03 g/m^3)[14] but no data were submitted on the concentration of halofuginone HBr in the dust and on the particle size of the dust. In the absence of data, for the purpose of the inhalation exposure assessment, it is assumed that the concentration of halofuginone HBr in the dust is the same as in the additive (6.3 g/kg corresponding to the maximum value of the specification proposed by the applicant) and it is also assumed that 100% of the particles in the dust is in the respirable fraction. The potential exposure of users by handling the additive to inhaled halofuginone was calculated according to the Technical Guidance on User safety (EFSA FEEDAP Panel, 2012b) and reported in Appendix B. The halofuginone concentration in the inhaled air could be calculated as 0.189 mg/m^3, resulting in inhalation exposure of 0.0263 mg halofuginone HBr per person during an 8-h working day.

Considering the respiratory toxicity and the potential of halofuginone HBr to cause systemic toxicity, it is concluded that the exposure by inhalation indicates a risk to persons handling STENOROL®.

Conclusions on safety for the user

Halofuginone HBr is toxic by inhalation, dermal and ocular routes and is very irritant to both the eye and the skin. It is considered also a skin sensitiser. The same conclusions are applied to STENOROL®. Inhalation exposure is considered a risk to persons handling the additive. Since the lack

[54] Technical dossier/Section III/Reference 94.

of genotoxic potential of halofuginone HBr has not been adequately demonstrated, it should be considered as an additional potential concern to users handling the additive.

3.2.6. Safety for the environment

The active ingredient under assessment is not a physiological/natural substance of established safety for the environment. The additive is also not intended for companion animals only. Consequently, according to Regulation (EC) No 429/2008, the Phase I assessment has to be continued to determine the predicted environmental concentration (PEC), according to the proposed conditions of use in chickens for fattening. In Phase I and Phase II, initially a total residues approach will be taken, meaning that the PECs will be calculated, based on the assumption that the additive is excreted 100% as parent compound.

The FEEDAP Panel evaluated the new studies provided in the dossier and re-assessed the studies already considered in its previous opinion (EFSA, 2003).

3.2.6.1. Phase I

STENOROL® contains 0.6% halofuginone HBr as the active substance and is proposed to be used in feed for chickens and turkeys for fattening at a level up to 3 mg halofuginone HBr/kg complete feed, equivalent to 2.51 mg halofuginone/kg complete feed. The environmental risk assessment is performed on halofuginone, which is the substance expected to be excreted and, therefore, to reach the environment (see Section 3.2.1).

Physico-chemical properties

The physico-chemical properties of halofuginone are summarised in Table 10.

Table 10: Physico-chemical properties of halofuginone

Property	Value	Unit
Octanol/water partition coefficient(1) (log K_{ow} 25°C)	1.06 (pH 5) 1.27 (pH 7) 2.58 (pH 9)	–
Water solubility(2) (20°C)	3.58 6.63 (pH 5) 1.83 (pH 7) 1.52×10^{-2} (pH 9)	g/L
Dissociation constant (25°C)(3) (pKa)	8.07	–
Vapour pressure(4) (VP)	8.1×10^{-7}	Pa

(1): Technical dossier/Section III/Reference 105 and 106.
(2): Technical dossier/Supplementary information March 2015/Reference 4.
(3): Technical dossier/Supplementary information March 2015/Reference 4.
(4): Technical dossier/Supplementary information March 2015/Reference 16.

Fate and behaviour

Fate in soil

Adsorption/desorption in soil

A study[55] on adsorption/desorption was conducted in accordance with OECD guideline 106 on five soils. Table 11 reports the characterisation of the soils used to determine the adsorption/desorption of halofuginone.

[55] Technical dossier/Supplementary information March 2015/Reference 11.

Table 11: Characterisation of the soils used to determine the adsorption/desorption of halofuginone

Soil	Soil 2.1	Soil 2.2	Soil 2.3	Soil 2.4	Soil 6S
Clay < 0.002 mm (%)	3.0 ± 1.0	6.6 ± 1.3	8.9 ± 1.5	27.1 ± 0.2	40.6 ± 1.5
Silt 0.002–0.05 mm (%)	9.3 ± 1.1	12.1 ± 1.3	28.7 ± 4.5	39.8 ± 1.1	37.0 ± 1.6
Sand 0.05–2.0 mm (%)	87.6 ± 0.9	81.3 ± 2.3	62.5 ± 4.7	633.2 ± 1.3	22.4 ± 1.7
Texture class	Sand	Loamy sand	Sandy Loam	Loam	Clay
pH in 0.01 M $CaCl_2$	5.1 ± 0.4	5.5 ± 0.1	6.7 ± 0.3	7.1 ± 0.2	7.1 ± 0.1
Moisture content (% dry basis)	0.52	1.82	1.21	3.87	3.99
Total organic carbon (%)	0.68 ± 0.15	1.93 ± 0.20	0.99 ± 0.08	2.53 ± 0.65	1.66 ± 0.14
Total nitrogen content (%)	0.04 ± 0.01	0.17 ± 0.02	0.08 ± 0.02	0.25 ± 0.05	0.18 ± 0.02
Cation exchange capacity (meq 100 g^{-1})	4.0 ± 1.0	10.0 ± 0.8	10 ± 2.0	29.0 ± 6.2	23.0 ± 6.0

Table 12 reports the Freundlich adsorption ($K_{F(ads)}$) and desorption ($K_{F(des)}$) coefficients, regression constant and the Freundlich adsorption/desorption coefficient corrected for soil organic carbon content (K_{Foc}) for halofuginone in soils.

Table 12: Freundlich adsorption and desorption coefficients, regression constant and K_{OC} for halofuginone in soils

Soil	$K_{F(ads)}$ (1)	1/n	K_{FOC} (2)	$K_{F(des)}$ (1)	1/n
Sand	25.34	0.918	3727	35.09	0.930
Loamy sand	95.32	0.905	4939	113.3	0.788
Sandy loam	116.4	0.877	11759	118.3	0.806
Loam	289.9	0.936	11457	309.1	0.905
Clay	1,289	1.105	7,7375	999.3	1.021
Arithmetic mean	363.1	0.948	21,851	315.0	0.890
Geometric mean	160.0	0.945	11,392	170.8	1.021

(1): $\mu g^{1-1/n}$ $mL^{1/n}/g$).
(2): mL/g.

The pK_a and the solubility studies indicate that halofuginone is a cation below pH 8 and shows increased solubility and decreased sorption at low pH. OECD guideline 106 requires a wide range of pH in order to evaluate the adsorption of the substance in its ionised and unionised forms. The lowest pH in the given soil sorption study was 5. To take into account also acidic soils, the FEEDAP Panel identified the lowest K_{Foc} value of 3727 L/kg as the most appropriate to calculate PEC in Phase I.

Degradation in soil

A study[56] on the degradation of halofuginone in soil was conducted in accordance with OECD guideline 307.

The degradation rate of halofuginone was determined in four soils whose properties are reported in Table 13. Halofuginone was applied at a nominal rate of 40 μg/kg to these four soils and incubated up to 122 days in aerobic conditions in the dark at a temperature of 20°C; soil 2.1 was analysed also in sterile condition.

Table 13: Characterisation of the soils used to determine the biodegradation of halofuginone

Soil	Soil 2.1	Soil 2.2	Soil 2.3	Soil 6S
Clay < 0.002 mm (%)	3.0 ± 1.0	6.6 ± 1.3	8.9 ± 1.5	40.6 ± 1.5
Silt 0.002–0.05 mm (%)	9.3± 1.1	12.1 ± 1.3	28.7 ± 4.5	37.0 ± 1.6
Sand 0.05–2.0 mm (%)	87.6 ± 0.9	81.3 ± 2.3	62.5 ± 4.7	22.4 ± 1.7
Texture class	Sand	Loamy sand	Sandy Loam	Clay
pH in 0.01 M$CaCl_2$	5.1 ± 0.4	5.5 ± 0.1	6.7 ± 0.3	7.1 ± 0.1
Moisture content (% dry basis)	0.52	1.82	1.21	3.99

[56] Technical dossier/Supplementary information March 2015/Reference 14.

Soil	Soil 2.1	Soil 2.2	Soil 2.3	Soil 6S
Total organic carbon (%)	0.68 ± 0.15	1.93 ± 0.20	0.99 ± 0.08	1.66 ± 0.14
Total nitrogen content (%)	0.04 ± 0.01	0.17 ± 0.02	0.08 ± 0.02	0.18 ± 0.02
Cation exchange capacity (meq 100 g^{-1})	4.0 ± 1.0	10.0 ± 0.8	10 ± 2.0	23.0 ± 6.0

The soil disappearance time (DT_{50} and DT_{90}), all recalculated according single first-order kinetics (SFO), is reported in Table 14 together with the arithmetic and geometric means. Since the DT_{50} derived in sterilised conditions shows almost no degradation, the aerobic soil degradation may be considered principally related to biotic processes.

Table 14: Soil biodegradation rate of halofuginone in soils

Soil	DT_{50} (days)	DT_{90} (days)
Soil 2.1	94.4	314
Soil 2.2	48.5	161
Soil 2.3	28.6	94.9
6S	60.9	202
Arithmetic mean	58	193
Geometric mean	53	176
Sterile 2.1	437	> 1,000

The arithmetic mean DT_{50} of 58 days is considered the reference value for the calculation of exposure. This value, normalised to 12°C using the Arrhenius equation,[57] corresponds to a DT_{50} of 123 days.

Conclusions on fate and behaviour

A K_{oc} of 3727 L/kg and a DT_{50} of 123 days at 12°C will be used for the Phase I assessment of the exposure in the different environmental compartments.

Predicted environmental concentrations (PECs)

The calculated PEC initial values for chickens for fattening and turkeys are given in Table 15. The highest dose recommended for chicken for fattening (3 mg halofuginone hydrobromide/kg complete feed, equivalent to 2.51 mg halofuginone/kg complete feed) was considered for calculation of the initial PECs. Results show that chickens represent the worst-case exposure, which covers also turkeys.

Table 15: Initial Predicted Environmental Concentration (PECs) of halofuginone in soil, groundwater, surface water and sediment

Input	Value	
Dose (mg/kg feed)	2.51	
Molecular weight (g/mol)	414.68	
Vapour pressure (Pa)	8.1×10^{-7}	
Solubility (mg/L) at pH 7	1,830	
K_{oc} (L/kg)	3,227	
DT_{50} in soil at 12°C (days)	123	
Output	**Chickens**	**Turkeys**
PEC_{soil} (µg/kg)	13	11.6
$PEC_{groundwater}$ (µg/L)	0.17	0.16

In Phase I, PEC trigger values are exceeded both for soil and for groundwater; a risk assessment for environment, according Phase II, is therefore required.

[57] The temperature correction was performed according to the scientific opinion of the Panel on Plant Protection Products and their Residues on a request from EFSA related to the default Q10 value used to describe the temperature effect on transformation rates of pesticides in soil (EFSA, 2007).

3.2.6.2. Phase II

Exposure assessment

PECs calculation refined in Phase II

Considering the DT_{50} value of 123 days, a recalculation of the different PECs was performed to take into account the possible accumulation during years. The $PEC_{plateau}$ for the different compartments is reported in Table 16. Since the calculated PECs are higher for chicken for fattening, all the evaluation for environment will be referred to this species, which cover also turkey for fattening.

Table 16: Predicted Environmental Concentration at plateau of halofuginone in soil, groundwater, surface water and sediment.

Output	Value
$PEC_{soil\ plateau}$ (μg/kg)	15
$PEC_{groundwater\ plateau}$ (μg/L)	0.23
$PEC_{surface\ water\ plateau}$ (μg/L)	0.08
$PEC_{sediment\ plateau}$ (μg/kg)	14

PEC_{soil} refined for metabolism

Since Halofuginone HBr is excreted unchanged at a large extent in chicken and turkey excreta (4.2.1.), no refinement based on metabolism is possible.

$PEC_{groundwater}$ refinement

To refine $PEC_{groundwater}$, the inequality described in the Guidance for assessing the safety of feed additives for the environment (EFSA, 2008a), related to the requirements for the K_{OM} (= K_{oc}/1.7) as a function of the FOCUS leaching concentration, was applied. Considering the DT_{50} at 20°C of 58 days and the K_{oc} of 3,727 L/kg, halofuginone is considered not to pose any risk to groundwater. This behaviour is also confirmed by the FOCUS calculation provided by the applicant.[58]

FOCUS PEARL version 4.4.4 was used to estimate $PEC_{groundwater}$ for the treatment of chickens for fattening and turkeys using an application rate of 0.039 kg/ha for chickens for fattening (corresponding to 2.51 mg/kg halofuginone) and 0.035 kg/ha for turkeys, the lowest K_{oc} value of 3,727 L/kg and a DT_{50} of 53.6 days, which is almost the same value of the recalculated geometric mean. The two recommended scenarios for avian feed additives were used, namely Jokioinen and Piacenza, and the crop was winter cereal. All other default parameters from the FEEDAP guidance on the environment (EFSA, 2008a) were used. All the calculated $PEC_{groundwater}$ values were below 0.001 μg/L indicating that halofuginone present in STENOROL® is not expected to leach into groundwater.

Conclusions on PEC used for risk assessment

The following exposure values are used for the risk assessment: PEC_{soil} of 15 μg/kg, $PEC_{surface\ water}$ of 0.08 μg/L and $PEC_{sediment}$ 14 μg/kg.

Ecotoxicity studies

Toxicity to soil organisms

Effects on plants

A GLP compliant study following OECD guideline 208 was performed to investigate the effect of halofuginone on terrestrial plants.[59] A sandy loam soil was treated with halofuginone at seven concentrations and seeds from six species were sown (monocotyledon species *Allium cepa* and *Hordeum vulgare*, and dicotyledon species *Raphanus sativus*, *Solanum lycopersicum*, *Cucumis sativa* and *Helianthus annuus*). Seedlings were allowed to emerge and grow for 14 days following emergence of 50% of the control plants under glasshouse conditions. The endpoints determined were the effects on emergence/survival, shoot length, shoot fresh weight and shoot dry weight biomass (Table 17). The study was valid, control seedling emergence was ≥ 70% for all species (actual 73.3–100%) and the mean survival of emerged control seedlings was ≥ 90% for the duration of the study (actual 95.5–100%). Fresh

[58] Technical dossier/Supplementary information August 2016/Reference 8.
[59] Technical dossier/Supplementary information March 2015/Reference 13.

weight biomass was the most sensitive endpoint for all species apart from *R. sativus* were shoot dry weight biomass was the most sensitive. Overall, *S. lycopersicum* was the most sensitive species for the endpoint fresh weight biomass with median effective concentration (EC_{50}) value of 12.6 mg/kg.

Table 17: EC_{50} values for shoot length, shoot fresh and dry weight based on nominal concentration of halofuginone (mg/kg soil dry weight (dw))

Endpoint	*A. cepa* (Onion)	*R. sativus* (Radish)	*S. lycopersicum* (Tomato)	*H. vulgare* (Barley)	*C. sativa* (Cucumber)	*H. annuus* (Sunflower)
EC_{50} Shoot length	51.3	908.8	890.5	389.4	1,022.0	722.4
EC_{50} Fresh weight	18.2	437.1	12.6	58.4	257.8	429.8
EC_{50} Dry weight	32.5	432.1	16.3	149.4	267.3	510.6

Effect on earthworms

The applicant re-submitted the same two studies that were assessed in 2003 (EFSA, 2003).[60] The FEEDAP Panel re-evaluated the information available following the requirements of the guidance to assess the safety of the feed additive for the environment (EFSA, 2008a) and concluded that the data submitted cannot be used for the assessment due to the following limitations: (i) in the first study, the standard control (soil) was not tested while the negative one (soil enriched with the solvent) showed a mortality higher than 50%, (ii) in the second study, the positive controls (with standard reference substance) did not perform as expected (showing low mortality) so the sensitivity of the laboratory culture has not been proved, and also the number of test treatments as well as the range of concentrations tested (only nominal values shown) is limited (concentrations high enough to cause effect have not been tested).

Effects on soil micro-organisms

A GLP compliant study following OECD guideline 216 was performed to investigate the effect of halofuginone on soil micro-organisms.[61] A sandy loam soil was treated with halofuginone at a rate of 13 and 130 μg/kg soil dry weight, equivalent to PEC_{soil} and 10 × PEC_{soil}. Control and treated soils were incubated for 28 days and subsamples were taken on 0, 8, 15 and 28 days after treatment and analysed for the nitrate concentration. The study is valid, variation in nitrate concentration of control replicates was less than 15% (actual ≤ 12.3%) for all time points. Nitrate formation rate deviations from the controls were less than 25% for the PEC_{soil} and the 10 × PEC_{soil} treatments, calculated using both the incremental and overall method, at 28 days after treatment.

Halofuginone has no long-term influence on the nitrogen transformation functionality of soil.

Toxicity to aquatic organisms

Effect on algae

A GLP compliant study following OECD guideline 201 was performed to investigate the effect of halofuginone on cyanobacteria.[62] *Anabaena flos-aquae*, freshwater cyanobacteria species was exposed to 2.56, 6.40, 16.0, 40.0 and 100 mg halofuginone HBr/L (equivalent to 2.13, 5.33, 13.3, 33.3 and 83.3 mg halofuginone/L) for up to 72 h. Concentrations of halofuginone were stable throughout the exposure period with measured concentrations within 5% of nominal concentrations. Therefore, the evaluation of biological endpoints was performed using nominal concentrations. The test meets the validity criteria resulting in inhibitory effect on the growth rate at 72 h E_rC_{50} (median effective concentration which results in 50% reduction in growth rate) of 43 mg/L and NOEC (no observed effect concentration) of 13.3 mg/L.

The FEEDAP Panel notes that the study provided is performed on cyanobacteria and the conclusions from this study cannot be extrapolated to algae.

[60] Technical dossier/Section III/Reference 114 and 115.
[61] Technical dossier/Supplementary information March 2015/Reference 12.
[62] Technical dossier/Supplementary information March 2015/Reference 15.

Effect on crustaceans

The applicant re-submitted the same two studies that were assessed in 2003 (EFSA, 2003).[63] The FEEDAP Panel re-evaluated the information available following the requirements of the Guidance to assess the safety of the feed additive for the environment (EFSA, 2008a) and concluded that the data submitted cannot be used for the assessment due to major limitation in study designs and reporting: in one of the two studies,[64] the exact experimental conditions or validity criteria were not provided; in both studies, exposure concentrations were not verified analytically.

Effect on fish

The applicant submitted four studies investigating the acute toxicity of halofuginone to fish.[65] These studies were already evaluated in 2003 (EFSA, 2003). The FEEDAP Panel re-evaluated the information available following the requirements of the Guidance to assess the safety of the feed additive for the environment (EFSA, 2008a) and concluded that the data submitted cannot be used for the assessment due to major limitations in test design and reporting: the exact experimental conditions or validity criteria were not provided (all studies) and exposure concentrations were not verified analytically (all studies).

Effect on sediment-dwelling organisms

No data on sediment-dwelling organisms were submitted. A log K_{ow} < 3 indicates that sediment effect assessment is not needed (ECHA, 2008).

Conclusions on the ecotoxic effect on soil, water and sediment

For the terrestrial compartment, studies are available for plants, earthworms and micro-organisms. The studies on plants and micro-organisms are performed according the proper guidance documents and are valid. The plant study indicated that *S. lycopersicum* is the most sensitive species showing an EC_{50} of 12.6 mg/kg for fresh weight biomass; the study on micro-organisms showed that halofuginone has no long-term influence on the nitrogen transformation functionality of soil; the two earthworm studies submitted show major limitations and cannot be used for the risk assessment.

For the aquatic compartment, studies on aquatic invertebrates and fish cannot be used for the assessment due to major limitations of the tests submitted. The test performed on cyanobacteria is valid and showed an inhibitory effect on the growth rate at 72 h E_rC_{50} of 43 mg/L and NOEC of 13.3 mg/L; however, the conclusions from this study cannot be extrapolated to algae.

Risk characterisation

Due to the major limitation in some of the ecotoxicological studies, the FEEDAP Panel cannot establish predicted no effect concentrations (PNECs) for earthworm and for aquatic organism. Consequently, no conclusions can be drawn on the safety of the additive for the environment.

Bioaccumulation and risk for secondary poisoning

No data on bioaccumulation of halofuginone were submitted. The octanol/water partition coefficient (K_{ow}) values indicate that the substance is unlikely to bioaccumulate. Therefore, risk for secondary poisoning for worm/fish eating birds and mammals is not likely to occur.

3.2.6.3. Conclusions on safety for the environment

The fate and behaviour in the environment was evaluated for halofuginone, which is the substance expected to be excreted and, therefore, to reach the environment. Predicted environmental concentrations have been calculated for halofuginone in the different environmental compartments. No concern for groundwater is expected. Due to the major limitations in some of the ecotoxicological studies, the FEEDAP Panel cannot establish predicted no effect concentrations for earthworm and for aquatic organisms. Consequently, no conclusions can be drawn on the safety of the additive for the environment.

These conclusions apply to chickens for fattening and extended to turkeys.

[63] Technical dossier/Section III/Reference 103 and 104.
[64] Technical dossier/Section III/Reference 104.
[65] Technical dossier/Section III/Reference 104, 107, 108, 109.

3.3. Efficacy

For coccidiostats under re-evaluation, efficacy data should derive from two types of target animal experiments: a) natural/artificial infection to simulate use conditions (e.g. floor pen studies with poultry), at least one of the locations should be in the EU, b) actual use conditions in field trials, all should be done in the EU within the last 5 years. Anticoccidial sensitivity tests (AST) could replace field trials provided they follow the criteria mentioned in the relevant guidance document on coccidiostats and histomonostats (EFSA FEEDAP Panel, 2011a).[66]

3.3.1. Efficacy in chickens for fattening

3.3.1.1. Floor pen studies in chickens for fattening

Three floor pen trials in chickens for fattening, conducted between 2011 and 2016, were submitted.[67] The study design of those trials is described in Table 18. In each trial, 1-day-old male chickens (Ross 308) were penned and distributed into three treatments, an uninfected untreated control group (UUC), an infected untreated control group (IUC) and an infected treated group (IT). The IT group received feed (wheat, corn, soybean-based diet in trial 1 and wheat, barley and soya-based diet in trial 2 and 3) containing 2 mg halofuginone HBr/kg feed, the lowest dose applied. The intended dietary halofuginone HBr concentration was analytically confirmed (see Table 18). In the infected groups of trials 2 and 3, all birds were inoculated with recent field isolates of pathogenic *Eimeria* species, the seeder bird model was used in trial 1. The experimental diets were fed for 39 days in trial 1, and 35 days in trials 2 and 3 followed by a withdrawal period of 1, 5 and 5 days, respectively. Animal health and mortality were monitored daily. Feed intake and body weight of the animals were measured, feed to gain ratio was calculated. Samples of excreta were analysed for oocyst excretion. Intestinal lesions were scored on two birds per pen in trial 1 and on three birds per pen in trials 2 and 3, following the method of Johnson and Reid (1970) (0 = no lesion, 1 = very mild, 2 = mild, 3 = moderate and 4 = severe).

In each trial, the pen was the experimental unit for the zootechnical parameters; for lesion scores and mortality, the experimental unit was the individual bird. In trial 1, the data were analysed by analysis of variance (ANOVA). In trial 2, performance data were analysed using ANOVA and Duncan's multiple range test for group comparison, whilst intestinal lesion scores were analysed using nonparametric Kruskal–Wallis test and oocyst counts by a two-sample t-test. In trial 3, performance parameters and mortality were analysed by the generalised lineal model, followed by a t-test, while intestinal lesion scores and oocysts counts were analysed by non-parametric tests (χ^2 from Kruskal–Wallis test) followed by a Wilcoxon Mann–Whiney U-test. The level of significance was set at $p < 0.05$.

Table 18: Experimental design of floor pen studies with chickens for fattening fed STENOROL®

Trial (year of conduct)	Replicates per treatment (birds per replicate)	Inoculum characteristics				Feed analysis halofuginone HBr (mg/kg feed)(1)
		Date and country of isolation	Intended dose (number of oocysts) and strain per bird		Day and mode of inoculation	
1 (2011)	12 (12)	2011 The Netherlands	98,000	*E. acervulina*	Day 14 6 birds per pen via syringe	2.0/1.9/3.5
			24,000	*E. maxima*		
			20,000	*E. tenella*		
			4,000	*E. praecox*		
			2,000	*E. mitis*		

[66] The FEEDAP Panel stated in its guidance for the preparation of dossiers for coccidiostats and histomonostats (EFSA FEEDAP Panel, 2011a) that studies with artificial infection would be preferred over field trials due to their inherent weaknesses. These short-term studies should use field strains of *Eimeria*, recently confirmed as pathogenic/resistant by a sensitivity test or recognised problems in the poultry operation (confirmed by veterinary certificate). The *Eimeria* field strains should ideally undergo one, but in any case not more than two passage(s) before use in such trials.

[67] Trial 1: Technical dossier/Supplementary information March 2015/Reference 17. Trial 2: Technical dossier/Supplementary information March 2015/Reference 18. Trial 3: Technical dossier/Supplementary information August 2016/Reference 9.

Trial (year of conduct)	Replicates per treatment (birds per replicate)	Inoculum characteristics				Feed analysis halofuginone HBr (mg/kg feed)(1)
		Date and country of isolation	Intended dose (number of oocysts) and strain per bird		Day and mode of inoculation	
2 (2013)	8 (13)	2013 Belgium	38,800	*E. acervulina*	Day 15 orally via gavage	1.57/1.62
			19,200	*E. maxima*		
			4,000	*E. mitis*		
			4,400	*E. tenella*		
			1,600	*E. praecox*		
3 (2016)	14 (40)	2014 Spain	100,000	*E. acervulina*	Day 14 orally via feed	2.1/2.0/2.02
			20,000	*E. tenella*		
			50,000	*E. maxima*		

(1): In trial 1, birds received starter diet from day 0 to 14, grower diet from day 15 to 35 and finisher diet from day 36 to 40. In trial 2, birds received starter diet from day 0 to 21 followed by grower diet until study completion. In trial 3, birds received starter diet for 2 weeks, grower diet for another 2 weeks, followed by a finisher diet until study completion.

In trials 1 and 3, mortality was very low (6 out of 432 and 33 out of 1680 birds, respectively). In trial 2, mortality was observed already on day 2 of the experiment; 22 birds out of 312 died in the first week. Since yolk sac infection was considered a possible reason, antibiotic treatment with lincomycin and spectinomycin (0.16 g/L) via water for drinking was applied 3 days after study start, for 5 days. Total mortality amounted to 34 birds without differences between the groups; a necropsy report was not provided.

In all trials, birds from the UUC groups revealed none or negligible intestinal lesion scores (ILS). In trial 1, significant lower ILS of seeder birds was observed in IT birds compared to IUC birds 6 days after inoculation due to *E. acervulina* (1.0 vs 2.3) and *E. maxima* (0.3 vs 0.6); no significant differences were found in contact birds between the two groups 13 days after inoculation. In trial 2, significant lower ILS was observed in IT birds compared to IUC birds 6 days (caecum 0.4 vs 1.7) and 13 days after inoculation (upper small intestine 0 vs 0.6; caecum 0 vs 0.3). In trial 3, significant lower ILS was observed in IT birds compared to IUC birds 6 days after inoculation in all intestinal segments (upper intestine 1.9 vs 0; middle intestine 1.8 vs 0; caecum 2.2. vs 0). No lesions were found in any animals 12 days after inoculation. Detailed results of ILS are reported in Appendix C.

In trial 1, oocyst excretion in the UUC group increased over time, probably due to poor hygiene management. Differences in the oocyst excretion between IT and IUC are therefore not considered since they could also be influenced by cross-contamination. In trial 2, UUC birds remained free of oocysts throughout the whole study period. A significantly lower excretion of oocysts was found in IT birds when compared to IUC birds at day 13 and day 20 post-inoculation. In trial 3, no oocysts were detected in samples from IT and UUC pens at sampling days 6 and 12, whereas a considerable oocyst excretion (*E. acervulina*, *E. maxima* and *E. tenella*) was observed in the IUC group. No oocysts were detected in the excreta of all groups 19 days post-inoculation. In Table 19, the performance parameters are reported. In trials 1 and 2, no significant differences were observed between IUC and IT birds for all performance parameters. In trial 3, IT birds had significantly higher feed intake, final body weight, weight gain and better feed to gain ratio than IUC birds.

Table 19: Performance parameters of chickens for fattening fed STENOROL® in floor pen studies(1)

	Feed intake (g/day)	Final body weight (g)	Weight gain (g/day)	Feed to gain ratio
Trial 1				
UUC	138	2,801^{a}	69^{a}	1.73^{a}
IUC	134	2,698^{b}	66^{b}	1.79^{b}
IT	135	2,728^{b}	67^{b}	1.79^{b}
Trial 2				
UUC	84^{b}	2,458^{b}	–	1.41^{a}
IUC	76^{a}	2,059^{a}	–	1.54^{b}
IT	75^{a}	2,073^{a}	–	1.49^{ab}

	Feed intake (g/day)	Final body weight (g)	Weight gain (g/day)	Feed to gain ratio
Trial 3				
UUC	98*	2,279*	64*	1.53*
IUC	94	2,163	61	1.56
IT	100*	2,397*	67*	1.49*

Means with an * are significantly different from IUC ($p < 0.05$).
a,b: means in a column in a trial are significantly different ($p < 0.05$).
(1): Results refer to day 40 for trial 1 and to day 35 for trials 2 and 3.

3.3.1.2. Anticoccidial sensitivity tests in chickens for fattening

Three anticoccidial sensitivity tests performed in 2013–2015 were submitted.[68] The experimental design is described in Table 20. The birds were randomly allocated to the treatment groups (UUC, IUC, IT). The IT group received feed (wheat, maize and soya bean meal-based diet) containing 2 mg halofuginone HBr/kg feed, the lowest dose applied. The intended dietary halofuginone HBr concentration was analytically confirmed (Table 20). Animal health and mortality were monitored. Feed intake and body weight of the animals were measured, feed to gain ratio was calculated. Samples of excreta were analysed for oocyst excretion. Intestinal lesions were scored following the method of Johnson and Reid (1970) (0 = no lesion, 1 = very mild, 2 = mild, 3 = moderate and 4 = severe).

In AST-1, the statistical analysis followed ANOVA for performance parameters and when significant differences were encountered, Tukey's test was used for group comparison. Lesion scores were statistically analysed using Mann–Whitney U test, oocyst counts by two-sample T-test and mortality by Firth's logistic regression. In AST-2 and AST-3, linear regression models were used for all parameters except the mortality that was analysed using the Cox's proportional hazards model. In all three studies, significance was set at $p < 0.05$.

Table 20: Experimental design of ASTs with chickens for fattening using STENOROL®

Study (year of conduct)	Replicates per treatment (birds(1) per replicate)	Inoculum characteristics				Anticoccidial treatment(2) (days of life)	Feed analysis halofuginone HBr (mg/kg feed)
		Date and country of isolation	Intended dose (number of oocysts) and strain per bird		Day of inoculation		
AST-1 (2013)	3 (6)	2013 Scotland	194,800	*E. acervulina*	15	13–22	1.6
			25,000	*E. maxima*			
			20,500	*E. tenella*			
			11,500	*E. praecox*			
AST-2 (2015)	8 UUC 8 IUC 6 IT (5)	3/2012 Belgium	148,800	*E. acervulina*	15	13-21	1.5
			17,600	*E. maxima*			
			31,200	*E. tenella*			
			68,000	*E. mitis*			
AST-3 (2015)	8 (5)	6/2015 Belgium	64,000	*E. acervulina*	15	13-21	2.05
			13,000	*E. tenella*			
			9,000	*E. maxima*			
			3,000	*E. mitis*			
			3,000	*E. necatrix/ E. praecox*			

(1): Male Ross PM3 in AST-1 and male Ross 308 in AST-2 and AST-3.
(2): Birds in the IT group were fed a basal diet supplemented with STENOROL®. Animals in the control groups UUC and IUC received the same basal diet without inclusion of the coccidiostat.

In AST-1, the absence of mortality in UUC and IT groups and a rather high coccidiosis-related mortality in the IUC group (38.9%) indicate coccidiostatic efficacy of the additive. In AST-2 and AST-3, mortality was very low (one bird in AST-3 and four birds in AST-2) and not related to treatments.

[68] AST-1: Technical dossier/Supplementary information March 2015/Reference 19. AST-2: Technical dossier/Supplementary information August 2016/Reference 10. AST-3: Technical dossier/Supplementary information August 2016/Reference 11.

The results of ASTs are summarised in Table 21.

In AST-1, ILS due to *E. maxima* and *E. tenella* were significantly reduced in the IT group compared to IUC. Although the total oocyst excretion per bird and day was not influenced by the treatment, no *E. maxima* oocysts were excreted in the IT group compared to 1.4 × 10^6 oocysts per bird per day in the IUC. In AST-2, when comparing IT with IUC, significantly lower ILS was seen due to *E. tenella* but higher ILS due to *E. acervulina* and no effects were seen on oocyst excretion. In AST-3, ILS due to *E. acervulina* and *E. tenella* were significantly lower in IT than in IUC; oocyst excretion was not significantly affected by the treatment. Regarding the zootechnical performances, significant positive results observed in AST-1 and AST-3 were considered as a consequence of the coccidiostatic action of the additive. In contrast, positive effects on zootechnical performance seen in AST-2 were not considered since no effect on the main study endpoint was demonstrated.

Table 21: Results of anticoccidial sensitivity tests in chickens for fattening fed STENOROL®

Group	Oocyst excretion(1)	Lesion scores (Ea/Em/Et)	Body weight (g)	Feed intake (g/day)	Weight gain (g or g/day)	Feed to gain ratio
AST-1	**D22**	**D22**	**D22**	**D13–22**	**D13–22**	**D13–22**
UUC	0^b	$0^a/0^a/0^a$	948^a	91^a	549^a	1.49^a
IUC	96^a	$2.6^b/1.9^c/3.6^c$	606^b	68^a	205^c	3.23^c
IT	194^a	$2.6^b/1.1^b/2.3^b$	701^b	72^a	299^b	2.18^b
AST-2	**D19–21**	**D21**	**D21**	**D15–21**	**D15–21**	**D15–21**
UUC	0*	0.02*/0.2*/0	790*	98*	60*	1.62
IUC	5.81	1.24*/0.87/0.66*	599*	73*	28*	2.78*
IT	5.93	2.28/0.72/0	743	81	49	1.73
AST-3	**D19–21**	**D21**	**D22**	**D15-22**	**D15-22**	**D15-22**
UUC	0	0.32*/0.36*/0	953	109	75*	1.46*
IUC	5.6	2.54*/1.33/0.79*	858*	106	61	1.73
IT	5.2	1.75/1.54/0.04	937	112	67	1.62

Ea: *E. acervulina*; Em: *E. maxima*; Et: *E. tenella.*
a, b, c: means with different letters in a column are significantly different ($p < 0.05$).
*: IT mean significantly different from IUC mean and/or IT mean significantly different from UUC mean ($p < 0.05$).
(1): In trial 1, number of oocysts excreted per day per bird × 10^6; in trials 2 and 3 log OPG (oocyst per gram excreta).

3.3.1.3. Synopsis on efficacy studies in chickens for fattening

Significant positive effects of the additive were observed in two floor pen trials, whereas in a third trial, no clear effect could be observed (trial 1). In this trial, reduction of ILS was seen only on seeder birds and not in contact birds; a further increase in oocyst excretion in UUC suggests insufficient hygiene management. This trial is therefore not considered further.

Two ASTs (AST-1 and AST-3) showed significant improvements by the treatment in intestinal lesion scores and no effect was observed on oocyst excretion. In a third AST (AST-2), the variable effects seen in lesion scores could not be considered as demonstrative for the coccidiostatic efficacy.

In summary, two floor pen trials and two ASTs are indicative for the coccidiostatic efficacy of the additive in chickens for fattening.

3.3.2. Efficacy in turkeys for fattening

3.3.2.1. Floor pen studies in turkeys for fattening

Three floor pen studies in turkeys for fattening, conducted between 2013 and 2016, were submitted.[69] In each study, turkeys were penned and distributed to the experimental groups (UUC, IUC, IT). The IT groups received feed containing 2 mg halofuginone HBr/kg feed, the lowest dose applied. In trials 1 and 3, wheat and soya bean meal-based diets were fed; for trial 2, no information was provided on the composition and the nutrient contents of the diets for the starter and grower phase. In the infected groups of trials 1 and 3, all birds were inoculated with field isolates of pathogenic *Eimeria* species; the seeder bird model was used in trial 2. The experimental design is

[69] Trial 1: Technical dossier/Supplementary information March 2015/Reference 20. Trial 2: Technical dossier/Supplementary information March 2015/Reference 21. Trial 3: Technical dossier/Supplementary information August 2016/Reference 12.

summarised in Table 22. The experimental diets were fed for 84 days. The intended dietary halofuginone HBr concentration was analytically confirmed (see Table 22). Animal health and mortality were monitored daily. Feed intake and body weight of the animals were measured, feed to gain ratio was calculated. Samples of excreta were analysed for oocyst excretion. In trial 2 also lesion scoring was performed using the Repérant scoring system (score from 0 (no lesions) to 4 (severe lesions)).

Table 22: Experimental design of floor pen studies with turkeys for fattening fed STENOROL®

Trial No (year of conduct)	Replicates per treatment (birds(1) per replicate)	Inoculum characteristics				Feed analysis halofuginone HBr (mg/kg feed)
		Date and country of isolation	Intended dose (number of oocysts) and strain per bird		Day and mode of inoculation	
1 (2014)	10 for IUC and IT/9 for UUC (23 to 26)	2008 France	72900	*E. meleagrimitis*	Day 14 via feed	2.1/2/1.9
			65,400	*E. gallopavonis* + *E. adenoeides*		
			83,700	*E. meleagridis*		
2 (2013)	8 (25)	2013 UK	26,700	*E. meleagrimitis*	Day 16 via syringe (8 birds per pen)	2.1/1.8/2.0
			4,300	*E. adenoeides*		
			1,800	*E. dispersa*		
3 (2016)	12 (25)	2014 Austria	68,628	*E. meleagrimitis*	Day 14 via feed	1.9/2.1/1.7
			1,151	*E. meleagridis* + *E. gallopavonis*		
			13,968	*E. adenoeides*		

(1): Female BUT10 in trials 1 and 3, male Converter hybrid in trial 2.
(2): In trial 1, birds received starter diet in crumble form from day 0 to 28, grower feed in pellets from day 28 to 56 and finisher feed as pellets until day 84. In trial 2, birds received starter diet in mash feed from day 0 to 33 followed by grower diets as pelleted feed (day 33–55 and 56–84). In trial 3, birds received starter diet in crumble form from day 0 to 27, grower feed in pellets from day 28 to 56 and finisher feed as pellets until day 84.

In trial 1, the data were subjected to analysis of variance (ANOVA). A two-sided significance level $\alpha = 0.05$ was used. Differences between IT and IUC groups were established by least significant differences (LSD) test for continuous variables and by Wilcoxon test for categorical data. In trial 2, generalised linear models (ANOVA) were used for normally distributed variables (feed intake, feed to gain ration and log-transformed oocysts/g of faeces) followed by (LSD) test for group comparisons; mixed models were used for live weight and average daily weight gain, and Kaplan–Meier survival analysis was used for mortality. In trial 3, continuous variables were analysed with the mixed linear model. Categorical data (e.g. mortality) and oocysts counts were analysed using the non-parametric exact Wilcoxon two-sample test. The level of significance was set at $p < 0.05$.

In trial 1, mortality was low (0.9, 2 and 1.6% in UUC, IUC and IT groups, respectively). The inoculum used was at least six times passaged before administration and may have lost at least a considerable part of its pathogenicity. This assumption is supported by the final bodyweight of the IUC group which does not show any depression due to the infection (UUC 7,402 g vs IUC 7,491 g). The IUC group showed also a significantly better feed to gain ratio than UUC (2.14 vs 2.19). The FEEDAP Panel notes that Stenorol® increased significantly final body weight in comparison to the UUC group (7,699 g vs 7,402 g), which might indicate a growth promoting effect of the additive. Although oocyst excretion was significantly reduced by the treatment on days 7, 11 and 13 post-inoculation (Appendix D), considering the above (low pathogenicity of the inoculum, growth promoting effect), the Panel concludes that this study provides only supporting evidence of the efficacy.

In trial 2, coccidiosis-related mortality of the IUC group was significantly higher than in the IT group (28 vs 3, each out of 200). In trial 3, total mortality was low and not different between the treatments (seven birds in IT and IUC groups, each) and not coccidiosis related.

In trial 2, oocyst excretion was counted on days 7, 11, 17 and 28 post inoculation. The number of oocysts per gram (OPG) of excreta was significantly reduced in the IT group compared to the IUC group on day 7 (from 56,700 to 22,975) and 28 post inoculation (from 41,150 to 6,525). In trial 3, oocyst excretion was counted on days 7, 11, 13, 41 and 69 post inoculation. Total oocyst excretion was significantly reduced in the IT group compared to the IUC group on day 7 (from 5,754 to 1,202) and on day 11 post inoculation (from 891 to 89). The excretion decreased over time and no oocyst

was detected on day 69 post inoculation in any of the groups. Detailed results of oocyst excretion are reported in Appendix D.

Intestinal lesion scores were examined only in trial 2, on days 5 (seeder birds), 11 (contact birds) and 17 (contact birds) post inoculation. The intestinal lesion scores for IT and IUC were significantly higher than in UUC on days 5 and 17 post inoculation. No difference was observed between IT and IUC birds.

In trial 2, performance data are difficult to interpret since final body weight was significantly higher in the IT group (8,926 g) compared to UUC (8,679 g) and feed to gain ratio was significantly better for IUC (1.56) to UUC (1.77) and IT (1.73). In trial 3, no significant differences in zootechnical parameters between IT, IUC and UUC were seen at the end of the study.

3.3.3.2. Anticoccidial sensitivity tests in turkeys for fattening

Three anticoccidial sensitivity tests performed in 2012–2016 were submitted.[68] The experimental design of the three studies is described in Table 23. The birds (male Grand Maker in AST-1 and male Converter Hybrid in AST-2 and AST-3) were randomly allocated to the treatment groups (UUC, IUC, IT). The IT group received feed containing 2 mg halofuginone HBr/kg feed, the lowest dose applied. The intended dietary halofuginone HBr concentration was analytically confirmed (Table 23). Animal health and mortality were monitored. In AST-1, the appearance of the excreta was assessed.[70] Feed intake and body weight of the animals were measured, feed to gain ratio was calculated. Samples of excreta were analysed for oocyst excretion. Intestinal lesions were also scored according to an in-house scoring scale in AST-1[71] and according to the Repérant scoring system in AST-2 and AST-3.

In AST-1, final body weight and weight gain were statistically analysed by a linear mixed model and differences between groups checked with a Tukey's test[72] while ILS was statistically examined by Mann–Whitney U test.[73] In AST-2 and AST-3, statistical analyses were performed on body weight, daily weight gain, daily feed intake, feed to gain ratio, total intestinal lesion score and OPG (oocyst concentration per gram excreta), using a linear regression model with treatment group as fixed effect. Intestinal lesion scores were analysed using ordinal regression models. Statistical significance was assessed at $p < 0.05$.

Table 23: Experimental design of ASTs with turkeys for fattening fed STENOROL®

Study (year of conduct)	Replicates per treatment (birds(1) per replicate)	Inoculum characteristics			Day of inoculation	Anticoccidial treatment(2) (days of life)	Feed analysis halofuginone HBr (mg/kg feed)
		Date and country of isolation	Intended dose (number of oocysts) per bird and strain				
AST-1 (2012)	6 (3)	11/2011 UK	50,000	*E. meleagrimitis*	23	21–32	1.7
				E. dispersa			
				E. adenoeides			
AST-2 (2016)	6 (6)	9/2015 Belgium	60,000	*E. meleagrimitis*	16	14–22	2.11
			500	*E. dispersa*			
			500	*E. adenoeides*			
AST-3 (2016)	6 (6)	3/2014 France	475,200	*E. meleagrimitis*	16	14–22	2.20
			7,200	*E. dispersa*			
			38,400	*E. adenoeides*			

(1): Male Grand Maker in AST-1 and male Converter Hybrid in AST-2 and AST-3.
(2): Birds in the IT group were fed a basal diet supplemented with STENOROL®. Animals in the control groups UUC and IUC received the same basal diet without inclusion of the coccidiostat.

Table 24 summarises the results of the ASTs.

No mortality occurred in any of the studies. In all ASTs, there was no significant effect of the additive on the oocyst excretion. In AST-1, lesions scores due to *E. meleagrimitis* and *E. adenoeides*

[70] According to an in-house scoring scale from 0 (normal drops) to 4 (liquid faeces with mucus, blood and pseudo-membranous material or caseous material).
[71] Score from 0 (no lesions) to 4 (severe lesions).
[72] Technical dossier/Clarification via e-mail January 2020/Efficacy/R-H-2012-04-stat.
[73] Technical dossier/Clarification via e-mail January 2020/Efficacy/Lesion scorings AST Turkeys reference 22_MG280120.

were significantly reduced by the treatment. The consistency of the excreta in the IT and IUC groups was altered compared to UUC, the droppings were soft or very soft but never diarrheic. In AST-2 and AST-3, the lesion scores in the IT group were significantly lower than in the IUC group.

In AST-1, body weight gain in the sensitive period (day 21–29) was significantly depressed by inoculation and partially restored by the anticoccidial treatment. Feed to gain ratio showed the same picture, but data were not statistically examined.

In AST-2 and AST-3, body weight gain and feed to gain ratio were significantly depressed by inoculation and significantly improved by the anticoccidial treatment.

Table 24: Results of anticoccidial sensitivity tests in turkeys for fattening fed STENOROL®

Group	Oocyst excretion(2)	Lesion scores (Ea/Em)	Body weight (g)	Daily feed intake (g)	Weight gain(1) (g)	Feed to gain ratio
AST-1	**D28–32**	**D29**	**D29**		**D21–29**	**D21–29**
UUC	0	0/0	872[a]	–	428[a]	1.68
IUC	2,600,000	1.27/3.07	674[b]	–	231[b]	2.32
IT	2,690,000	0.27*/2.13*	775[c]	–	332[c]	1.76
AST-2	**D22**	**D22**	**D22**	**D16–22**	**D16–22**	**D16–22**
UUC	0*	0.4*	703*	70*	54*	1.29*
IUC	120,793	2.9	576	51	33	1.54
IT	129,734	2.0*	680*	65*	50*	1.31*
AST-3	**D22**	**D22**	**D22**	**D16-22**	**D16-22**	**D16-22**
UUC	45*	0.2*	706*	70*	54*	1.32*
IUC	65,380	3.0	508	50	22	2.28
IT	17,806	1.5*	685*	67*	51*	1.31*

–: not reported.
a, b, c: Means in a column in a study are significantly different ($P < 0.05$).
*: IT/UUC mean with * is significantly different from IUC mean ($P < 0.05$).
(1): In AST-1 mean weight gain per bird over the period indicated. In AST-2 and AST-3 mean daily weight gain per bird.
(2): In AST-1 daily oocysts output over 5 days. In AST-2 and AST-3 oocysts per gram faeces (OPG).

3.3.3.3. Synopsis of efficacy studies in turkeys for fattening

Two floor pen studies showed efficacy of the additive by reducing oocyst excretion. One of them showed also reduced coccidiosis-related mortality. A third floor pen study (trial 1) was considered as supportive only.

In the ASTs, no mortality occurred, and oocyst excretion was not reduced by the treatment. However, the intestinal lesion scores were significantly reduced by the additive in all three ASTs. In all ASTs, the growing performance was improved by the treatment during the week following the inoculation. This improvement can be considered as supportive to conclude on the coccidiostatic action of the additive.

In summary, two floor pen trials and three ASTs are indicative for the coccidiostatic efficacy of the additive in turkeys for fattening.

Conclusions on efficacy for the target species

The FEEDAP Panel is not in the position to conclude on the coccidiostatic efficacy of STENOROL® in chickens for fattening and turkeys for fattening due to the insufficient number of studies with positive results.

3.4. Post-market monitoring

Field monitoring of *Eimeria* spp. resistance in chickens for fattening and turkeys to halofuginone HBr should be undertaken, preferably during the latter part of the period of authorisation.

4. Conclusions

The FEEDAP Panel is not able to conclude on the safety of STENOROL® for chickens and turkeys for fattening at the highest proposed use level of 3 mg halofuginone HBr/kg complete feed. No incompatibilities or interactions with feedingstuffs, carriers, other approved additives or medicinal

drugs are expected. Halofuginone HBr does not have antimicrobial activity at the highest dose proposed; it is not expected to exert adverse effects on chicken gut microbiota (including shedding of enteropathogens) or select for resistance and cross-resistance with other antimicrobials.

The Panel cannot conclude on the genotoxic potential of halofuginone HBr since an appropriate *in vivo* follow-up to exclude the mutagenic effect of the compound was not available. Therefore, the FEEDAP Panel cannot conclude on the safety of halofuginone HBr for the consumer.

Halofuginone HBr is toxic by inhalation, dermal and ocular routes and is very irritant to both the eye and the skin. It is considered also a skin sensitiser. The same conclusions are applied to STENOROL®. Inhalation exposure is considered a risk to persons handling the additive. Since the lack of genotoxic potential of halofuginone HBr has not been adequately demonstrated, it should be considered as an additional potential concern to users handling the additive.

The fate and behaviour in the environment was evaluated for halofuginone, which is the substance expected to be excreted and, therefore, to reach the environment. Predicted environmental concentrations (PECs) have been calculated for halofuginone in the different environmental compartments. No concern for groundwater is expected. Due to the major limitations in some of the ecotoxicological studies, the FEEDAP Panel cannot establish predicted no effect concentrations (PNECs) for earthworm and for aquatic organisms. Consequently, no conclusions can be drawn on the safety of the additive for the environment. These conclusions apply to chickens for fattening and turkeys for fattening.

The FEEDAP Panel is not in the position to conclude on the coccidiostatic efficacy of STENOROL® in chickens for fattening and turkeys for fattening due to the insufficient number of studies with positive results.

Documentation as provided to EFSA/Chronology

Date	Event
04/11/2010	Dossier received by EFSA. STENOROL® (halofuginone hydrobromide) submitted by Huvepharma NV
08/03/2012	Reception mandate from the European Commission
15/11/2012	Application validated by EFSA – Start of the scientific assessment
25/01/2013	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: Methods of analysis*
15/02/2013	Comments received from Member States
06/03/2013	Additional request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003. *Issues: Characterisation, Safety for the consumer, Safety for the user, Safety for the environment, Efficacy*
17/11/2014	Additional request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003. *Issues: Safety for the target species, Safety for the consumer, Safety for the user, Safety for the environment*
25/03/2015	Reception of supplementary information from the applicant
29/06/2015	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003. *Issues: Characterisation, Safety for the target species, Safety for the environment, Efficacy*
08/07/2015	Reception of supplementary information from the applicant – *Methods of analysis*
29/07/2015	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
19/08/2016	Reception of supplementary information from the applicant - Scientific assessment re-started
27/11/2019	Request of clarification to the applicant via email – *Tolerance and efficacy studies*
27/01/2020	Reception of reply to clarification request including additional information
01/07/2020	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment
30/09/2020	Opinion withdrawn by the FEEDAP Panel. Amended opinion adopted by the FEEDAP Panel

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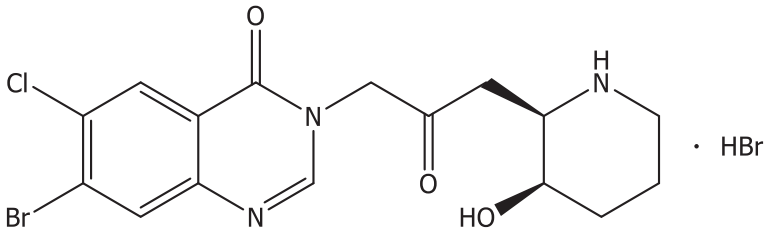
Abbreviations

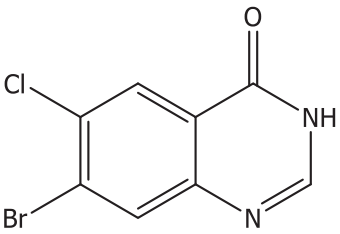
ADI	acceptable daily intake
ANOVA	analysis of variance
AST	anticoccidial sensitivity test
bw	body weight
CAS	Chemical Abstracts Service
CV	coefficient of variation
CVMP	Committee for Medicinal Products for Veterinary Use
DM	Dry matter
DW	Dry weight
DT50	disappearance time 50 (the time within which the concentration of the test substance is reduced by 50%)

DT90	disappearance time 90 (the time within which the concentration of the test substance is reduced by 90%)
DM	dry matter
EC	European Commission
ECHA	European Chemicals Agency
EMA	European Medicines Agency
EURL	European Union Reference Laboratory
EC50	median effective concentration
ErC50	median effective concentration which results in 50% reduction in growth rate
FACE	Feed Additive Consumer Exposure
FOCUS	FOrum for Co-ordination of pesticide fate models and their USe
GLP	Good Laboratory Practice
GC-MS	gas chromatography-mass spectrometry
HPLC	high-performance liquid chromatography
LOQ	limit of quantification
K_{ow}	water partition coefficient
K_{OC}	Adsorption/desorption coefficient corrected for soil organic carbon content
LC50	Median lethal concentration
MIC	minimum inhibitory concentration
MRL	maximum residue limit
MW	molecular weight
NOAEL	no observed adverse effect level
RH	relative humidity
NOEC	No effect concentration
OECD	Organisation for Economic Co-operation and Development
OPG	Oocysts per gram
pKa	Acid dissociation constant
PEC	Predicted environmental concentration
PNEC	Predicted no effect concentration
SFO	Single first order
UDS	Unscheduled DNA synthesis (OECD TG 486)
WoE	weight of evidence
TGR	transgenic rodent assay (OECD TG 488)

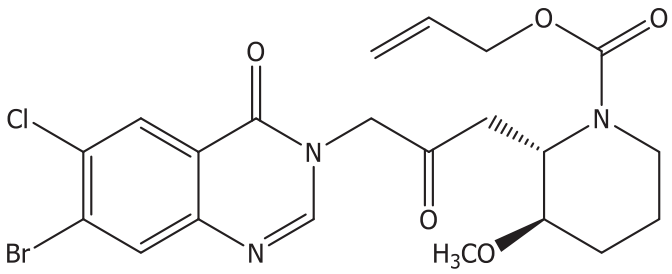
Appendix A – Chemically related impurities in halofuginone HBr



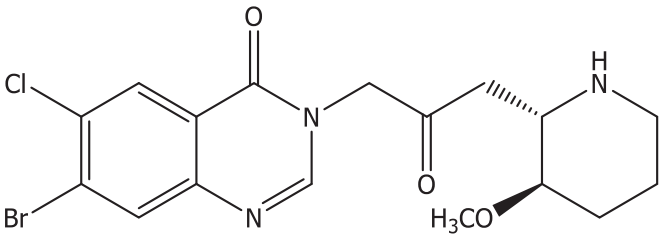
cis-isomer of halofuginone HBr



cebrazolone



melylcebegine



methoxy cebegine

Appendix B – Estimation of user exposure to halofuginone HBr from the additive STENOROL®, including consideration of using a filter mask FF P2 or FF P3 as a preventative measure

Calculation	Identifier	Description	Amount	Source
	a	Halofuginone HBr in the dust (mg/g)	6.3	Technical dossier
	b	Dusting potential (g/m^3)	0.03	Technical dossier
a × b	c	Halofuginone HBr in the air (mg/m^3)	0.189	
	d	N° of premixture batches prepared/ working day	10	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012b)
	e	Time of exposure per production of one batch (s)	20	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012b)
d × e	f	Total duration of daily exposure/ worker (s)	200	
	g	Uncertainty factor	2	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012b)
f × g	h	Refined total duration of daily exposure/worker (s)	400	
h/3 600	i	Refined total duration of daily exposure (h)	0.11	
	j	Inhaled air per hour (m^3)	1.263	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012b)
j × i	k	Inhaled air during exposure (m^3)	0.14	
c × k	l	Halofuginone HBr inhaled during exposure per 8-h working day (mg)	0.0263	
n/10	o	Halofuginone HBr inhaled per 8-h working day (mg) reduced by filter mask FF P2 (reduction factor 10)	0.0026	
n/20	p	Halofuginone HBr inhaled per 8-h working day (mg) reduced by filter mask FF P3 (reduction factor 20)	0.0013	

Appendix C – Lesion scores for different *Eimeria* species or at different intestinal segments at different study days in floor pen trials in chickens for fattening

	6 days PI(1)			13 days PI(2)		
	E. acervulina	***E. maxima***	***E. tenella***	***E. acervulina***	***E. maxima***	***E. tenella***
Trial 1						
UUC	0^a	0^a	0^a	0^a	0^a	0
IUC	2.3^c	0.6^b	0^a	1.8^b	0.5^b	0
IT	1.0^b	0.3^a	0.3^b	1.5^b	0.3^b	0
	Upper small intestine	**Middle small intestine**	**Caecum**	**Upper small intestine**	**Middle small intestine**	**Caecum**
Trial 2						
UUC	0	0	0	0	0	0
IUC	1.3	0.7	1.7	0.6	0.5	0.3
IT	1.2	0.4	0.4*	0*	0.2	0*
Trial 3						
UUC	0*	0*	0*	0	0	0
IUC	1.9	1.8	2.2	0	0	0
IT	0*	0*	0.1*	0	0	0

PI: post-inoculation.
a, b, c: means with different letters in a column are significantly different ($p < 0.05$).
*: Means significantly different from IUC mean ($p < 0.05$). In trial 2, comparison of IUC vs IT; in trial 3, comparison of IUC vs UUC and IT were performed.
(1): Seeder birds in trial 1.
(2): Contact birds in trial 1. In trial 3, 12 days PI.

Appendix D – Oocyst excretion at different study days in floor pen trials in turkeys for fattening

	6 days PI	11 days PI	13 days PI	42 days PI	69 days PI
Trial 1					
UUC	–	–	–	–	–
IUC	295,000	28,800	47,900	93.3	–
IT	23,400*	1,260*	501*	–	–

PI: post-inoculation.
*: Means significantly different from IUC mean ($p < 0.05$).

	7 days PI	11 days PI	17 days PI	18 days PI
Trial 2				
UUC	0^{c}	0^{b}	$8,850^{b}$	$9,775^{b}$
IUC	$56,700^{a}$	$20,375^{a}$	$45,850^{a}$	$41,150^{a}$
IT	$22,975^{b}$	$7,700^{a}$	$92,050^{a}$	$6,625^{b}$

PI: post-inoculation.
a, b, c: Means with different letters in a column are significantly different ($p < 0.05$).

	6 days PI	11 days PI	13 days PI	41 days PI	69 days PI
Trial 3					
UUC	–	–	–	–	–
IUC	5,754	891	166	83	–
IT	1,202*	89*	87*	71	–

PI: post-inoculation.
*: Means significantly different from IUC mean ($p < 0.05$).

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis

In the current application, authorisation is sought for *STENOROL®*, under article 10 as a feed additive under the category 'coccidiostats and histomonostats', according to the classification system of article 6 of Regulation (EC) No 1831/2003. Authorisation is sought for *chickens for fattening* and *turkeys*. *STENOROL®* consists of *halofuginone hydrobromide* as active substance (6 g/kg), povidone (10 g/kg) and macrogolglycerol ricinoleate as excipients (20 g/kg) in a corn cobs carrier. *STENOROL®* is intended to be incorporated directly in *feedingstuffs* or through *premixtures*. The Applicant proposed a concentration range in *feedingstuffs* (from 2 to 3 mg active substance per kg) and several maximum residue limits (MRLs) for *halofuginone hydrobromide* in liver and kidney. As these MRLs are not set by Commission Regulation (EC) No 37/2010, the EURL evaluated the correspondent methods of analysis.

For the quantification of *halofuginone hydrobromide* in the *feed additive* the Applicant submitted a single laboratory validated and further verified method based on high-performance liquid chromatography coupled with ultraviolet detection (HPLC-UV). The following performance characteristics were recalculated by the EURL based on the experimental data provided: a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 0.9% to 1.8% and a *recovery rate* (R_{Rec}) ranging from 97% to 102%. Based on these satisfactory performance characteristics, the EURL recommends for official control, this HPLC-UV method to quantify *halofuginone hydrobromide* in the *feed additive*.

While the Applicant submitted a single laboratory validated method for the quantification of *halofuginone hydrobromide* in *feedingstuffs*, the EURL identified instead the ring-trial validated Community method based on HPLC-UV. The following performance characteristics were reported: a relative standard deviation for *reproducibility* (RSD_R) ranging from 14% to 18%, R_{Rec} ranging from 74% to 88%, and a limit of quantification (LOQ) equal to 1 mg/kg *feedingstuffs*. Based on the performance characteristics presented, the EURL recommends for official control the Community method to quantify *halofuginone hydrobromide* in *feedingstuffs*.

For the quantification of *halofuginone hydrobromide* in *premixtures,* the Applicant submitted a single laboratory validated and further verified HPLC-UV method based on the Community procedure for *feedingstuffs*. The following performance characteristics were recalculated by the EURL based on experimental data obtained using samples containing 100–600 mg/kg: a precision (relative standard deviation for *repeatability* (RSD_r) and RSD_{ip}) of 4.9%, and RRec ranging from 88% to 100%. Based on these satisfactory performance characteristics, the EURL recommends for official control the HPLC-UV method for the quantification of *halofuginone hydrobromide* in *premixtures*.

For the quantification of *halofuginone hydrobromide* in target tissues (liver and kidney), the Applicant submitted a single laboratory and further verified method based on reversed-phase HPLC coupled to a triple quadrupole mass spectrometer (RP-HPLC-MS/MS) in electrospray ionisation mode (ESI) using matrix matched standards. Based on the performance characteristics presented, the EURL recommends for official control the RP-HPLC-MS/MS method proposed by the Applicant or any equivalent other analytical methods complying with the requirements set by Commission Decision 2002/657/EC, to enforce the MRLs of *halofuginone hydrobromide* in the target *tissues*.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.