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Safety and efficacy of ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) for all animal species

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)

This scientific output, published on 20 November 2015, replaces the earlier version published on 18 November 2015[1]

Abstract

The additive ethoxyquin contains ≥ 91 % ethoxyquin, ≤ 8 % ethoxyquin polymers and ≤ 3 % *p*-phenetidine. It is intended for use in all animal species as an antioxidant at a maximum content of 50 mg/kg complete feed. Ethoxyquin is rapidly absorbed after oral administration. Ethoxyquin oxidation in feed materials and in animals leads to four main compounds: 2,4-dimethyl-6-ethoxyquinoline, ethoxyquin N-oxide, ethoxyquin quinone imine, and ethoxyquin dimer (detected only in fishmeal and in salmon). Ethoxyquin itself is not genotoxic or carcinogenic, and does not cause developmental toxicity. The lowest NOAEL (based on studies in rats and dogs) is 2 mg/kg body weight per day. The genotoxic profile of the dimer reflects that of ethoxyquin. Ethoxyquin quinone imine shows structural alerts for mutagenicity, carcinogenicity and DNA binding; no conclusion on the absence of genotoxicity of ethoxyquin quinone imine is possible. *p*-Phenetidine is a recognised possible mutagen. Concentrations of 50 mg ethoxyquin/kg and 11 mg ethoxyquin/kg complete feed might be considered as potentially safe for chickens and breeders and for dogs, respectively. No conclusion on potential safe levels for other poultry, pigs, ruminants, fish and cats is possible. Overall, when considering the presence of *p*-phenetidine in the additive, no conclusion on any safe level of the additive for target animals can be drawn. An assessment of safety for the consumer is prevented by the lack of exposure data, the absence of a safe level of exposure and the presence of *p*-phenetidine in ethoxyquin. The respirable mist of ethoxyquin is of low toxicity. Ethoxyquin is not a dermal irritant, but is considered a potential irritant to eyes and other mucous membranes and a skin sensitiser. No conclusion on the safety for the environment can be made. Ethoxyquin is a potent antioxidant; however, no data confirm its efficacy at the proposed use level.

Keywords: ethoxyquin, *p*-phenetidine, ethoxyquin quinone imine, antioxidant, genotoxicity, toxicity, safety

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[1] An editorial amendment was carried out that does not materially affect the contents or outcome of this Scientific Opinion. The ammednment is on page 5, footnote 3 completing the list of companies supporting the application. To avoid confusion, the original version has been removed from the EFSA Journal, but is available on request, as is a version showing all the changes made.

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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 ethoxyquin for all animal species.

The additive ethoxyquin contains at least 91 % ethoxyquin, ≤ 8 % ethoxyquin polymers, ≤ 3 % *p*-phenetidine and ≤ 0.02 % acetone. It is intended to be used in feed for all animal species with a maximum content of 50 mg/kg complete feed.

Ethoxyquin is rapidly absorbed after oral administration. Ethoxyquin oxidation in feed materials and in animals leads to four main compounds: 2,4-dimethyl-6-ethoxyquinoline, ethoxyquin N-oxide, ethoxyquin quinone imine, and ethoxyquin dimer (detected only in fishmeal and in salmon).

Ethoxyquin itself is not carcinogenic, and it does not cause developmental toxicity in the offspring. The lowest no observed adverse effect level (NOAEL) observed in the available studies on rats and dog is 2 mg/kg body weight (bw) per day. Ethoxyquin showed positive results in some *in vitro* studies on cultured mammalian cells; however these outcomes were not confirmed *in vivo*. The FEEDAP Panel concludes that ethoxyquin itself is not genotoxic. The toxicological profile of the ethoxyquin dimers, present in feed and animal tissues, is considered to reflect that of the precursor monomers. No conclusion on the absence of genotoxicity of ethoxyquin quinone imine is possible.

Ethoxyquin quinone imine shows structural alerts for mutagenicity, carcinogenicity and DNA binding. Ethoxyquin quinone imine is negative in a bacterial reverse mutation assay but the results of an *in vitro* micronucleus assay do not allow the exclusion of clastogenicity. Consequently, no conclusion on the absence of genotoxicity of ethoxyquin quinone imine is possible.

p-Phenetidine, an impurity of the additive ethoxyquin, is a possible mutagen. The few available toxicological studies do not allow the identification of an overall NOAEL for *p*-phenetidine.

Considering the dietary levels of ethoxyquin apparently tolerated by growing chickens and breeders reported in the literature (500–750 mg/kg feed), the proposed maximum concentration of 50 mg ethoxyquin/kg might be considered as potentially safe for chickens and breeders. Since no studies comparable to a tolerance study were available, an extrapolation to other poultry (including laying hens) is not possible. The safety of the use of ethoxyquin in feed for piglets is not demonstrated by a tolerance study. No conclusion on the safety of EQ for other pigs is possible. Owing to the absence of data, no conclusions can be made on the safety of ethoxyquin for calves, cattle for fattening, dairy cows, sheep and goats. Considering the data gaps in the available studies, no safe dietary level for fish, including salmonids, can be derived. Using default values for body weight and feed intake and applying an uncertainty factor of 10 (for individual and breed variability) to the NOAEL of 2 mg ethoxyquin/kg bw per day from a chronic dog study, 11 mg ethoxyquin/complete feed is derived as the maximum potentially safe ethoxyquin concentration in complete feed for dogs. No data were available for cats.

Considering that the additive ethoxyquin contains *p*-phenetidine, a possible mutagen, the FEEDAP Panel cannot conclude on any safe level of ethoxyquin in feed for target animals.

An estimate of consumer exposure to ethoxyquin-related residues in tissues and products from animals treated with ethoxyquin is not possible owing to considerable data gaps. An assessment of the safety for the consumer is prevented by the lack of a safe level of exposure and the presence of *p*-phenetidine in the currently measured quantities in the additive.

The user may be exposed by inhalation to a mist of the additive. However, the respirable mist of ethoxyquin is of low toxicity. Ethoxyquin is not a dermal irritant, but should be considered as a potential irritant to eyes and other mucous membranes and as a skin sensitiser.

Since the ecotoxicity of ethoxyquin to the soil and the sediment compartments cannot be assessed owing to lack of data, no conclusion on the safety for the environment resulting from the use of ethoxyquin as a feed additive for all animal species can be drawn.

Ethoxyquin is a potent antioxidant; however, the studies presented do not confirm its efficacy at the proposed use level of 50 mg/kg complete feed.

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003[2] 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 ANTOXIAC EEIG (Antioxidants Authorisation Consortium European Economic Interest Grouping)[3] for re-evaluation of the product ethoxyquin (6-Ethoxy-1,2-dihydro-2,2,4-trimethylquinoline), when used as a feed additive for all animal species (category: Technological additives; functional group: antioxidants).

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. According to Article 8 of that Regulation, 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. The particulars and documents in support of the application were considered valid by EFSA as of 1 December 2010.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA shall 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 the efficacy of the product ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline), when used under the proposed conditions of use (see Section 3.1.3).

1.2. Additional information

The additive ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) is currently authorised in the EU for use in all animal species and categories. Ethoxyquin was also authorised in the EU as pesticide until 2009, when the authorisation for its use was withdrawn.[4]

It is a legal requirement of the United Nations International Maritime Organisation (IMO) that 'Stabilization of fishmeal shall be achieved to prevent spontaneous combustion by effective application: of between 400 and 1000 mg/kg (ppm) ethoxyquin, or liquid BHT (butylated hydroxy toluene); or between 1000 and 4000 mg/kg (ppm) BHT in powder form at the time of production' (IMO, 2014) and that: 'fish scrap of fish meal shall contain at least 100ppm of antioxidant (ethoxyquin) at the time of consignment' (UN, 2014).

The Scientific Committee on Animal Nutrition (SCAN) issued an opinion on the safety of ethoxyquin for dogs (EC, 1993). The Scientific Committee for Food (SCF) issued an opinion on the safety of ethoxyquin used for the treatment of scald in apples and pears (EC, 1975). The Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment (JMPR) has delivered several opinions on the safety of ethoxyquin (FAO, 1969; 1998; 2005). EFSA issued a conclusion on the peer

[2] 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.

[3] On 13/3/2013, EFSA was informed by the applicant that ANTOXIAC EEIG was liquidated on 19/12/2012 and their rights as applicant were transferred to FEFANA asbl (EU Association of Specialty Feed Ingredients and their Mixtures). Avenue Louise, 130A, Box 1, 1050 Brussels, Belgium. Companies: Industrial Técnica Pecuaria, S.A, Barcelona, Spain; Raschig GmbH, Germany; Novus Europe NV/SA, Brussels, Belgium; Norwegian Seafood Federation, Oslo, Norway; LUCTA S.A., Barcelona, Spain; Jiangsu Zhongdan Group Co., Ltd, Qiwei Town, Taixing City, Jiangsu Province, China. During the course of the evaluation, the applicant notified that the companies Jiangsu Zhongdan Group Co. and LUCTA S.A. are no longer defending this additive within the current application.

[4] Commission Decision 2008/941/EC of 8 December 2008 concerning the non-inclusion of certain active substances in Annex I to Council Directive 91/414/EEC and the withdrawal of authorisations for plant protection products containing these substances. OJ L 335, 13.12.2008, p. 91

review of the pesticide risk assessment of ethoxyquin (EFSA, 2010) and a reasoned opinion on the review of the existing maximum residue levels (MRLs) for ethoxyquin (EFSA, 2013).

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 ethoxyquin as a feed additive. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003 and the applicable EFSA guidance documents.

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, other scientific reports and experts' elicitation knowledge, to deliver the present output.

EFSA has verified the EURL report as it relates to the methods used for the control of the ethoxyquin in animal feed/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 ethoxyquin is in line with the principles laid down in Regulation (EC) No 429/2008[7] and the relevant guidance documents: Guidance on technological additives (EFSA FEEDAP Panel, 2012a), Technical guidance: Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008a, revised in 2009), Guidance for the preparation of dossiers for the re-evaluation of certain additives already authorised under Directive 70/524/EEC (EFSA, 2008b, revised in 2009), Guidance for the preparation of dossiers for additives already authorised for use in food (EFSA FEEDAP Panel, 2012b), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012c), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012d).

3. Assessment

Ethoxyquin (EQ) was originally developed by the rubber industry to prevent rubber from cracking as a result of the oxidation of isoprene. Because of its high antioxidant efficiency and stability, it was further developed for use as a preservative in animal feeds because it protects lipids against peroxidation and stabilizes fat-soluble vitamins (A and E). It acts in a similar way to other synthetic antioxidants (e.g. butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA)) by quenching free radical formation. Presently, ethoxyquin is used primarily as an antioxidant in canned pet food and in feed intended for farmed fish or poultry. It is also used to preserve freshly produced fishmeal against (auto) oxidation and self-ignition.

Ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) is currently authorised[8] in the EU as an antioxidant in feed for all animal species (except dogs) with a maximum content of 150 mg/kg complete feed (alone or in combination with BHA and/or BHT). In feed for dogs, the authorised maximum content is 100 mg/kg complete feed (the combination with BHA and/or BHT should not exceed 150 mg/kg complete feedingstuffs). Ethoxyquin is not authorised for use in food.

Ethoxyquin has undergone several national and international assessments, particularly since 1990, when reports on adverse effects in different target animals, mainly dogs, reached the public (US NTP,

[5] FEED dossier reference: FAD-2010-0141.
[6] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2010-0141-ethoxyquin%20.pdf
[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] List of the authorised additives in feedingstuffs published in application of Article 9t (b) of Council Directive 70/524/EEC concerning additives in feedingstuffs(2004/C 50/01).

1990). The authorisation for its use as pesticide in preventing scalding of pears after harvest was withdrawn in the EU in January 2009.[9] EFSA then conducted a peer review of ethoxyquin. Based on a Draft Assessment Report provided (DAR) (Germany, 2007), EFSA identified a data gap and a critical area of concern in the toxicology and the residue sections, which mentioned 'impurity 7' but for which no data on genotoxicity and ecotoxicity were provided (EFSA, 2010). A risk assessment for the consumer and for operator and worker exposure could not be conducted. In 2013, EFSA assessed the Maximum Residue Limit (MRL) established by the Codex Alimentarius Commission (EFSA, 2013). It was found that the Codex Maximum Residue Limit for Pesticide (CXL) was not adequately supported by data and a possible risk to consumers was identified.

The current application is for ethoxyquin as technological additive, functional group antioxidants, for use in feed of all animal species. The maximum feed contents foreseen in the initial application applied were in accordance to the current authorisation; however, during the course of the assessment, the applicant reduced these maximum contents. Ethoxyquin is now proposed to be applied for all animal species with a maximum content of 50 mg/kg complete feed (the combination with BHA and/or BHT should not exceed 150 mg/kg complete feedingstuffs).

3.1. Characterisation

3.1.1. Characterisation of the additive

The additive is produced by the reaction obtained from heating a mixture of *p*-phenetidine and acetone. Water is then added and the system is adjusted to alkaline conditions. The product phase is then distilled under vacuum. The lower boiling fraction, which contains mainly *p*-phenetidine, is separated, while the main fraction is collected in a tank, mixed and packed.

The additive is a yellow-brown, nearly odourless, liquid, with a surface tension of 58.5 mN/m (90 % saturated solution) at 20 ºC and a viscosity of 150 centistokes at 25 °C. Its solubility in water at 20 ºC is < 1 g/L.

It contains by specification at least 91 % ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline; Chemical Abstracts Service (CAS) number 91-53-2; European Inventory of Existing Commercial chemical structures (EINECS) number 202-075-7; chemical formula $C_{14}H_{19}NO$ and molecular weight 217.30), and not more 8 % of ethoxyquin polymers. The additive is also specified to contain ≤ 3 % *p*-phenetidine (CAS no 156-43-4, EINECS no 205-855-5, molecular weight 137.18) and ≤ 0.02 % acetone. The structural formula of ethoxyquin is shown in Figure 1.

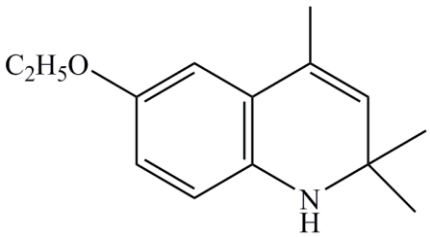

Figure 1: Structural formula of ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline)

Data on batch-to-batch consistency were provided for a total of 27 batches from three producers.[10] The concentration of ethoxyquin varied from 91.2 to 99.7 %, that of *p*-phenetidine from 0.4 to 1.4 %, that of ethoxyquin polymers from 0 to 7.7 % and the concentration of acetone from 0.02 to 0.08 %, the last exceeding the proposed specified limit in eight batches. Three batches (one from each manufacturer) were analysed to further characterise the ethoxyquin fraction,[11] which consisted of 95.4, 99.5 and 100 % ethoxyquin and 4.6, 0.5 and 0 % ethoxyquin polymers. A direct comparison of these data with the specification is not possible, since both data sets use different dimensions.

[9] Commission Decision 2008/941/EC.
[10] Supplementary Information February 2014/Annexes Qi specifications; Supplementary Information October 2014/Annexes Qi batch-to-batch.
[11] Supplementary Information February 2014/Annexes Qii polymers.

A reduced data set (15 batches out of the above 27, five for each producer) included also ethoxyquin-related substances (such as ethoxyquin dimers and ethoxyquin monomers), the concentration of which ranged from 0 to 7.7 %, as determined by the difference from 100 of the sum of ethoxyquin + *p*-phenetidine + acetone.[12] The ethoxyquin fraction in these batches consisted of 88.3–100 % ethoxyquin and 0–11.7 % ethoxyquin polymer, with marked differences between producers (average of five batches per producer: 88.8, 95.8 and 99.9 % ethoxyquin).

One producer provided data on the content of four compounds (monomers)—DH-ethoxyquin, iso-ethoxyquin, 2-4-dichlorophenoxyacetic acid (2,4-D) and impurity X1—in 30 batches and reported concentrations in the range of 0.61–0.82, 1.26–1.64, 0.45–0.89 and 1.18–1.52 area %, respectively.[13]

Heavy metals and arsenic were measured in 11 batches (from three producers).[14] Arsenic concentration was < 0.1 mg/kg, and the concentrations of lead, mercury and cadmium were < 0.05 mg/kg. The concentrations of dioxin and the sum of dioxins and dioxin-like polychlorinated biphenyls (PCBs) in nine batches (from three producers) were < 0.26 ng WHO-PCDD/F-TEQ/kg and < 0.44 ng WHO-PCDD/F-PCB-TEQ/kg, respectively.[15]

The applicant has developed methods to reduce the *p*-phenetidine content in ethoxyquin.[16] The results, obtained using laboratory-scale methods, indicate that it is possible to produce ethoxyquin with a *p*-phenetidine concentration as low as 40 mg/kg (0.004 %).

3.1.2. Stability and homogeneity

Shelf life

A total of nine batches of the additive (three batches each from three different producers) were stored in closed containers at room temperature for varying storing periods.[17] One batch was stored for 1 year, a total of four batches (two producers) were stored for 2 years, and the remaining four batches (two producers) were stored for 3 years. At the end of the storage periods the samples were analysed for ethoxyquin concentration. The results showed in all cases no loss of ethoxyquin.

Stability in premixture and feedingstuffs

The stability of antioxidants in premixtures and feedingstuffs cannot be demonstrated by analysing the concentration of these substances after a certain period of storage since they are degraded by their action. This has been extensively documented for ethoxyquin, ethoxyquin dimer (EQDM) and ethoxyquin quinone imine (EQI), these being the major degradation products in fishmeal and fish feed, and which also exhibit antioxidant activity (see section 3, Mode of action).

The persistence of the ethoxyquin effect was studied by measuring its antioxidant activity over time (Parrish and Patterson, 1987). The vitamin A concentration (from a gelatine-stabilised formulation) in a commercial poultry vitamin–mineral premixture was used as an end point and measured after 1, 3 and 5 months of storage. Ethoxyquin was added at concentrations of 0, 0.1 and 2.5 %. The premixtures were stored under different conditions (ambient: 25–27 °C, 50–70 % relative humidity (RH); accelerated: 44 °C, 70–75 % RH).

Control samples and those containing 0.1 % ethoxyquin showed almost complete loss of vitamin A after 5 months' storage (recovery rate < 4 % under ambient conditions and 0 % under accelerated conditions). In contrast, in the premixtures containing 2.5 % ethoxyquin, the recovery of vitamin A after 3 and 5 months was 56 and 41 %, respectively, under ambient conditions and 24 and 14 %, respectively, under accelerated conditions.

The persistence of the ethoxyquin effect in a feed material was measured by Kirkland and Fuller (1969) using the iodine number as an end point for unsaturated double bonds. The authors added 0 and 750 mg ethoxyquin/kg to a poultry by-product meal (two production batches). The poultry by-

[12] Supplementary Information October 2014/Annexes Qi polymers 2aii.
[13] Supplementary Information February 2014/Annex Qi ETX specifications.
[14] Supplementary Information October 2014/Annexes Qi ETX heavy metals; Annexes Qi ETX specifications.
[15] Supplementary Information February 2014/Annexes Qi ETX specifications; Annex Qiii ETX Dioxins and PCBs.
[16] Supplementary Information June 2015/Annex 01 Modified manufacturing conf; Annex 02 Report *p*-Phenetidine; Annex 03 Method *p*-Phenetidine Draft.
[17] Technical dossier/ Section II/Annex Annex_II_4_1; Supplementary Information February 2015/Annexes Qv ETX Shelf-life.

product meal was then stored in polyethylene-lined multi-wall paper bags for up to 12 (one batch) or 24 weeks at room temperature. Starting at 4 weeks of treatment, ethoxyquin-treated samples showed consistently higher iodine numbers than the control samples; however, the difference reached significance only for one batch at 12 weeks after treatment (64 vs. 57). A comparable study was carried out with a complete poultry feed containing 5 % of the above feed material, either treated with ethoxyquin (corresponding to 37.5 mg ethoxyquin/kg complete feed) or untreated. The use of the treated poultry by-product in the complete diet (with 5 % added maize oil) resulted in a higher iodine number after 6 (74 vs. 66) and 10 weeks of storage (71 vs. 62).

Stability during pelleting

Ethoxyquin was added to a dog feed (two batches) at concentrations of 0, 150 and 300 mg ethoxyquin/kg (analysed values: 124 and 250 mg/kg (batch 1) and 125 and 255 mg/kg (batch 2)).[18] Ethoxyquin recovery after pelleting (at 70–75 °C) was approximately 89 % and 95 % in the diets supplemented with 150 and 300 mg/kg, respectively.

Homogeneity

The homogeneous distribution of ethoxyquin was studied in dry dog feed. Ethoxyquin was added to three different batches each at a concentration of 0, 150 or 300 mg/kg.[19] The three batches were analysed for ethoxyquin. The batches with the same intended ethoxyquin concentration showed similar levels (108, 111 and 108 mg/kg for the batches with 150 mg ethoxyquin added/kg feed, and 227, 223 and 230 mg/kg for the batches with 300 mg ethoxyquin added/kg feed).

3.1.3. Conditions of use

Ethoxyquin is intended to be used in feed for all animal species with a maximum content of 50 mg/kg complete feed (the combination with BHA and/or BHT should not exceed 150 mg/kg complete feedingstuffs). No withdrawal period is proposed.

3.2. Mode of action

3.2.1. *In vitro*

Chemical mechanisms of ethoxyquin oxidation have been reported (Battjes et al., 1991; Brannegan, 2000), giving rise to four main compounds, successively 2,4-dimethyl-6-ethoxyquinoline, EQI N-oxide, EQI and EQDM. These compounds were identified (not quantified) when ethoxyquin was put into the presence of oxidising compounds such as ter-butylperoxide, methyl-linoleate, fishmeal and fish feed (Thorisson, 1992). EQDM and EQI exhibited a significant antioxidant activity, representing 63 % and 44 % of ethoxyquin activity, respectively (de Koning and van der Merwe, 1992).

De Koning and van der Merwe (1992) developed an analytical method based on gas chromatography (GC) to separate and identify EQ derived oxidized products using purified reference compounds. Ethoxyquin was added to five commercial fishmeals prepared from different kind of fish at concentrations of 400 mg ethoxyquin/kg in four meals and 1000 mg ethoxyquin/kg in one meal; the meals were stored at 25°C for 1 to 2 years. After 1 day's storage (two meals), EQI concentration was only 2 mg/kg, and no EQDM was identified. Very small and variable amounts of EQI (2–39 mg/kg meal) were formed, the concentration of which peaked after 59–244 days and then progressively diminished. Larger amounts of EQDM (up to 120 mg/kg meal) were formed progressively and eventually plateaued after 1 year's storage; they matched or even exceeded the concentrations of residual ethoxyquin in four of the five meals studied.

In a study by de Koning (1996), untreated fresh pilchard meal and anchovy meal were each divided into three portions. One portion was treated with 400 mg ethoxyquin/kg, one with 400 mg EQI/kg and one with 400 mg EQDM/kg. All portions were stored for 1 year at 25 °C; bimonthly GC analysis of pilchard meal showed that neither ethoxyquin nor EQI was detectable from day 238 onwards,

[18] Technical dossier/Section II/Annex_III_2_2_5_1_4b
[19] Technical dossier/Section II/Annex_III_2_2_5_1_4b

whereas EQDM was still present at a dose of 84 mg/kg (55 mg/kg after 1 year); analysis of anchovy meal after 1 year found concentrations of 253 mg EQDM/kg, 48 mg ethoxyquin/kg and 10 mg EQI/kg.

In a study by He and Ackman (2000a), ethoxyquin containing a very small amount of EQI (0.5 %) as an impurity was added to freshly produced herring fishmeal at a concentration of 400 mg/kg, then stored at room temperature or 50 °C for 4 months. Determination of ethoxyquin, EQI and EQDM was carried out monthly using an analytical method (high-performance liquid chromatography, HPLC) limiting ethoxyquin oxidation; no EQI (additional) or EQDM (limit of detection (LOD) = 5 mg EQDM/kg) was formed after 2 months' storage under either conditions; during the 4 months' storage at room temperature EQI remained at variable low levels (1.5 to 3.7 mg/kg) and EQDM concentration increased to 9 mg/kg, whereas at 50 °C EQI decreased to 1.5 mg/kg and EQDM remained undetectable; ethoxyquin concentration decreased by about 9 % and 48 % at room temperature and 50 °C, respectively. In the same study, one commercial fishmeal treated with ethoxyquin (400 mg/kg) and two commercial fish feeds (FF1 and FF2) containing ethoxyquin (150 mg/kg) were stored at room temperature or at 50 °C for 4 months; during the 4 months' storage at room temperature, the EQI content of fishmeal declined from 2.7 to 1.5 mg/kg and the EQDM concentration increased from 6 to 12 mg/kg, whereas in FF1 and FF2 the EQI content declined from 1.9–2.4 to 0.8–0.7 mg/kg and the EQDM concentrations only slightly increased from 14–8 to 17–10 mg/kg; at 50 °C, EQI decreased more rapidly and EQDM increased to a lesser extent; ethoxyquin concentration decreased in fishmeal by about 10 % and 72 % at room temperature and at 50 °C, respectively, and in FF1 and FF2 by 4 % and 41 %, at room temperature and at 50 °C, respectively.

These studies indicate that: (i) EQI may be generated during storage of fishmeal, (ii) EQDM concentrations increase slowly regardless of storage conditions, (iii) ethoxyquin is lost more quickly from fishmeals than from fish feeds and this loss is drastically increased at higher temperature; and (iv) the sum of EQI plus EQDM does not explain the ethoxyquin decrease during storage, which indicates that other ethoxyquin-derived compounds may be formed or that ethoxyquin interaction with feed constituents may take place.

3.2.2. *In vivo*

Ethoxyquin has proven to be very effective in preventing lipid oxidation, being able to scavenge a number of strong oxidant radical species (e.g. hydroxyl- and peroxyl radicals) which are spontaneously formed in feedstuffs during that process. In doing so, it may limit the consumption of natural antioxidants such as carotene, xanthophylls and lipid-soluble vitamins such as retinol and tocopherols. It should be noted that also the oxidation products/metabolites of ethoxyquin, including EQI, ethoxyquin-nitroxide and EQDM, also have antioxidant activity (de Koning, 2002).

The antioxidant activity of ethoxyquin has been demonstrated under *in vivo* conditions (Tavarez et al., 2011). The addition of ethoxyquin to the diet has been reported to decrease oxidative stress-related parameters and to increase natural antioxidant serum levels in fish (Hung et al., 1981) and poultry (Wang et al., 1997). At the molecular level, ethoxyquin is able to activate the Nrf2 signalling pathway, resulting, primarily, in an increase in the transcription of detoxifying enzymes such as certain glutathione-S transferase (GSTs) isoenzymes or NAD(P)H: quinone oxidoreductase 1 (NQO1), but also resulting in many other beneficial changes (e.g. an increase in glutathione (GSH) synthesis and regeneration) (Kensler and Wakabayashi, 2010). Overall, the above effects have been associated in experimental species with ethoxyquin chemoprotective effects toward known carcinogens, most notably aflatoxin B1 and dimethyl benzanthracene (DMBA) (Ito et a., 1986; Manson et al., 1997; Bammler et al., 2000). While acting as chemoprotective agents against model carcinogens in certain organs, ethoxyquin can increase the carcinogenic potency of some carcinogens (see Section 5.1.1 and Appendix A).

The antioxidant activity of ethoxyquin is concentration dependent. It is generally accepted that, in common with other antioxidants, it may act as a pro-oxidant when present at high concentrations in feedstuffs. Some of its oxidation products (e.g. phenoxyl radicals, ethoxyquin nitroxide), owing to their reactive nature, are thought to be responsible for a number of adverse effects in laboratory and target species, and are probably also involved in causing genetic and cell damage (Błaszczyk et al., 2013). Hepatic and renal damage is reported in experimentally exposed animals; renal damage is reported to be related to several biochemical effects, including interferences with the mitochondrial

electron transport and inhibition of ATPases and of renal organic anion and cation transport (Reyes et al., 1995).

3.3. Absorption, distribution, metabolism and excretion (ADME)

3.3.1. Ethoxyquin

Mammals

Studies to determine the metabolic fate of EQ, performed in mammals (rats, mice, dogs) with ^{14}C-labelled ethoxyquin, have been assessed previously (FAO, 1998; US NTP, 1990) and reviewed recently (Błaszczyk et al., 2013). The main conclusions are the following: (i) ethoxyquin is rapidly and extensively absorbed after oral administration; (ii) ethoxyquin has a short half-life in plasma (e.g. 23 minutes in rat); (iii) urinary and faecal excretion account for two-thirds and one-third of total excretion, respectively; an extended biliary excretion occurs; (iv) after oral or intravenous administration of ethoxyquin, trace amounts of unchanged ethoxyquin are found in the urine and faeces, whereas about 30 % of the administered radioactivity is found in the bile; and (v) total residues are found, in decreasing order of concentration,,in liver, kidney and adipose tissue, with only trace amounts of ethoxyquin being found in muscle. The metabolic pathways, derived from the studies of Burka et al. (1996), are given in Appendix A and can be summarised as follows: (i) in both rats and mice O-de-ethylation at C-6 appears to be the main metabolic route, resulting in 6-hydroxy-2,2,4-trimethyl-1,2-dihydroquinoline (6-hydroxy-ethoxyquin) as the major urinary metabolite; five minor metabolites (four hydroxylated ethoxyquins and one dihydroxylated ethoxyquin; hydroxylation at C-8 has been unambiguously demonstrated) have been identified; about 20–40 % of urinary metabolites are glucuronidated (the preferred route in mice) or sulphated (the preferred route in rat); (ii) in the bile, ethoxyquin is a minor component (< 5 %) and three ethoxyquin–GSH conjugates have been isolated, of which two have been identified as diastereoisomers at C-4 derived from the epoxidation at C-3/C-4 and one is the result of either epoxidation at C-7/C-8 or the direct addition of GSH to EQI.

A study was performed to compare the metabolic fate of ethoxyquin administered orally to sheeps and rats (Kim et al., 1992). Ethoxyquin and a hydroxylated metabolite with the same mass spectrum were found in the urine of both species. Moreover, a dihydroxylated ethoxyquin metabolite was present in the urine of rats. No identification of the metabolites was provided.

Fish

Tissues (liver and muscle) from salmon fed for 12 weeks with fishmeal-containing diets supplemented with ethoxyquin (0, 18, 107 or 1 800 mg/kg) and from wild salmon (11–1 800 mg ethoxyquin/kg feed) (Bohne et al., 2006, 2007a) were analysed for the presence of the main degradation products of ethoxyquin (EQDM, EQI and de-ethylated ethoxyquin (DEQ)). An HPLC method with fluorescence detection was used to separate and quantify ethoxyquin and derived compounds extended to those exhibiting similar spectral characteristics. Ethoxyquin, EQDM, DEQ and EQI were identified in the liver and muscle, EQI being below the limit of quantitation (LOQ) in most samples. Overall, 14 compounds were separated, including a major unidentified compound in muscle. The authors noted a correlation between the concentrations of ethoxyquin, EQDM, DEQ and the unidentified compound in muscle and the amount of ethoxyquin fed. They concluded on their 'possible metabolic origin'. It is noteworthy that in these studies salmon received a control feed containing small amounts of ethoxyquin (11 mg/kg) and a supplemented feed containing also EQDM (up to 8 %). EQDM is more lipophilic than ethoxyquin (log P = 7.39 vs. 3.93) and prone to accumulation in the tissues of rats (Ørnsrud et al., 2011) as well as fish. Since no isotopically labelled ethoxyquin was used in these studies, the presence of EQDM (and EQI to a much lesser extent) in tissues of fish administered diets supplemented with ethoxyquin for a long period of time cannot be attributed exclusively to the biotransformation of ethoxyquin by fish.

Plants

When applied to the protection of pears against post-harvest fungi, ethoxyquin is metabolised to a set of metabolites different from those found in mammals, essentially methyl-ethoxyquin, dihydro-

ethoxyquin and dehydro-demethyl-ethoxyquin. Ethoxyquin dimer accounts for the highest quantity of ethoxyquin residues in pears about 8 weeks after treatment.

3.3.2. Ethoxyquin dimer (EQDM)

EQDM is formed during the storage of fishmeal treated with ethoxyquin (see section 3.1) It has been identified as a metabolite of ethoxyquin in fish, but not in mammals (see section 4.1.2).

In the study by Ørnsrud et al. (2011) rats were orally exposed for 90 days to EQDM at a dose of 12.5 mg/kg bw per day. The deposition of ethoxyquin and EQDM was measured in tissues and organs in the control and experimental groups at the end of the study. EQDM accumulated in adipose tissue (53 319 ± 2 824 mg/kg wet tissue) and to a lesser extent in liver (1 096 ± 135 mg/kg) and kidney (1 483 ± 219 mg/kg). EQDM was not detected in the tissues of the rats in the control group. Ethoxyquin was not detected in the tissues of rats in either group.

In the same study, the influence of EQDM on P450 enzymes was investigated. Hepatic Cyp1a1 mRNA was significantly reduced in rats fed EQDM, and hepatic Cyp2b1 mRNA was increased. EQDM increased Gstpi1 mRNA expression, but the activity level of this phase II enzyme was reduced. Biomarkers of liver and kidney function did indicate adverse effects of EQDM when F344 rats were fed 12.5 mg/kg bw per day. EQDM produced responses similar to those seen with the parent compound (ethoxyquin).

3.3.3. Ethoxyquin quinone imine (EQI)

EQI is formed when ethoxyquin comes into contact with the highly unsaturated fatty acids of fishmeal and to a much lesser extent complete feeds (see section 3.1). It has been identified as an ethoxyquin metabolite in fish (Bohne et al, 2007b and 2008) and it is likely to occur in rats and mice (Burka et al., 1996). No data are available concerning the metabolic fate of EQI administered orally to animals.

3.3.4. *p*-Phenetidine

Little is known about the fate of *p*-phenetidine in laboratory animals and in target species. In addition, most of the information is not derived from studies pertaining to the compound *per se* but as a main metabolite of its N-acetylated derivative, phenacetin. Phenacetin is a well-known analgesic that has been withdrawn from the market because it results in methaemoglobin (MetHb) formation, renal failure and renal carcinogenic activity occurring in patients ingesting large amounts of the drug (Group 1 carcinogen, IARC, 2012). The available studies are described in detail in Appendix B, the main findings are summarised below.

Metabolic reactions involve both the ethoxylic group ($-O\cdot C_2H_5$) and the amino group. The former undergoes a CYP-mediated O-de-ethylation yielding *p*-aminophenol, which is normally subjected to uridyl diphosphate (UDP)-glucuronosyltransferase-mediated glucuronidation, the resulting metabolites being excreted mainly via the urine (Büch et al., 1967).

Different metabolic pathways may involve the amino group. One pathway is N-glucuronidation; thus, the formation of *p*-phenetidine N-glucuronides has been documented in urines from dosed rabbits (Smith and Williams, 1949a). A further possibility is N-terminal acetyltransferases -dependent N-acetylation, leading to the formation of phenacetin (see above), which has been detected in the urine of treated rabbits (Smith and Williams, 1949b). The crucial step in *p*-phenetidine bioactivation is considered the oxidation of the amino group, giving rise to N-hydroxyphenetidine (also referred to as *p*-hydroxyaminophenetol), a reactive metabolite that is responsible for the onset of methaemoglobinaemia and haemolysis not only in rats administered this metabolite (Kiese, 1974, as cited by Jensen and Jollow, 1991), but also in human patients as a consequence of the chronic abuse of phenacetin (Jensen and Jollow, 1991). A further oxidation step to *p*-nitroso-derivatives (e.g. *p*-nitrosophenetol) has been reported in dogs (Baader et al., 1960)

In vitro studies indicate that in extrahepatic tissues, including renal medulla and urinary bladder, *p*-phenetidine bioactivation may be mediated by prostaglandin H synthetase (PGS), with the generation of reactive metabolites that can covalently bind kidney microsomal proteins (Larsson et al., 1985) and which also display genotoxic properties (Andersson et al., 1982). The absence in dogs of both NAT genes (Trepanier et al., 1998), which accounts for their known inability to N-acetylate aromatic

amines (Poirier et al., 1963), might be expected to favour the oxidative (bioactivating) pathways over the conjugative (detoxicating) ones, thus increasing dogs' susceptibility. A similar reasoning also applies to cats, whose poor acetylating ability, caused by the expression in cats of the gene NAT-1 only, is coupled with their inability to glucuronidate most structurally related aromatic substrates (e.g. phenacetin or acetaminophen), owing to the lack of the UGT-1A6 gene (Court, 2013).

3.4. Toxicological profile

3.4.1. Ethoxyquin and related substances

Ethoxyquin

Ethoxyquin has been tested in many toxicological studies, which are described in details in Appendix A. Full reports were not available for all of the studies and in such cases the FEEDAP Panel used the summaries prepared by other committees (FAO, 1969; FAO 1998; FAO, 2005; US EPA, 2004; Germany, 2007) that had access to the reports. Several of the studies investigated the effects of only one dose of ethoxyquin in modifying the toxicity of other substances and as such were of limited use for identifying a safe level of exposure to ethoxyquin. Of those investigating the toxicity of ethoxyquin *per se*, some were not performed to adequate protocols by today's standards. Nevertheless sufficient acceptable studies were available to allow the FEEDAP Panel to reliably identify dose levels that can cause toxicity in laboratory animals and dose levels causing no adverse effects. The most relevant toxicological studies are summarised below.

Ethoxyquin was slightly toxic to rats in acute oral studies with single-dose LD_{50} values of 1657 to 2040 mg/kg bw being reported, and post-mortems revealing adverse effects on several organs including liver, lungs and gastrointestinal tract. A dose of 500 mg/kg bw caused degeneration of hepatocytes in rats. In dogs given 50 mg/kg bw or more there was biliary stasis at all dose levels in males and at 100 mg/kg bw or more in females.

In sub-chronic oral toxicity studies in rats and dogs, the most commonly affected organs were the liver and kidneys in which pathological changes leading to necrosis were seen. Based on the lowest dose associated with hepatocellular necrosis (4 mg/kg bw per day) in a 90-day study in beagle dogs, the no observed adverse effect level (NOAEL) was set at 2 mg/kg bw/day. In rats, changes to the kidneys (tubular nephropathy and papillary necrosis) were seen in both sexes at doses of 200 mg/kg bw per day or greater; and histopathological lesions to the liver, lungs, stomach and spleen were also seen at higher doses. In one 90-day rat study, reddened or enlarged thyroids (at 200 mg/kg bw per day or greater) and inconsistently altered blood levels of associated hormones were found down to a dose of 40 mg/kg bw per day (elevated thyroid-stimulating hormone (TSH) was the only effect at this dose). The lowest NOAEL identified for rats was 20 mg/kg bw per day based on decrease in body weight.

When ethoxyquin was given to rats in chronic oral toxicity studies of 20 to 102 weeks duration, lesions in the liver, kidneys and thyroid were reported. Renal lesions (mainly hyperplasia of the transitional epithelium of the pelvis, and interstitial degeneration or necrosis of the papilla) were seen in males, but were not associated with any effect on urinary α2u-globulin concentrations. In females, the effects on the kidneys were less severe, with increased weights and lipofuscin deposition in the renal tubules but limited other evidence of renal toxicity at doses that caused renal toxicity in males. Ethoxyquin appeared to exacerbate spontaneous chronic progressive nephropathy (CPN) in male rats and to a lesser extent in females. Preneoplastic tubule hyperplasia was seen in some rats with pyelonephritis or advanced stages of CPN, but did not appear to be directly caused by ethoxyquin. The lowest NOAEL reported in any of the rats studies was 6.25 mg/kg bw per day based on increased weights of liver and kidneys at 12.5 mg/kg bw per day.

The chronic toxicity of ethoxyquin has been investigated in three dog studies of approximately 1 year duration and in one 5-year dog study. The 5-year study was not performed and reported to modern standards, using only one dose level, but nevertheless investigated haematological, clinical biochemistry and histopathology. The results showed no adverse effects at a dietary concentration of 300 ppm (equivalent to 15 mg/kg bw per day). One of the 1-year studies was not performed to modern standards and its results were uninterpretable in relation to effects of ethoxyquin because the dogs suffered an infection by *Histoplasma capsulatum*, which caused lesions in the liver and kidneys.

One of the other one-year dog studies showed no effects at any dose tested (0.9 to 3.6 mg/kg bw per day), other than a trace presence of an anisotropic pigment in Kupffer cells of the liver in one of four males given 3.6 mg/kg bw per day. This effect was not regarded as adverse and was not seen in females (no observed effect level (NOEL) = 1.8 mg/kg bw per day; NOAEL = 3.6 and 2.7 mg/kg bw per day for females and males, respectively). The remaining 1-year study used higher dose levels (3 to 100 mg/kg diet), but the group sizes were small (three dogs, both sexes). Dark coloured livers and reduced liver function, as measured by bromsulpthalein retention time, were seen at dietary concentrations of 10 mg/kg diet or greater, and these findings were associated with degeneration and fatty change in hepatocytes. The same groups of animals also showed swelling and histological changes to the kidneys, characterised by granulation of the cells of the collecting tubules and fatty accumulation. A NOAEL of 28 mg/kg bw per day was reported for this study.

No dedicated carcinogenicity studies were performed in rats or dogs. Several studies of the effects of ethoxyquin on the toxicity of other substances (mostly potent cancer inducers) on specific organ systems used a control group of rats given ethoxyquin (typically 0.8 % in the diet, equivalent to 720 mg/kg bw per day) for approximately 30 weeks. These control groups showed no evidence of carcinogenicity to the liver, kidneys, oesophagus, fore-stomach, ear duct, mammary glands or urinary bladder. When co-administered with cancer promoters, ethoxyquin reduced the prevalence of induced tumours at some sites (fore-stomach, mammary glands, liver), increased the prevalence at other sites (oesophagus, glandular stomach, descending colon, kidneys) and had no effect on prevalence at other sites (caecum, non-descending regions of the colon, lungs). In the urinary bladder, up to 0.5 % (250 mg/kg bw per day) of dietary ethoxyquin had no effect on the prevalence of tumours induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), but 0.8 % (720 mg/kg bw per day) increased the number of benign tumours and hyperplasia without increasing the number of malignant tumours. Thus, whilst it appears that ethoxyquin is not carcinogenic in itself, it can increase or reduce the carcinogenic potency of some carcinogens.

Reproduction toxicity was investigated in a series of studies, including multi-generation and developmental studies that were performed in rats, rabbits and dogs. Oral treatment of rats with ethoxyquin did not cause any developmental toxicity, but there were effects on other aspects of reproduction toxicology, including fertility, litter size, number of stillbirths and pup survival to weaning. The lowest NOAEL from the rat reproduction studies was 11 mg/kg bw per day. A two-generation reproduction study in dogs used only two oral dose levels: 2.5 and 6 mg/kg bw per day. Reproduction parameters were unaffected by the treatments, but there was a deposition of pigment (protoporphyrin IX) in periportal hepatocytes and Kupffer cells in the livers of parental females at both dose levels. The deposition of pigment was not regarded as an adverse effect in itself. Serum alanine aminotransferase was elevated in both sexes of parental dogs at 6 mg/kg bw per day, and serum alkaline phosphatase was also raised at occasional time points in parental and F_1-generation dogs in the same dose group. The parental and offspring NOAEL for this study was 2.5 mg/kg bw per day, based on these effects.

Ethoxyquin was tested in several *in vitro* and *in vivo* genotoxicity studies (described in detail in Appendix A). Negative results were reported in a bacterial reverse mutation assay, while the substance was positive in a gene mutation assay in L5178Y mouse lymphoma cells test and in a chromosome aberrations assay in Chinese hamster ovary (CHO) cells. Dose-dependent but statistically non-significant increases in chromosome aberrations were reported also in cultured human lymphocytes. Moreover, the substance and its dimer (see below) were positive in an *in vitro* Comet assay in cultured human lymphocytes

On the other hand, ethoxyquin was negative in two oral *in vivo* studies. However, the negative results reported in a micronucleus study in mouse bone marrow would not be conclusive because, in the absence of local toxicity at bone marrow, no evidence of target exposure was provided. However, ethoxyquin was also negative in an unscheduled DNA synthesis (UDS) assay in hepatocytes from rats treated *in vivo*, in which the presence of clear systemic toxicity warrants the exposure of the target cells.

Ethoxyquin quinone imine

The structure-activity analysis performed on EQI by the OECD quantitative structure–activity relationship (QSAR) toolbox revealed structural alerts for the formation of reactive oxygen species and for mutagenicity, carcinogenicity and DNA bindings.

The applicant submitted two *in vitro* mutagenicity studies with EQI (Appendix C). The bacterial reverse mutation assay gave negative results.[20] In the *in vitro* micronucleus assay with cytokinesis block EQI induced a dose-dependent, statistically significant, increase in the number of mono- and binucleated cells with micronuclei, both in the absence and in the presence of metabolic activation.[21] The authors of the study observed that the presence of micronuclei in mononucleated cells suggests that the test item might be considered aneugenic. However, while a contribution of aneuploidy to the evident increase of micronuclei is plausible, in the absence of the analysis of the micronuclei by fluorescence *in situ* hybridisation (FISH) it is not possible to exclude the possibility that, besides a likely aneugenic potential, the test item can also be clastogenic.

Ethoxyquin dimers

The dimer formed by the combination of two ethoxyquin molecules (EQDM) and the dimer formed by the combination of ethoxyquin and its quinolone derivative 2,2,4-trimetylquinolin-6-(2H)-one (EQQI) were investigated in an *in vitro* Comet assay in human lymphocytes (Augustyniak et al. 2012). The cells were treated for 1 hour with EQDM or EQQI at doses ranging from 0.125 µM to 100 µM, only in the absence of metabolic activation, and used directly in a Comet analysis. Only one sampling time was used. EQDM induced a significant increase in the percentage of DNA in comet tails at doses of 0.5 µM or more, while cytotoxicity (as assessed by trypan blue exclusion assay) was observed only at 100 µM. EQQI showed a significant genotoxic effect at doses of 0.5 µM and above and was cytotoxic at doses of 1.0 µM or higher. Hydrogen peroxide used as positive control performed as expected.

The application of OECD QSAR toolbox to EQDM showed no structural alert for genotoxicity, while the QSAR profile of EQQI reflects the structural alerts of the EQI monomer.

3.4.2. *p*-Phenetidine

According to the OECD (1994) Screening Information Data Set (SIDS) Report, the oral LD_{50} of *p*-phenetidine in rats and mice is 580 mg/kg bw and 530 mg/kg bw, respectively, while for rabbits a value of 7 000 mg/kg bw could be established. A 28-day repeat dose toxicity study was performed in F344 rats of either sex (n = 5) and reported only as abstract (Sato et al., 1991). Animals were administered *p*-phenetidine (dissolved in olive oil) by oral gavage at doses of 0 (control), 10, 40, or 160 mg/kg bw per day for 28 days. Two further groups, either untreated or treated with the highest dosage, were sacrificed 14 days after treatment withdrawal. No gender differences in the toxic response were noticed. MetHb was detected only at the highest dose level (160 mg/kg bw per day). A reduction in the number of erythrocytes, and increased number of reticulocytes, an increase in the urinary levels of urobilinogen, increase in spleen weight, extramedullary haematopoiesis and haemosiderosis as well as myeloid hyperplasia were observed in both sexes in the group treated with 40 and 160 mg/kg bw per day. Such lesions were no longer detected in animals allowed to recover for 14 days. Based on the above findings a NOAEL of 10 mg *p*-phenetidine/kg bw per day could be established.

Effects on reproduction were assessed in an unpublished Good Laboratory Practice (GLP) study described in a OECD (1994) SIDS report. Phenetidine doses of 0 (vehicle corn oil), 3, 12, 50 and 200 mg/kg bw per day were administered by oral gavage to the male rats for 42 days and from the 14th day before mating until day 3 of lactation to the female rats. Copulation index and fertility index were not affected at any dose levels. All the dams in the 200 mg/kg bw per day group died on days 23 to 25 of pregnancy. A decrease in gestation index was observed in the groups administered 50 and 200 mg *p*-phenetidine/kg bw per day. A decrease in survival index was noticed in the F_1 offspring with a calculated NOAEL for this effect of 50 mg *p*-phenetidine/kg bw per day. The NOAEL for this study was 12 mg/kg bw per day, based on decreased gestation index at 50 mg/kg bw per day or more (OECD, 1994).

[20] Supplementary Information July 2015/Annex 05
[21] Supplementary Information July 2015/Annex 06

MetHb formation was investigated in dogs of different ages after a single exposure to *p*-phenetidine (12 mg/kg bw). Animals older than 1 year were much more sensitive (about ten-fold higher increase in MetHb) than those aged 3–4 months; the MetHb peak was reached 2 hours after dosing and no differences were observed between oral (gavage) and intravenous (i.v.) administration (Baader et al., 1960). In cats treated once i.v. with 8.57 mg phenetidine/kg bw, the peak of MetHb formation (47 %) also occurred 2 hours after dosing (McLean et al., 1969).

In two repeated inhalation toxicity studies, groups of 10 rats per sex were exposed nose-only to vapour and/or aerosol atmospheres of *p*-phenetidine at mean analytical concentrations of 38.2, 133.0 or 1247.6 mg/m^3 for 6 hours/day for 5 days, and of 11.1, 86.2 or 882.6 mg *p*-phenetidine/m^3 for 6 hours/day, 5 days/week, for 4 weeks. Based on the erythrocytotoxicity occurring at 86.2 mg/m3 and above, including the apparent reactive changes in bone marrow (increased erythropoiesis) and spleen (increased erythroclasia), the NOAEL in the 1-week study was 38.2 mg/m^3 air and the NOAEL in the 4-week study was 11.1 mg/m^3.

p-Phenetidine is a primary aromatic amine. It is currently classified by Regulation (EC) No 1272/2008 as a 'substance which causes concern for humans owing to the possibility that it may induce heritable mutations in the germ cells of humans' (Germ cell mutagenicity: Muta. 2). The OECD QSAR Toolbox analysis showed structural alerts for DNA binding, *in vitro* mutagenicity (Ames test), *in vivo* mutagenicity (micronucleus) and carcinogenicity, as expected for a primary aromatic amine.

Several genotoxicity studies, both *in vitro* and *in vivo*, are available (described in details in Appendix B). In summary, the substance was positive for mutagenicity effect in *Salmonella strain* TA100 cultures with S9 mix, while equivocal results were reported without S9 and in the strain TA98 only with S9. In contrast, in another bacterial study (Nohmi, 1985), *p*-phenetidine was positive only in the absence of metabolic activation. Positive results for mutagenicity obtained in Chinese hamster CHL cells are mentioned in the OECD (1994) SIDS report. An *in vivo* micronucleus assay in mouse bone marrow showed positive results in females after oral administration of the highest dosage.

3.4.3. Conclusions on the oral toxicity of the additive ethoxyquin

Taken as a whole, the toxicological results indicate that ethoxyquin itself is not carcinogenic or genotoxic, and does not cause developmental toxicity in offspring. The lowest NOAEL observed in the available studies in rats and dogs is 2 mg/kg bw per day.

Ethoxyquin showed positive results in some *in vitro* studies on cultured mammalian cells; however, these outcomes were not confirmed *in vivo*, in particular in a UDS study in rat hepatocytes in which the presence of a clear systemic toxicity reflects exposure of the target cells. The positive outcomes reported only *in vitro* are probably due to the pro-oxidant activity of the antioxidant that occurs under *in vitro* experimental conditions. The FEEDAP Panel concludes that ethoxyquin itself is not genotoxic. The toxicological profile of the ethoxyquin dimers is considered to reflect that of the precursor monomer.

EQI showed structural alerts for the formation of reactive oxygen species and for mutagenicity, carcinogenicity and DNA binding. EQI was negative in a bacterial reverse mutation assay but in an *in vitro* micronucleus assay it induced dose-dependent statistically significant increases in the number of mono- and binucleated cells with micronuclei. Although a contribution of aneuploidy to the induction of micronuclei is plausible, the possibility that EQI could also be clastogenic cannot be excluded. Consequently, the FEEDAP Panel is not in a position to conclude on the absence of genotoxicity of EQI.

In view of the limited data, an overall NOAEL for *p*-phenetidine cannot be identified. *p*-Phenetidine is a possible mutagen.

3.5. Safety

3.5.1. Safety for target species

Considering that the additive ethoxyquin contains *p*-phenetidine, a possible mutagen, no conclusion on any safe level in feed can be made for target animals, particularly for those fed the additive for their lifetime, such as dogs and cats and reproducing animals.

The following considerations on potentially safe levels of ethoxyquin for target animals are based on one tolerance study in piglets, a near-tolerance study in Atlantic salmon, two other fish studies, a review of literature in poultry, and the toxicity studies in dogs.

No one of these studies with ethoxyquin was qualified by design and purpose to examine the effect of *p*-phenetidine on the health and welfare of target animals. Consequently, conclusions on potentially safe levels of ethoxyquin are subject to the reservation that effects of the possible mutagen *p*-phenetidine were not taken into account.

Safety for chickens for fattening and breeders

No specific tolerance studies were provided. The applicant instead submitted an expert review on selected experiments from literature in which chickens and breeders were fed different levels of dietary ethoxyquin (none of the studies included the proposed use level).

Gassner et al. (1960) studied the toxicity of ethoxyquin in young growing chickens (White Leghorn) from four days of age until 12 weeks of age (ethoxyquin doses: 0, 7.5,15, 30, 75 (1.5× the maximum proposed content), 750 (12.5×) mg/kg) as well as in breeding hens from 1 day to 70 weeks of age (ethoxyquin doses: 0, 75, 750 mg/kg feed). These studies used nutritionally adequate diets, and investigated animal health in an adequate number of individual birds (e.g. 70 birds per treatment group in the 12-week chicken study). In the chicken study, no differences in mortality were observed. The highest 6-week weight was observed in the control group, but there were no significant differences between the groups; nor was there any difference in body weight at 12 weeks. However, the best feed to gain ratio was achieved in the control group (3.32), the ration in the ethoxyquin groups ranging from 3.56 to 3.70 ($P > 0.05$). Further chronic toxicity studies in breeding hens showed no significant effect of ethoxyquin at 7.5, 75 or 750 mg/kg feed on any factors studied including growth, feed consumption, liveability, egg production, fertility of eggs, hatchability of eggs and growth and liveability of progeny. Histological examination of liver, spleen, kidney, ovary, oviduct, and thyroid of the breeding hens showed no changes that correlated with treatment. Studies on male fertility were inconclusive, probably because of the relatively small numbers of males used.

A 50-week study determined if ethoxyquin (750 mg/kg feed up to week 38, 3000 mg/kg feed for week 38 to 50) was as effective as vitamin E in restoring or maintaining fertility in male poultry fed a basal diet low in vitamin E and high in linoleic acid (Kuhns and Arscott, 1969). Adult single-comb White Leghorn males (n = 42) were administered various experimental treatments for a 50-week period. The basal diet was based on maize, soybean meal and safflower oil. In the absence of vitamin E and ethoxyquin fertility and sperm concentration were reduced; both dosages of ethoxyquin or vitamin E fed after 38 weeks of vitamin E depletion restored fertility and semen concentration to values comparable to males fed vitamin E or ethoxyquin continuously; the lower dosages of vitamin E (32 mg/kg feed) or ethoxyquin maintained fertility and sperm concentration over the 50-week experiment; and no statistically significant differences were observed in semen volume, hatchability of fertile eggs, feed consumption, body weights or dead sperm.

Studies by Bailey et al. (1996) in young chicks from day-old to 6 or 8 weeks of age used up to 1000 mg/kg ethoxyquin in nutritionally adequate diets, and found no signs of toxicity on any health or performance parameter except significantly higher relative liver weights in the groups receiving 1000 mg ethoxyquin/kg feed in the second (8-week) study. The relative liver weights were 2.8 %, 2.7 %, 2.6 % and 3.1 %, in the groups receiving 0, 125, 500 and 1 000 mg ethoxyquin/kg feed.

The studies of Cabel et al. (1988) and Dibner et al. (1996) demonstrated the benefits of ethoxyquin (at up to 125 mg/kg feed) in improving zootechnical performance in chicks fed diets containing oxidised fat.

The influence of diet on the toxicity of ethoxyquin in chicks was studied by March et al. (1968). The toxicity of ethoxyquin was significantly greater in chicks fed a diet that was low in protein. Ethoxyquin at 2500 mg/kg feed fed from 4 to 9 weeks of age did not depress growth in chicks fed a basal diet containing 20 % protein, but did suppress growth in chicks fed a diet containing 18 % protein and 8 % safflower oil. Ethoxyquin at 250 mg/kg feed did not affect chick growth adversely when added to either feed. In further experiments, the energy density of basal diets was varied by addition of different levels and sources of dietary fat. Ethoxyquin at 2 500 mg/kg feed depressed growth in chicks by 3.5 weeks of age when fed diets containing 19.3 % protein and 8 % oleic acid or safflower oil. This

effect was not seen in chicks fed diets containing 21 % protein and 5 % tallow, or in chicks fed these three diets containing only 500 mg ethoxyquin/kg feed. In another experiment, ethoxyquin at 2 500 mg/kg feed depressed growth by 4.5 weeks of age in chicks fed basal diets containing 21 % protein and the basal diet containing 10 % safflower oil, 10 % soybean oil or 10 % lard. This effect was not observed in chicks fed these diets containing only 250 mg ethoxyquin/kg feed. In a further experiment, ethoxyquin at 2 500 or 1 250 mg/kg feed depressed growth in chicks by 4 weeks of age. The chicks had been fed diets containing17 or 23 % protein, with or without 10 % lard. In the last experiment, ethoxyquin at 2 500 mg/kg feed depressed growth and feed-to-gain ratio in chicks of 3 weeks of age. The chicks had been fed diets containing 17, 21 or 23 % protein and 10 % dextrose or 10 % lard. In these studies ethoxyquin did not adversely affect the performance of young chicks when added to feed at doses of up to 500 mg/kg. When fat supplementation of the diet enhanced ethoxyquin toxicity, the effect appeared to result from an increase in the energy-to-protein ratio of the diet rather than from the fat itself. The concentration of ethoxyquin in the livers of chicks fed 2 500 mg ethoxyquin/kg feed for 6 weeks was significantly higher when the dietary level of protein was 17 % than when the level was 23 %.

Ohshima et al. (1996) evaluated the use of alfalfa leaf extract treated with different concentrations of ethoxyquin, and added to basal diets to give target concentrations of 0, 50, 125, 350, 600 or 1 400 mg ethoxyquin/kg complete feed. The basal diets were isoenergetic, and contained 12 % crude protein (CP), supplemented with amino acids and other nutrients to meet the requirements of young single-comb White Leghorn chicks. Eight chicks of 1 week of age were allocated to each of the six treatments for a period of 2 weeks. On day 21, body weight gain and feed intake of chicks were measured, and gain to feed ratio calculated. At study end, chicks were sacrificed to collect blood samples, livers, breast skin, and legs. During the short experimental period, no significant differences in growth, final body weight, gain to feed ratio, liver weight, relative liver weight, haematocrit or toe web pigmentation were detected between the negative control birds and birds treated with ethoxyquin. Compared with the negative control group, chick performance increased up to 125 mg ethoxyquin/kg feed, and thereafter declined. Blood plasma concentrations of total cholesterol, high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol were lowest in animals fed 50 and 125 mg ethoxyquin/kg diet. The LDL to total cholesterol ratio tended to be higher with increasing dietary ethoxyquin.

A commercial broiler company experienced a sudden increase in mortality in four flocks at two separate locations (Leong and Brown, 1992). Affected houses had red dust on inside walls and on the equipment. Affected chickens were 10 to 18 days old. Gross pathological examination revealed pale, swollen kidneys, dark-brown enlarged livers and urates in joint spaces. Histological examination showed multifocal proximal tubular necrosis in the kidneys, dilated sinusoids in the livers, biliary hyperplasia and accumulation of brown pigment in hepatocytes and bile ducts. One starter ration was analysed and found to contain 6 500 mg ethoxyquin/kg. Clinical signs and histological lesions were reproduced experimentally with 12 500 mg ethoxyquin/kg feed.

Safety for pigs

A total of 96 piglets (Piétrain × (Landrace×Duroc)) were allocated at two per pen to a total of 48 pens. Group size was 12 (replicates) × 2 (per pen) piglets.[22] Pigs were fed, after an adaptation period of 8 days (initial body weight: 9.3 kg), a basal diet supplemented with 0, 150 (3×), 750 (12.5×) or 1500 (30×) mg ethoxyquin/kg, for 6 weeks. The test item (Capsoquin N) contained 66.6 % ethoxyquin. A prestarter diet (barley, soybean, fishmeal, whey powder; 20.6 % CP and 14.0 MJ metabolisable energy (ME)/kg) was fed, probably for days 1–14, followed by a starter diet (barley, soybean; 18.7 % CP and 13.8 MJ metabolisable energy (ME)/kg) until study completion. The analysed ethoxyquin concentrations in the supplemented diets were 107, 640 and 1137 mg/kg prestarter and 118, 565 and 1129 mg/kg starter, respectively. Body weight and feed intake were measured in biweekly intervals and feed to gain ratio was calculated.

Blood samples from eight piglets per group were collected at the end of the trial. The blood samples were analysed for haematology[23] and clinical biochemistry.[24]

[22] Supplementary Information February 2014/Annex Qvii ETX safety piglets tolerance trial.
[23] Haematocrit, erythrocytes, haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and leucocytes count.

After finishing the trial, 24 piglets (six/group) were euthanized (by an intravenous overdose of sodium pentobarbital) for necropsy.

All parameters were analysed using the generalised linear model (GLM) procedure; the model used included experimental treatment and block as main effects. Means were compared by the Student-Newman–Keuls test. For productive parameters a replicate corresponded to pen data and for haematology and blood biochemistry individual data were used.

One piglet in the intermediate-dose group died on day 22 of an unknown cause. The zootechnical results in each group are described in Table 2.

Table 1: Data of a 6-week tolerance study with piglets (12 × 2 per treatment)

Ethoxyquin (mg/kg diet)		Final body weight (kg)	Average daily feed intake (g)	Average daily gain (g)	Feed to gain ratio
Intended	Analysed*				
0	0	36.0[a]	958[a]	637[a]	1.51
150	113	34.0[b]	873[b]	589[b]	1.48
750	653	35.3[ab]	916[ab]	620[ab]	1.48
1500	1133	33.8[b]	868[b]	584[b]	1.49

* Means of the analytical values of prestarter and starter diets

[ab] Figures with different letter superscripts in one column are significantly different ($P < 0.05$)

In general, piglets in the low- and high-ethoxyquin groups showed a significantly lower body weight, daily gain and feed intake than the control animals. A tendency towards reduced body weight was seen as early as 2 weeks ($P < 0.09$) and became significant after 4 weeks ($P < 0.02$). Pigs in the groups treated with low and high doses of ethoxyquin grew less than animals in the control and intermediate ethoxyquin dose groups. This could be a resultant of the lower average daily feed intake which was observed during the first 2 weeks and overall ($P < 0.05$). During the second and third periods, a tendency for a reduced feed intake was also observed ($P < 0.15$). Animals in the low- and high-ethoxyquin groups consumed less feed than those in the control and the intermediate ethoxyquin groups. Consequently, no statistical differences in feed-to-gain ratio during each period of the study or overall were observed. An influence of ethoxyquin on feed palatability in piglets could not be ruled out.

No statistical differences in packed cell volume, red blood cell (RBC) count, haemoglobin or white blood cell count (WBC) were observed between experimental treatments, and all values were in the physiological range for these parameters in growing pigs. Similar results were observed for blood biochemical parameters. Total protein, albumin, urea, bilirubin and creatinine concentrations were similar between treatments ($P > 0.05$) and their absolute values fell in the normal range for swine. No significant differences were observed between experimental treatments ($P > 0.10$) in the enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyltransferase (γGT), and they rank in the normal range for pigs of the same age, genotype, gender or live weight. Necropsies did not reveal any gross abnormality of external or internal organs, including those of pigs in the high-ethoxyquin group.

The results indicate that ethoxyquin, even at 113 mg/kg feed, affected feed intake and growth performance, as the 10-fold dose also did. However, this significant effect was not observed at the five-fold dose. No effect of dietary ethoxyquin on necropsy findings or on blood haematology and clinical biochemistry was observed.

Safety for ruminants

No data were available to assess the safety of ethoxyquin for calves, cattle for fattening, dairy cows, sheep and goats.

[24] Alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (gamma-GT), protein, albumin, urea, creatinine and bilirubin.

Safety for fish

The applicant did not submit specific tolerance studies on salmonids but did submit studies on salmon and other fish published in the literature.

Bohne et al. (2008) conducted a 12-week feeding trial (followed by 2 weeks' feed deprivation) in 900 Atlantic salmon (*Salmo salar*). Fish were kept in 15 flow-through indoor tanks each stocked with 60 fish with an initial weight of 460 ± 80 g (mean ± SD). Water salinity and temperature were monitored during the trial and were 27 ± 2 ‰ and 9.5 ± 0.8 °C, respectively. Dietary treatments consisted of five diets fed to fish in triplicate tanks, a control group (feed contained 11 mg ethoxyquin/kg), and groups fed 18, 107 (2×) and 1 800 (36×) mg ethoxyquin/kg feed. A group containing 15 000 mg ethoxyquin/kg (300×) was also included, but, since fish refused to eat the diet, this group was omitted from the study. The diets contained by analysis 43.3 % CP and 30.1 % total lipids. Survival, body weight, weight gain and feeding rate were measured in fish from each replicate tank. At the end of the feeding period, there were no deaths and there were no significant differences in fish growth rate or feed-to-gain ratio. No samples were taken for haematology, clinical blood chemistry or histopathology.

Wang et al. (2010) investigated the effects of ethoxyquin on survival, growth, feed utilisation and body composition of large yellow croaker, *Pseudosciaena crocea*. Five experimental diets were formulated to contain graded levels of ethoxyquin (0, 50, 150 (3×), 450 (9×) and 1 350 (27×) mg/kg, not analytically confirmed). Each diet was randomly fed to fish in three sea cages, each stocked with 60 fish (with an initial body weight of 7.8 ± 0.7 g) for 10 weeks. Survival was more than 93 %, irrespective of dietary ethoxyquin levels. Final body weight and specific growth rate were significantly lower ($P < 0.05$) in fish fed 1 350 mg ethoxyquin/kg than in the other treatment groups. There was no significant difference in feed intake or feed-to-gain ratio among dietary treatments and no significant differences were found in the moisture, protein, or ash content of fish among dietary treatments. The lipid content of fish did vary significantly depending on treatment; however, there was no dose–response relationship. The condition factor of fish fed the diet containing 1 350 mg ethoxyquin/kg was significantly lower than that of fish in the other groups. The hepato-somatic index decreased significantly in a dose-dependent manner from 1.3 % to 1 % in the control and the highest treatment group respectively. However, the viscero-somatic index of fish did not vary among treatments.

Yamashita et al. (2009) examined the effect of ethoxyquin on immunity in tilapia (*Oreochromis niloticus*). Duplicate tanks of 17 fish were fed a control diet (containing ethoxyquin-free fishmeal; ethoxyquin in feed not analysed) or a spiked diet (approximately 400 mg ethoxyquin/kg, not analytically confirmed) for 1 month. Five fish were sampled from each tank after 2 weeks and 1 month. Blood samples were taken for analysing phagocytic and antibacterial activity and four fish from each experimental treatment ($n = 8$ per treatment) were dissected and tissues (thymus, head, kidney, spleen and liver) taken for histopathological observations. Phagocytic activity in the fish fed the control diet was significantly higher after 1 month, whereas the activity in fish exposed to ethoxyquin remained the same. The antibacterial activity of whole blood was significantly lower in experimental fish than in the control fish after 2 weeks' and 1 month's exposure to ethoxyquin. Histopathological examination showed pyknosis in the liver of fish fed ethoxyquin (not quantified), which visually appeared to progress with duration of dietary exposure. Few lesions were observed in immune system-related organs such as thymus, head kidney and spleen (data not shown). No significant difference in mortality was seen after 30 days' dietary exposure to feed containing 400 mg ethoxyquin/kg; growth was not measured.

Safety for dogs

No tolerance study in dogs was provided. However, from toxicity studies, a NOAEL of 2 mg/kg body weight was derived (see section 5.1.1). Using the default values for body weight and feed intake (EFSA FEEDAP Panel, 2012b) and applying an uncertainty factor of 10 (for individual and breed variability), 11 mg ethoxyquin/kg complete feed could be derived as the maximum safe ethoxyquin concentration in complete feed for dogs.

Safety for cats

No tolerance studies or toxicity data are available for cats, which are expected to excrete the additive and possibly some of its metabolites at a lower rate than other mammalian species considering the poor ability to glucuronidate aromatic substrates.

Conclusions on the safety of ethoxyquin for target animals

From a literature review it could be concluded that at least 750 mg ethoxyquin/kg feed is tolerated by growing chickens (Leghorn type, no modern breed of chicken for fattening) and breeders. However, the reduction in ethoxyquin tolerance seen with protein-reduced diets (absolutely or by increased energy density) established lower tolerated levels (up to 500 mg ethoxyquin/kg). It appears that the maximum proposed dietary concentration of 50 mg ethoxyquin/kg is potentially safe for chickens and breeders. Since no studies comparable to a tolerance study with closely graded dietary ethoxyquin levels were available, an extrapolation to other poultry (including laying hens) is not possible.

The safety of the use of ethoxyquin in feed for piglets was not demonstrated by a tolerance study. No clear reason for the reduction in feed intake and growth at 113 mg ethoxyquin/kg diet could be derived from the experiment; however, an influence of ethoxyquin on feed palatability in piglets should be taken into consideration. Therefore, no conclusion on the safety of ethoxyquin for pigs is possible.

Owing to the absence of data, no conclusions could be drawn on the safety of ethoxyquin for calves, cattle for fattening, dairy cows, sheep and goats.

Atlantic salmon appeared to tolerate 1 800 mg ethoxyquin/kg feed for 3 months based on zootechnical parameters as did large yellow croaker fed 450 mg ethoxyquin/kg for 10 weeks. Considering the data gaps in the available studies, no safe dietary level for fish, including salmonids, could be derived.

The Panel concludes that ethoxyquin at 11 mg ethoxyquin/kg complete feed is potentially safe for dogs. No safe level can derived for cats and other pets.

Considering that the additive ethoxyquin contains *p*-phenetidine, a possible mutagen, the FEEDAP Panel cannot conclude on any safe level of ethoxyquin in feed for target animals.

3.5.2. Safety for the consumer

Residue status of ethoxyquin in animal tissues and products

Limited data are available on the metabolic fate and residues of ethoxyquin in target animal species, with the exception of fish. Consequently, in the framework of this assessment, the FEEDAP Panel considered ethoxyquin as the marker residue by default in mammals. Since EQDM is the major residue in fish and is considered toxicologically equivalent to ethoxyquin, the sum of ethoxyquin plus EQDM was used to calculate the contribution of fish to consumer exposure. Owing to the uncertainties concerning (i) the presence of significant amounts of EQI in feedingstuffs, (ii) the lack of metabolic studies in the target species and laboratory animals and (iii) the lack of data on residue in tissues and products, this metabolite was not taken into consideration in this assessment.

Poultry

A study of the carry-over of ethoxyquin from the diet to tissues and eggs of chickens and laying hens was performed on birds fed complete feed containing 125 mg ethoxyquin/kg (Hobson Frohock, 1982). Twenty 1-day-old chickens received the supplemented feed until slaughter (8 weeks). Twelve 18-week-old laying hens were fed the supplemented feed, and 30 weeks later the eggs were collected daily for 4 weeks. The hens were then slaughtered and samples of muscle and tissues were collected. Ethoxyquin concentration in the eggs was in the range 0.016 to 0.054 mg/kg whole egg (average 0.031 mg/kg), essentially found in the yolk. Ethoxyquin concentration in the muscle (leg and breast) of the chickens and laying hens was < 0.005 mg/kg (LOQ). Ethoxyquin concentration in the liver was 0.063 and 0.048 mg/kg in chickens and laying hens, respectively; it was highest in the fat, at 0.215 and 0.238 mg/kg in chickens and laying hens, respectively. The presence of ethoxyquin metabolites/oxidation products was not investigated.

The ethoxyquin content of chicken tissues was determined in animals fed from day 10–15 until day 40 a diet containing 150 mg ethoxyquin/kg (Dvinskaya et al., 1979). After 25–30 days ethoxyquin was determined in tissues and amounted to 0.282 mg/kg liver, 0.034 mg/kg leg muscle, 0.29 mg/kg visceral fat and 0.226 mg/kg skin; it was not detected in breast. No ethoxyquin residues were found after 2 weeks' withdrawal.

Cow milk

A lactating cow was administered a meal containing 155 mg ^{14}C-ethoxyquin (biologically stable labelling). Milk was collected for 84 hours and total radioactivity measured. The radioactivity reached a maximum value 36 hours after treatment which corresponded to 0.036 mg ethoxyquin/L (Wilson et al., 1959). In another study in which ethoxyquin was fed to three dairy cows for 7 days at doses of 150 mg or 1500 mg/kg diet (dry matter), ethoxyquin concentrations in milk measured with a fluorimetric method were approximately 0.050 mg/L and 0.125 mg/L in the low- and high-dose groups, respectively (Dunkley et al., 1967). Three cows were fed a diet containing 150 mg ethoxyquin/kg for 13 days, the milk was collected three times during the week before the experiment, every day for the 13 days of the treatment and during a 5-day withdrawal period. A qualitative approach was followed and the quantification was limited to one value of less than 0.007 mg ethoxyquin/L milk (average value from three animals) and 0.1 to 0.2 mg of total unidentified ethoxyquin-related compounds/L (Dunkley et al., 1968).

Salmon

In a study by Bohne et al. (2008) (described in detail in section 5.1.4), ethoxyquin and EQDM residues in the flesh of Atlantic salmon (*Salmo salar*) were determined after a 3-month feeding period and a 2-week depuration period. Analysis of ethoxyquin in feeds was performed at the beginning (11, 18, 107 and 1761 mg/kg) and the end (9, 18, 96 and 932 mg/kg) of the experimental period. Analysis of EQDM in feeds at the end of the experimental period found concentrations of 0.15, 0.18, 9.5 and 7.0 mg EQDM/kg, respectively. Five fish from each tank were randomly sacrificed on days 0, 3, 7, 14, 28 and 84 during the feeding period and on days 3, 7 and 14 during the depuration period (days 0, 84 and at end of depuration period for the control group). Frozen samples of homogenised muscle were protected from the light then thawed and protected from oxidation by submersion in a reaction blend consisting of ethanol, sodium hydroxide, saturated EDTA, ascorbic acid and pyrogallol. Ethoxyquin and EQDM were extracted (using hexane) and their concentration determined by HPLC separation and fluorimetric measurement. The results are given in Table 3. Although preventive measures were taken by the authors (intended use of ethoxyquin-free fishmeal, commercial ethoxyquin source with purity > 97 %, low-temperature storage of feeds), the control feed contained small amounts of ethoxyquin at the beginning of the experiment (EQDM not measured). The ethoxyquin-supplemented diets contained considerable amounts of EQDM at the end of the experimental period.

Table 2: Concentration of ethoxyquin and ethoxyquin dimer (EQDM) (µg/kg fresh weight ± SD; n = 15) in Atlantic salmon fillets following 84 days exposure to graded levels of EQ in feed and following a 14-day depuration period (adapted from Bohne et al. 2008).

Initial ethoxyquin in mg/kg feed	**11**	**18**	**107**	**1761**
Final (day 84) ethoxyquin (EQDM) in mg/kg feed	9 (0.15)	18 (0.18)	96 (7.5)	932 (7.0)
At the end of feeding period				
Ethoxyquin (µg/kg fillet)	18 ± 0.4	43 ± 5	410 ± 13	2249 ± 275
EQDM (µg/kg fillet)	683 ± 59	512 ± 61	560 ± 214	2503 ± 361
Sum of ethoxyquin + EQDM (µg/kg fillet)	701 ± 59	555 ± 66	967 ± 82	4752 ± 587
After depuration				
Ethoxyquin (µg/kg fillet)	3.0 ± 0.4	4.5 ± 0.4	10 ± 1.4	25 ± 9
EQDM (µg/kg fillet)	545 ± 49	996 ± 223	951 ± 116	4432 ± 219
Sum of ethoxyquin + EQDM (µg/kg fillet)	548 ± 49	1000 ± 273	961 ± 118	4667 ± 858

Even on the first day of the study, muscle tissue contained substantial levels of ethoxyquin and EQDM (14 µg ethoxyquin/kg and 2326 µg EQDM/kg). After 3 months, no differences in EQDM content were

observed between the control group and the groups receiving low and the intermediate doses (mean about 600 µg/kg fillet). The ethoxyquin concentration in fillet reflected dietary exposure. After depuration, the ethoxyquin content declined in all groups by a factor of 6, 10, 40 and 90 in the groups receiving 11, 18, 107 and 1761 mg ethoxyquin/kg initial feed, respectively. At the same time, EQDM increased in the two groups fed the intermediate and the high ethoxyquin concentrations such that no difference in the sum of ethoxyquin and EQDM owing to the depuration period could be seen. The increase by a factor of 2 in the sum of ethoxyquin and EQDM in the low-dose group during depuration cannot be explained.

Data from surveys

Concentrations ranging between 0.05 and 0.30 mg EQDM/kg liver and between 0.25 and 1.27 mg/kg muscle were reported for rainbow trout (*Oncorhynchus mykiss*) and between 0.43 and 0.92 mg/kg muscle for Atlantic salmon (*Salmo salar*). These data originate from a study (He and Ackman, 2000a) carried out to improve the analytical method for EQDM in muscle and liver of fish farmed in seawater (three samples each).

Ethoxyquin, EQDM and DEQ were analysed, using HPLC separation and fluorimetric detection, in fillets of Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic cod (*Gadus morhua*) sampled at fish farms and fish-processing plants in Norway (Lundebye et al., 2010). EQDM was far (one to two orders of magnitude) more abundant than ethoxyquin in all fish species, with the exception of cod, in which it was below the LOQ; the DEQ concentration was very low and similar in all fish species analysed. The variability of the results was very high, due in part to the reduced number of animals sampled (Table 4).

Table 3: Concentrations (µg/kg wet weight) of ethoxyquin and its metabolites ethoxyquin dimer (EQDM) and de-ethylated ethoxyquin (DEQ) in fillets from Norwegian farmed fish: Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic cod (*Gadus morhua*) (adapted from Lundebye et al., 2010).

	Ethoxyquin	EQDM	DEQ	Sum of ethoxyquin+metabolites
Salmon (n=24)				
Mean (µg/kg fillet)	55	730	1.4	786
SD (µg/kg)	46	290	0.4	
Range (µg/kg)	13–167	332–1 450	< 0.04–2.0	
Trout (n=16)				
Mean (µg/kg)	39	760	1.5	800
SD (µg/kg)	19	430	0.2	
Range (µg/kg)	9–65	180–1 700	<0.04–1.8	
Halibut (n=15)				
Mean (µg/kg)	16	720	1.3	737
SD (µg/kg)	7.2	725	0.5	
Range (µg/kg)	4.6–25	110–2 593	0.4– .4	
Cod (n=4)				
Mean (µg/kg)	9.5	< 0.2	1.2	11
SD (µg/kg)	2.8		0.3	
Range (µg/kg)	5.9–11.9		0.9–1.6	

A survey of ethoxyquin and EQDM contents of various whole fish (fresh and smoked) and fish products (fillets) from the Swiss market was carried out (Ortelli et al., 2011). Samples of wild fish (n=9), fish from organic farming (n=7) and fish from aqua farming (n=66) were analysed using ultra-performance-liquid chromatography hyphenated to time-of-flight mass spectrometry as analytical method. The results are available only as graphs. About 96 % of fish from aqua farming (organic or not) contained low amounts of ethoxyquin (mean value: 40 µg/kg, maximum value: 300 µg/kg) and much higher quantities of EQDM (mean value: 160 µg /kg, maximum value: 700 µg/kg).

Consumer exposure to ethoxyquin

Any estimate of consumer exposure following Regulation (EC) No 429/2008 has to include total residue data for muscle, liver, kidney, skin (with natural proportion of fat), dairy milk and eggs. The data should be obtained from studies using the highest proposed dose in complete feed. The EFSA FEEDAP Panel Guidance on consumer safety (EFSA FEEDAP Panel, 2012c) defines a minimum number of samples from which data should be obtained.

In particular, data that allow the determination of the ratio of marker residue to total residue are required.

When comparing the data that are required with the data available, the FEEDAP Panel notes that:

(i) All available studies lack figures for total residues. In all studies except studies of fish, only ethoxyquin, the parent compound, was measured.

(ii) In the study of salmon, the major residue in flesh was not identified.

(iii) No attempt to determine metabolites was made in studies on poultry, eggs and milk.

(iv) No data were provided concerning a potential marker residue and the ratio of marker to total residue.

(v) As the application is for all animal species, data on one mammalian species, on poultry and on fish would be required. No data for mammalian tissues were available.

(vi) The number of cows required for milk analyses is eight; the available data come from only three cows.

(vii) The studies were conducted with ethoxyquin concentrations in feed of > 100 mg/kg, whereas the maximum proposed concentration is 50 mg/kg.

(viii) No information is available on the presence of residues of *p*-phenetidine in tissues and products of animal origin.

The FEEDAP Panel concludes that the available data base do not allow the estimation of consumer exposure to total ethoxyquin residues.

Assessment of consumer safety

EFSA (EFSA, 2010) could not set reference values (i.e. acceptable daily intake (ADI)) for ethoxyquin, owing to the limited toxicological package. No substantially new data were available to the FEEDAP Panel since that assessment. Therefore, the FEEDAP Panel is not in a position to propose a reference value.

An estimate of the exposure of consumers to ethoxyquin-related residues and *p*-phenetidine in tissues and products from animals treated with ethoxyquin is not possible owing to considerable data gaps. The presence of *p*-phenetidine in the currently measured quantities in the additive and the formation of EQI in feed and animals additionally prevent the identification of a safe level of consumer exposure.

3.6. Safety for the user

Full reports of most of the studies provided were not available. The following description is mainly based on the summaries given in the DAR (Germany, 2007).

3.6.1. Effects on the respiratory system

The acute inhalation toxicity of ethoxyquin has been tested in two studies. In one study, the test material was the pesticide Xedaquine 52 % Drench, which contains 500 g ethoxyquin/L. Five Sprague–Dawley rats were exposed nose-only for 4 hours to a respirable dust of Xedaquine. There was no mortality during exposure or during the 14-day observation period that followed and the rats showed no clinical signs. Body weight was not affected and autopsies revealed no gross abnormalities. In the other study, a solution of ethoxyquin was tested as an aerosol. Five Sprague–Dawley rats of each sex were exposed (whole body) to an atmosphere containing 1.97 mg ethoxyquin/L for an

unspecified time followed by an observation period of 14 days. Six of the 10 treated rats had tremors following exposure. One female died as a result of the treatment. The remaining animals showed only very minor transient decreases in body weight gain and autopsies revealed no gross pathology. It was concluded that the LC_{50} of ethoxyquin is greater than 1.97 mg/L (equal to 1527 mg/kg, assuming that 1 L of air has a mass of 1.29 g), which is classed as Category 4 in the OECD's Globally Harmonised System of Classification and labelling of Chemicals (GHS).

3.6.2. Effects on eyes and skin

Dermal irritation was tested in three studies in rabbits and one in rats. The protocols of the studies appeared to be in line with OECD Guideline 404. All of the tests found only mild transitory irritation following skin exposure to ethoxyquin or, in one rabbit study, an ethoxyquin-based pesticide (Xedaquine 52 % Drench) not triggering a classification as an irritant.

Eye irritation was tested in three rabbit studies. One of the studies used an ethoxyquin-based pesticide (Xedaquine 52 % Drench) as the test material and found that the treatment caused conjunctival redness (but no other effect) which had resolved at 72 hours post treatment. The other two studies tested undiluted ethoxyquin: one of these studies found that the treatment initially caused moderate erythema, slight oedema and copious discharge, but all symptoms had disappeared at 72 hours post treatment in all animals; the other study found the treatment caused redness and chemosis of the conjunctiva and that the conjunctival redness persisted until 72 hours post treatment in one out of six animals. The results of the latter study require the test material to be classified as an eye irritant.

The skin sensitisation potential of ethoxyquin has been tested in guinea-pigs using a Buehler test and that of the ethoxyquin-based pesticide Xedaquine 52 % Drench has been tested in a Magnusson-Kligman test in guinea-pigs. The protocols of the studies appeared to be in line with OECD Guideline 429. The Buehler test of Xedaquine 52 % Drench gave positive results at challenge in 16 out of 20 animals tested, but also gave positive results in 3 out of 20 control animals. Rechallenge with 1 % and 3 % mixtures of Xedaquine 52 % Drench in mineral oil resulted in 2/20 and 15/20 positives, respectively, compared with 0/10 in controls. This was interpreted as a positive result for Xedaquine 52 % Drench, but it was not clear whether this result was due to the ethoxyquin or another ingredient of the pesticide. The maximisation test of a 50 % solution of ethoxyquin gave a positive response in 1 of 12 treated animals (and no positives in controls), which was interpreted as indicating that ethoxyquin has a weak sensitising potential. Considering the results of the two studies together, it is concluded that the possibility that ethoxyquin is a skin sensitiser cannot be discounted.

A number of field cases have been reported concerning the onset of severe allergic contact dermatitis in processors and handlers of animal feed (Burrows, 1975; Van Ecke, 1977; Alanko et al., 1998; Rubel and Freeman, 1998). Positive results in patch tests have been recorded in affected workers given challenge concentrations of as little as 0.01 % ethoxyquin in petrolatum (Zachariae, 1978).

3.6.3. Conclusions on user safety

In the absence of data, it is assumed that workers may be exposed by inhalation to a mist of the additive. However, the results of acute inhalation toxicity studies indicated that respirable mist of ethoxyquin itself is of low toxicity.

The results of skin irritation tests of ethoxyquin and an ethoxyquin-based pesticide in rabbits and rats indicated that ethoxyquin is not a skin irritant.

The results of one out of two eye irritation tests of ethoxyquin in rabbits indicated that ethoxyquin was irritant to eyes. A further test suggested that an ethoxyquin-based pesticide is not an eye irritant. It would be prudent to treat ethoxyquin as a potential irritant to eyes and other mucous membranes.

The results of a Buehler test of an ethoxyquin-based pesticide showed the pesticide to be a skin sensitiser. The results of a maximisation test (normally more sensitive that the Buehler test) showed that ethoxyquin was a weak skin sensitiser. In the light of reports of contact dermatitis in humans, ethoxyquin should be regarded as a skin sensitiser.

3.7. Safety for the environment

As a consequence of the use of ethoxyquin-containing feed additives, ethoxyquin is unavoidably released into the environment. When used in livestock feed, ethoxyquin and its metabolites excreted in the faeces will enter the soil environment when faeces are applied as fertiliser to land, in the form of manure, slurry or litter. This may present two main potential risks:

- Ethoxyquin accumulation within the topsoil to concentrations posing risks for non-target species in terrestrial environmental compartments.
- Leaching of ethoxyquin from soil to ground waters in concentrations for non-target species in the aquatic environmental compartment.

When used in aquaculture, feed additives, such as ethoxyquin, can be released directly to the broader aquatic environment around an aquaculture facility as waste feed or be taken up by fish and then excreted into the environment. The compartment of concern for fish farmed in cages is assumed to be the sediment, whereas for fish farmed in land-based systems the effluent flowing to surface water is considered to pose the main environmental risk.

3.7.1. Phase I assessment

Exposure assessment
The active ingredient is not a physiological/natural substance of established safety for the environment. A phase I assessment has to be conducted to determine the predicted environmental concentrations (PECs) of the feed additive in relevant environmental compartments.

The applicant supplied phase I assessments for ethoxyquin when used as a feed additive.

Additives for terrestrial animals

The PECs for ethoxyquin in soil and groundwater were calculated based on inclusion of 50 mg ethoxyquin/kg feed, and 100 % excretion. The PEC_{soil} ranged from 0.23 mg/kg for turkeys to 1.07 mg/kg for lambs for fattening. The $PEC_{groundwater}$ ranged from 2.18 to 4.15 mg/L. Since PEC_{soil} and $PEC_{groundwater}$ exceeded the trigger values of < 10 µg/kg and 0.1 µg/L, respectively, a phase II assessment is required for ethoxyquin.

Additives for aquatic animals

The PECs for ethoxyquin in sediment (PEC_{sed}) and in surface water from aquaculture (PEC_{swaq}) were calculated based on inclusion of 50 mg ethoxyquin/kg feed and 100 % excretion.

The PEC_{sed} was 0.106 mg/kg ww, above the trigger value of 10 µg/kg ww. The values of PEC_{swaq} were below the trigger value of 0.1 µg/L for salmon, trout and turbot, while it was 0.125 µg/L for seabass/seabream. Therefore, a phase II assessment is required for ethoxyquin.

Environmental fate

On the basis of its physico-chemical properties ethoxyquin may be considered as moderately volatile. In a hydrolysis study, ethoxyquin was unstable and underwent relatively rapid hydrolytic degradation forming four major unidentified transformation products. The half-life of ethoxyquin in pH 5, 7, and 9 solutions was 4, 7 and 9 days, respectively (Reynolds 1995, cited in DAR (Germany, 2007)). Ethoxyquin is not readily biodegradable based on a QSAR estimation, and the mobility of ethoxyquin in soil might be classified as low. No data were submitted on the degradation of ethoxyquin in either soil or sediment; consequently, PEC refinement for these environmental compartments was not possible.

3.7.2. Phase II assessment

Since the PEC_{soil} and PEC_{sed} for ethoxyquin exceed the trigger values, phase II assessment is required to assess the potential of ethoxyquin to affect non-target species in the environment. For the terrestrial environment studies should include toxicity to earthworms, three terrestrial plants and soil

microorganisms. For sea cages, toxicity of the feed additive should be tested in three marine sediment species.

The applicant described a toxicity test with ethoxyquin conducted on bobwhite quail (Palmer et al., 1996, cited in DAR (Germany, 2007)), rainbow trout (*Oncorhynchus mykiss*) and the cladoceran *Daphnia magna* (Drottar et al., 1996, cited in DAR (Germany, 2007)), and the freshwater alga *Pseudokirchneriella subcapitata* (Desjardins et al., 2006, cited in DAR (Germany, 2007)). The FEEDAP Panel did not consider these studies as relevant to assess the toxicity of ethoxyquin to soil- or sediment-dwelling organisms. Moreover, no data were available for the assessment of possible bioaccumulation of ethoxyquin and its metabolites. Consequently, the environmental risk of ethoxyquin to these environmental compartments could not be established.

Since the ecotoxicity of ethoxyquin to the soil and the sediment compartments could not be assessed, no conclusion on the safety for the environment could be made for the use of ethoxyquin as a feed additive for all animal species.

3.8. Efficacy

No typical efficacy studies were provided; instead, the applicant made available some publications in which ethoxyquin was used as an antioxidant in feeds and feed materials.

Romoser et al. (1968) and Romoser (1979) conducted two studies on the capacity of ethoxyquin to prevent the auto-oxidation of fishmeal. Heat generation in fishmeal was measured. It was shown that doses of 400 and 750 mg ethoxyquin/kg fishmeal effectively prevented auto-oxidation (i.e. maximum temperature of about 65 °C after 16 hours, untreated fishmeal > 100 °C after 20 hours). The temperature of the treated fishmeal declined further with time (< 30° C after 17 days).

A complete feed for chicken for fattening (21.5 % CP), based on maize, maize gluten meal, dehulled soybean meal and 4 % vegetable oil (40 % polyunsaturated fatty acids) was supplemented with 125 mg ethoxyquin/kg feed and stored for 6 weeks in galvanized metal cans exposed to sunshine (McGeachin et al., 1992). Maximum temperature in the cans ranged from 38 °C to 53 °C and relative humidity from 71 % to 84 %. Peroxides and malonaldehyde were measured in samples of oil extracted from stored feedstuffs. In ethoxyquin-supplemented feed, the levels of peroxides and malonaldehyde were similar during the whole study period. In the unsupplemented control feed, peroxides increased significantly after one week, and malonaldehyde after two weeks.

Stability studies are also suitable to demonstrate the efficacy of a technological additive (see 2.2).

Ethoxyquin at a dose of 2.5 % in a premixture was partially effective in preservation of vitamin A during 3 to 5 months' storage.

The protection of unsaturated double bounds of fatty acids could be shown during storage of a poultry complete feed containing 5 % poultry-by-product meal supplemented with 750 mg ethoxyquin/kg (37.5 mg/kg complete feed). After 6 and 10 weeks of storage, the iodine number (indicating fatty acids with double bounds) was higher than in the unsupplemented control feed.

4. Conclusions

Ethoxyquin itself is not genotoxic, carcinogenic, and does not cause developmental toxicity in the offspring. The toxicological profile of the ethoxyquin dimers, present in feed and animal tissues, is considered to reflect that of the precursor monomer. No conclusion on the absence of genotoxicity of EQI is possible.

p-Phenetidine, an impurity of the additive EQ, is a possible mutagen.

The proposed maximum concentration of 50 mg ethoxyquin/kg might be considered as potentially safe for chickens and breeders, but extrapolation to other poultry (including laying hens) is not possible. No conclusion on the safety of ethoxyquin for pigs, ruminants and fish can be drawn. The maximum potentially safe ethoxyquin concentration in feeds for dogs is 11 mg complete feed. The FEEDAP Panel cannot conclude on safe concentrations for cats and other pets. Considering that the additive ethoxyquin contains *p*-phenetidine, the FEEDAP Panel cannot conclude on any safe level of ethoxyquin in feed for target animals.

An estimate of consumer exposure to ethoxyquin-related residues in tissues and products from animals treated with ethoxyquin is not possible because there are considerable data gaps. An assessment of safety for the consumer is prevented by the lack of a safe level of exposure and the presence of *p*-phenetidine in the currently measured quantities in the additive.

Ethoxyquin itself has a low toxicity by inhalation and is not a dermal irritant, but should be considered a potential irritant to eyes and other mucous membranes and a skin sensitizer.

Since the ecotoxicity of ethoxyquin to the soil and the sediment compartments cannot be assessed owing to lack of data, no conclusion on the safety for the environment can be made for the use of ethoxyquin as a feed additive for all animal species.

Ethoxyquin is efficacious as an antioxidant in feed; however, the presented studies do not confirm its efficacy at the proposed use level of 50 mg/kg complete feed.

Remark

The conditions of use proposed by the applicant contain, as an extension to the maximum content of 50 mg ethoxyquin/kg complete feed: the combination with BHA and/or BHT should not exceed 150 mg/kg complete feedingstuffs. The FEEDAP Panel notes that the combination of one or two other antioxidants with ethoxyquin has not been assessed since the additives BHA and BHT are presently under re-evaluation.

Documentation provided to EFSA

1. Ethoxyquin for all animal species. September 2010. Submitted by FEFANA asbl.
2. Ethoxyquin for all animal species. Supplementary information. February 2014. Submitted by FEFANA asbl.
3. Ethoxyquin for all animal species. Supplementary information. October 2014. Submitted by FEFANA asbl.
4. Ethoxyquin for all animal species. Supplementary information. July 2015. Submitted by FEFANA asbl.
5. Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for Ethoxyquin for all animal species.
6. Comments from Member States received through the ScienceNet.

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Abbreviations

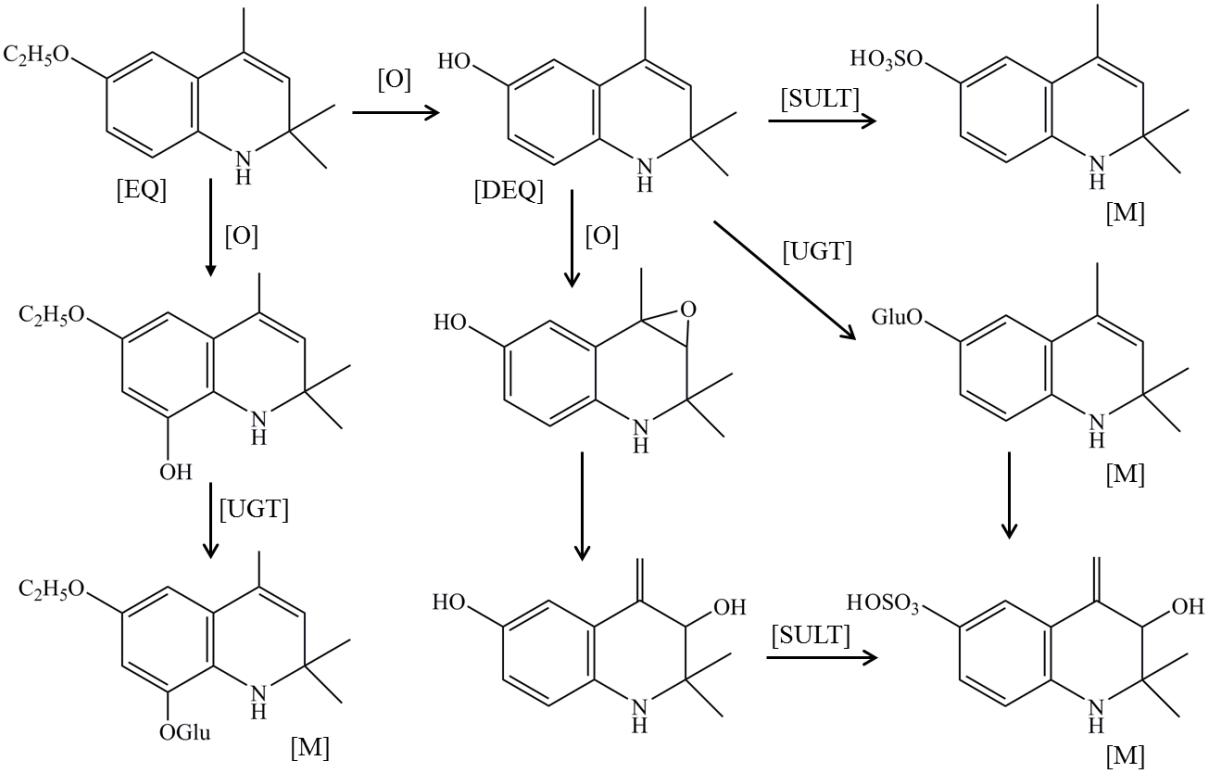
γGT	Gamma-glutamyl transferase
ADME	Absorption, distribution, metabolism and excretion
ALT	alanine aminotransferase
AP	alkaline phosphatase
AST	aspartate aminotransferase
BHA	butylated hydroxyanisole
BHT	butylated hydroxy toluene
BUN	Blood urea nitrogen
CAS	Chemical Abstracts Service
CHO	chinese hamster ovary
CP	Crude protein
CPN	chronic progressive nephropathy
CXL	codex maximum residue limit
DAR	Draft Assessment Report (prepared under Council Directive 91/414/EEC)
DEQ	De-ethylated ethoxyquin
DMBA	dimethyl benzanthracene
DNA	Deoxyribonucleic acid
EC	European Commission
EFSA	European Food Safety Authority
EINECS	European Inventory of Existing Commercial chemical structures
EQDM	ethoxyquin dimer
EQI	ethoxyquin qunone imine
EQQI	ethoxyquin/ethoxyquin quinolone derivative dimer
EU	European Union
EURL	European Union Reference Laboratory for Feed Additives
FAO	Food and Agriculture Organisation
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
GHS	Globally Harmonised System of Classification and labelling of Chemicals
GLM	Generalised linear model
GLP	Good laboratory practice
GSH	glutathione
HDL	high-density lipoprotein
HPLC	high-performance liquid chromatography
IARC	Intenational Agency for Research on Cancer
IMO	International Maritime Organisation
JMPR	Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment

LD_{50}	Lethal Dose, 50%
LDL	Low-density lipoprotein
LOD	limit of detection
LOQ	Limit of quantification
ME	Metabolisable energy
MJ	Mega Joule
MRLs	maximum residue levels
mRNA	Messenger ribonucleic acid
NOAEL	no observed adverse effect leve
NOEL	no observed effect level
OECD	Organisation for Economic Co-operation and Development
PCB	Polychlorinated biphenyls
PCDD	Polychlorinated dibenzo-p-dioxins
PCDF	Polychlorinated dibenzofurans
PEC	Predicted environmental concentrations
PGS	Prostaglandin synthetase
QSAR	quantitative structure-activity relationship
RH	Relative humidity
SCAN	Scientific Committee on Animal Nutrition
SCF	Scientific Committee for Food
SIDS	Screening Information Data Set
TEQ	Toxic equivalency factor
TSH	thyroid-stimulating hormone
UN	United Nations
US NTP	United States Nation Toxicology Program
WHO	World Health Organisation

Appendix A – Summary of the studies on metabolic fate and toxicological profile of ethoxyquin

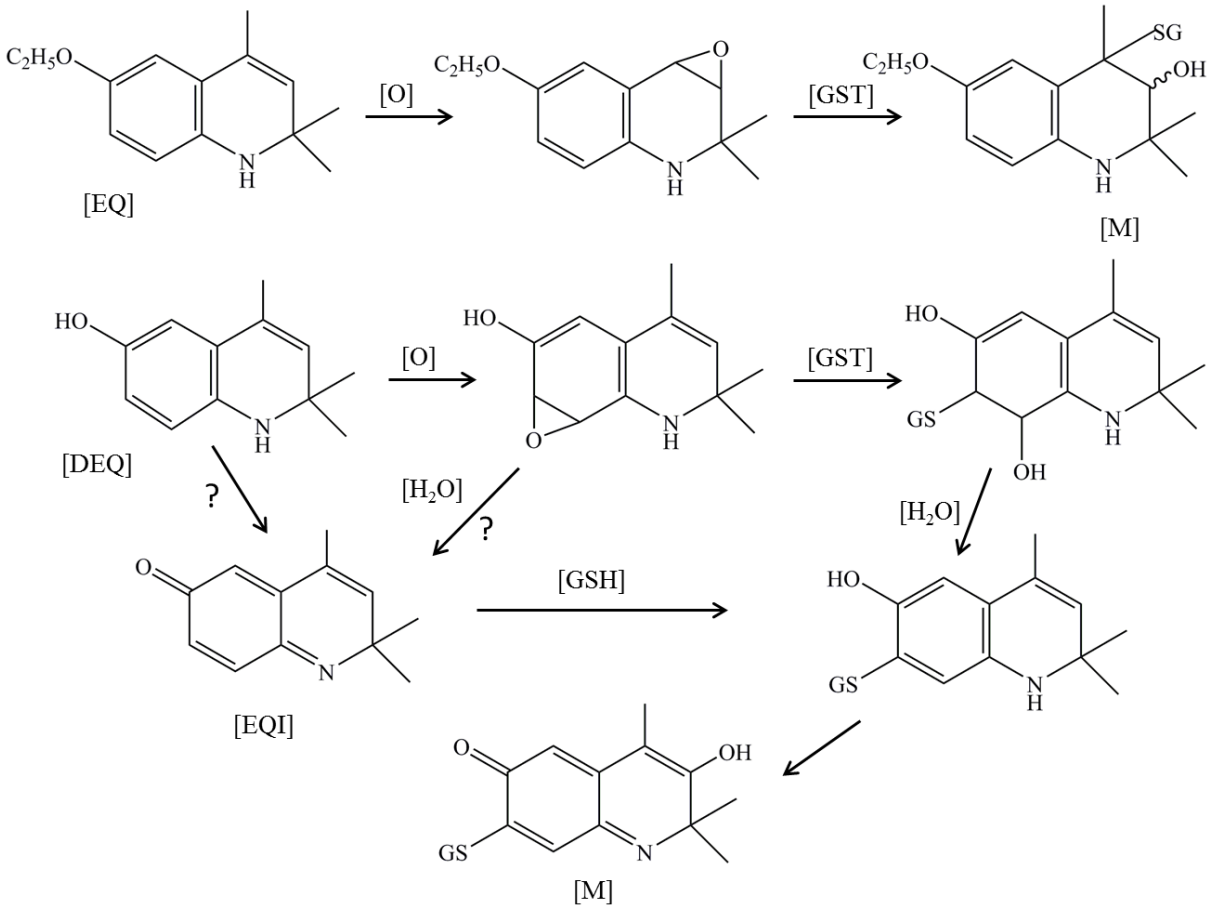
A.1. Metabolic fate

The metabolic pathways leading to the major urinary and biliary metabolites of ethoxyquin are described in Figure 2 and in Figure 3, respectively.



EQ, ethoxyquin; DEQ, de-ethylated ethoxyquin; O, oxidation; M, identified metabolites; SULT, sulphotransferase; UGT, glucuronosyl transferase.

Figure 2: Metabolic scheme for urinary metabolites of ethoxyquin in rat and mouse (adapted from Burka et al., 1996).



EQ, ethoxyquin; EQI, ethoxyquin quinone imine; DEQ, de-ethylated ethoxyquin; O, oxidation; M, identified metabolites; GST, glutathione transferase; GSH, glutathione.

Figure 3: Metabolic scheme for biliary metabolites of ethoxyquin in rat and mouse (adapted from Burka et al., 1996).

A.2. Toxicological profile

A.2.1. Acute toxicity

The acute toxicity of ethoxyquin has been investigated in several studies of rats that were summarised in DAR (Germany, 2007) and in US EPA (2004). Single-dose oral LD_{50} values of 1657 to 2040 mg/kg bw were reported, which are classified as 'slightly toxic' on the Hodge and Sterner scale and as 'Toxicity Category III' on the scale used by the US EPA. The reported signs of toxicity included reduced food intake, hypoactivity, ataxia, red staining around the eyes and urogenital area, piloerection, laboured respiration and death. Autopsies and/or histopathology showed gastrointestinal inflammation, haemorrhagic lungs, liver jaundice and red fluid in the urinary bladder.

A single oral administration of 500 mg ethoxyquin/kg bw to rats caused a slowly developing depression that lasted 3 days and degeneration of hepatocytes (Nafstad and Skaare, 1978, as cited by US EPA, 2004).

In an unpublished study claimed to be performed in accordance with GLP principles (FAO, 2005), groups of six male and six female beagle dogs were administered capsules containing ethoxyquin (purity 98.93 %) in a single oral dose of 50, 100 or 200 mg/kg bw. A concurrent control group received empty capsules on a comparable dietary regimen. Four animals of each sex per group were subjected to necropsy 24 hours after dosing, while the remaining two animals of each sex per group were assigned to a 14-day non-dosing recovery period. A statistically significant increase in total serum bilirubin was consistently observed 24 hours after dosing in animals of either sex at all tested dosages, but values returned to normal levels in the remaining animals (n = 2 for each sex) sampled

14 days after ethoxyquin administration. In dogs subjected to necropsies 24 hours after dosing, microscopic findings were confined to the liver and consisted of minimal to mild bile stasis in all dogs in treated with ethoxyquin at all doses. Bile stasis was characterised by spherical accumulations of bile in intrahepatic bile canaliculi and was consistent with increased total bilirubin concentration noted in serum chemistry profiles. In addition to bile stasis, deposits of hepatocellular glycogen were generally depleted in all dogs treated with 200 mg/kg bw. In the remaining dogs (n = 2 for each sex) which were killed for autopsy 14 days after dosing, lesions were restricted to the liver and consisted of minimal bile stasis in the liver in males treated with 50, 100 and 200 mg/kg bw ethoxyquin and in females treated with the higher ethoxyquin doses of 100 and 200 mg/kg bw.

The Environmental Protection Agency of the USA (US EPA) set an acute reference dose (acute RfD) for ethoxyquin of 0.3 mg/kg bw, set by applying a safety factor of 100 to a NOAEL of 3 mg/kg bw/day, which was the highest dose administered in a rabbit developmental toxicity study that showed no adverse effects.

A.2.2. Sub-chronic oral toxicity

A.2.2.1 Mouse studies

Female mice given 0.25 or 0.5 % (275 or 750 mg/kg bw per day) ethoxyquin or ethoxyquin hydrochloride in their diets for 20 days experienced a dose-dependent body weight loss, decreasing with time (Kim, 1985 as cited in US EPA, 2004). A significant ($P < 0.0001$ compared with controls) and dose-dependent increase in the prevalence of hepatic hypertrophy was seen with both substances. Liver weights in the high-dose ethoxyquin group were higher than those in the high-dose ethoxyquin hydrochloride group.

A.2.2.2 Rat studies

Munday et al. (1999) tested the effects of various substances, including ethoxyquin, on the toxicity of two naphthoquinones: 2-methyl-1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone. Three groups of six female Sprague–Dawley rats were given nine daily oral gavage doses of 250 mg/kg bw/day of ethoxyquin in maize oil. One of these groups received no further treatment, whereas the rats in other two groups were each given, 1 day later, a single oral dose of 3 mmol/kg bw 2-hydroxy-1,4-naphthoquinone or 6 mmol/kg bw 2-methyl-1,4-naphthoquinone. Groups of six control rats were given maize oil without ethoxyquin for 9 days followed by either no further treatment or treatment with 1,4-naphthoquinone or 2-methyl-1,4-naphthoquinone. An untreated control group of six rats was also used. One day later the rats were killed for necropsy and blood was taken for analysis for packed cell volume (PCV), haemoglobin (Hb), Heinz bodies, and plasma levels of urea and creatinine. Selected organs (liver, kidneys, spleen, gastrointestinal tract, heart, bladder, lungs) were weighed and examined microscopically. DT-diaphorase activity was measured in samples of the same organs. (DT-diaphorase is an enzyme that facilitates conjugation and excretion of naphthoquinones.) When ethoxyquin was given on its own, there was no effect on body weight gain, but weights of kidneys, liver and forestomach were increased. The activity of DT-diaphorase was increased in the liver, kidneys, lungs, glandular stomach, duodenum, jejunum, ileum, caecum and colon. The naphthoquinones caused haemolytic anaemia, as reflected by increased erythrocytic Heinz body formation, decreased blood packed cell volumes and haemoglobin levels, increased relative splenic weights, splenic sinusoidal engorgement, hepatic erythropoiesis and increased levels of iron in the spleen, liver and kidneys. The pretreatment with ethoxyquin increased the severity of the haemolytic activity of 2-hydroxy-1,4-naphthoquinone but protected against that of 2-methyl-1,4-naphthoquinone. The severity of renal lesions (tubular dilatation, necrosis, casts) caused by 2-hydroxy-1,4-naphthoquinone was unaffected by the pretreatment with ethoxyquin. An NOAEL for ethoxyquin was not identified from the results of this study, but the study was not designed for this.

When male Sprague–Dawley rats were fed ethoxyquin (3.15 mmol/kg bw per day, equivalent to 684 mg/kg bw per day) for 3 weeks at a concentration equal to the haemorrhagic LC_{50} (lethal concentration in 50 % of animals at 40 days) of butylated hydroxytoluene (BHT), ethoxyquin caused some animals to haemorrhage in a manner similar to that observed following BHT (Takahashi and Hiraga, 1978).

Ethoxyquin at 0.8 % (equivalent to 720 mg/kg bw per day), administered in drinking water to male F344 rats for 4 to 8 weeks, did not have any observable effect on mortality. However, ethoxyquin caused significant body weight reduction accompanied by food consumption decline at both intervals.

Water consumption was also reduced. Ethoxyquin did not cause any changes in urinary pH or sodium ion concentration or in any other urinary parameters examined (chlorine, potassium, calcium, phosphorus, magnesium, urine crystals). There were no microscopic or macroscopic abnormalities of the urinary bladder in rats examined at both intervals. Leafy or ropey microridges and/or short uniform microvilli were observed on the bladder epithelial surface. Urinary bladder DNA synthesis was assessed by bromodeoxyuridine (BrdU) incorporation and was found to be significantly increased in the ethoxyquin-treated rats compared with the untreated controls (Shibata et al., 1989).

In a range-finding study, groups of five Sprague–Dawley rats of each sex were given oral gavage doses (in maize oil) of 0, 50, 250, 500 or 1000 mg ethoxyquin/kg bw/day for 28 days (unpublished study summarised by US EPA, 2004). (A full report of this study was not available.) The ethoxyquin was of technical grade (98.2 % pure). It was claimed that the study was conducted in accordance with GLP. The protocol was broadly in line with OECD Guideline 407 (2008), but did not include the additional testing for neurotoxicity and immunotoxicity that were introduced in 1995. In the 1 000 mg/kg bw/day dose group, all of the animals died within the first 3 days of treatment, whereas all animals treated with 500 mg/kg bw/day survived until the end of the scheduled treatment period. Doses of 250 mg/kg bw/day or more resulted in adverse clinical signs, including salivation, yellow matting of fur and brown-coloured urine, and changes in haematological parameters (decrease in the RBC count, Hb level and PCV) and plasma biochemistry (increased total protein, globulin, cholesterol, phosphorus, potassium and calcium and decreased albumin–globulin (A/G) ratio) were seen. At 500 mg/kg bw/day or more, body weight gain was reduced and further changes in serum biochemistry were seen (increased bilirubin, blood urea nitrogen (BUN) and gamma-glutamyltransferase (γGT) and reduced glucose) in addition to those seen at 250 mg/kg bw/day. At 500 and 1 000 mg/kg bw/day, absolute and relative liver weights were increased in both sexes, and histopathological lesions were found in the kidneys (tubular dilatation, lymphocytic infiltration and regeneration of tubular epithelium). A low level of lymphocytic infiltration in the kidneys of the 50 and 250 mg/kg bw/day groups, in the absence of other effects, was not considered by the authors to be treatment related. In rats given 1 000 mg/kg bw/day, gross lesions were found in the gastrointestinal tract, kidneys and lymph nodes, and histopathological lesions were found in the kidneys, liver, lungs, stomach and spleen. The NOAEL for this study was 50 mg/kg bw/day, based on clinical signs, haematological changes and blood biochemistry changes at doses of 250 mg/kg bw/day or greater.

In the main study, groups of 10 Sprague–Dawley rats of each sex were given daily, by gavage, doses of 0, 20, 40, 200 or 400 mg ethoxyquin/kg bw in maize oil for 90 days. The ethoxyquin was of technical grade (98.2 % pure) (unpublished study summarised by US EPA, 2004). It was claimed that the study was conducted in accordance with GLP. It was broadly in line with OECD Guideline 408 (1998). Ophthalmoscopy was performed at the beginning and end of the treatment period and on week 12. Clinical pathology (haematology, serum biochemistry and urinalysis) was performed on samples taken at the end of the experiment. Autopsies and histopathology were performed on all animals. All rats survived until the end of the treatment period. Clinical signs were reported in both sexes in the 200 and 400 mg/kg bw/day dose groups: excessive salivation, yellow staining of the fur, red staining around the mouth and brown staining around the urogenital area. Body weight gain was reduced in males at doses of 40 mg/kg bw/day or more throughout the study. In females body weight gain was reduced at 200 mg/kg bw/day or more at some measurements, but was within 5 % of control values at termination. Haematological (decreased white blood cell count (WB), RBC, Hb, PCV, prothrombin time; and increased reticulocyte count) and serum biochemistry changes (increased bilirubin, γGT and cholesterol) were seen at 200 and 400 mg/kg bw/day. Total leucocyte count was also decreased in rats of both sexes in the 400 mg/kg bw/day group. Serum thyroxine (T4) was decreased in males receiving the highest dose, and thyroid-stimulating hormone (TSH) was elevated in males given 200 mg/kg bw/day and in both sexes given 40 mg/kg bw/day. Reddened or enlarged thyroids were seen in 5/10 male and 9/10 female rats given 200 mg/kg bw/day and in all rats at 400 mg/kg bw/day. Amber-coloured urine was reported in the 200 and 400 mg/kg bw/day groups and 'abnormal contents' were found in the urinary bladders of rats in the 400 mg/kg bw/day group. Absolute and relative weights of liver and kidneys were increased in rats of each sex in the 200 and 400 mg/kg bw/day groups. Histopathological lesions of the kidneys (nephropathy and hyaline droplets in cortical tubular epithelial cells for females only, and papillary necrosis and hyaline bodies in both sexes) were found in the rats in the 200 and 400 mg/kg bw/day groups. The NOAEL for this study was 20 mg/kg bw/day, based on reduced body weight at 40 mg/kg bw/day.

A.2.2.3 Dog studies

A 28-day dose-range-finding study was performed on groups of two male and two female beagle dogs given 0, 25, 50, 100 and 200 mg ethoxyquin/kg bw/day in orally administered gelatine capsules. The ethoxyquin was of technical grade (98.2 % pure) (unpublished study, summarised in FAO, 1998, and US EPA, 2004). It was claimed that the study was conducted in accordance with GLP. There is no OECD Guideline for 28-day toxicity testing in non-rodents. Urine was collected for analysis at unstated times. At the end of the treatment period, blood samples were taken for haematology and serum biochemistry. Autopsies and histopathology were performed on all animals. All of the dogs given 100 or 200 mg/kg bw/day and one female given 50 mg/kg bw/day had to be euthanised because of treatment-related poor condition, characterised by body weight loss, reduced food consumption, reduced activity, emaciation, decreased defecation and brown-coloured urine. Serum biochemistry changes in the euthanised dogs included increases in alkaline phosphatase (AP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, BUN and glucose and decreases in sodium, potassium, calcium, chloride and phosphorus. All euthanised dogs had gastrointestinal alterations (including reddened mucosa, stomach ulcers and enlarged lymph nodes), dark-coloured livers and atrophy of the thymus. Dark pigmentation of the liver was also noted in all treated rats that were killed at the scheduled time, after 90 days. Organ weights, haematology and urinalysis were unaffected by treatment. Reduced body weight gain and reduced food intake were seen in both sexes at 50 mg/kg bw/day and reduced body weight gain was seen in females given 25 mg/kg bw/day. In the animals that survived until the end of the scheduled treatment period, there were changes in serum biochemistry (including reductions in ALT, AST, glucose and calcium) at all dose levels tested. No haematological changes were seen. As there was reduced body weight gain in males and changes in serum biochemistry parameters in both sexes at all dosages tested, it was not possible to identify a NOAEL for this study.

In the main study, groups of five beagle dogs of each sex were given daily oral capsules delivering dosages of 0, 2, 4, 20 and 40 mg ethoxyquin/kg bw/day for up to 90 days. The ethoxyquin was of technical grade (98.2 % pure) (unpublished study, summarised in FAO, 1998, and US EPA, 2004). It was claimed that the study was conducted in accordance with GLP. It was in broad agreement with OECD Guideline 409. Treatment was stopped in the high-dose group after 49 days because of unacceptably high systemic toxicity, and the dogs in this group received empty capsules for the remainder of the planned 90-day treatment period. One female in this group had to be euthanised because of pneumonia. Blood and urine were taken for analysis prior to the start of dosing and during weeks 4 and 13 of treatment. Ophthalmoscopy was performed prior to the start of treatment and in week 12. Autopsies and histopathology were performed on all animals. One female in the high-dose group had to be euthanised on day 13 of treatment, but all other dogs survived until the end of the treatment period. Reduced food consumption and reduced body weight gain were seen at 20 and 40 mg/kg bw/day, and body weight loss was seen in the 40 mg/kg bw/day dogs at several times during the treatment period. Clinical signs were noted in the groups given 20 and 40 mg/kg bw/day, and included brown sclera, brown- or black-coloured urine, staining of the urogenital area, black mucoid faeces and emesis. Urinalysis showed no changes other than pigmentation. Haematological and ophthalmoscopic parameters were unaffected by treatment. Changes in serum biochemistry were seen at week 13 with dosages of 4 mg/kg bw/day or greater, with increases in AP, ALT, AST and γGT seen at 20 and 40 mg/kg bw/day, and only ALT affected at 4 mg/kg bw/day. Dark-coloured livers were noted in the 20 and 40 mg/kg bw/day groups at autopsy. Organ weights were unaffected by treatment. Microscopic examination showed lesions of the livers at dosages of 4 mg/kg bw/day or greater: pigmentation (described as 'porphyrin-class') of hepatocytes and Kupffer cells, hepatocellular vacuolation, bile duct proliferation (not at 4 mg/kg bw/day), and hepatocellular necrosis (minimal at 4 mg/kg bw/day. The NOAEL for this study was 2 mg/kg bw/day, based on hepatotoxicity seen at 4 mg/kg bw/day or greater.

A.2.3. Chronic toxicity and carcinogenicity studies

A.2.3.1 Mouse study

The parenteral carcinogenicity of several food additives, including ethoxyquin, was tested in infant Swiss mice (Epstein et al., 1970). Four subcutaneous doses of ethoxyquin, ranging from 1 to 10 mg/kg bw, were administered over the first 21 days of life. All dosing regimes caused acute toxicity. Surviving mice were sacrificed at 1 year of age and examined for tumours. A few of the mice developed lymphomas or pulmonary adenomas, but the prevalences of tumours were not statistically different from those found in solvent-treated control animals and, thus the results were equivocal as regards the carcinogenicity of ethoxyquin.

Dietary concentrations of 0.25 and 0.5 % ethoxyquin were reported to prolong the lifespan of mice (Rudra et al., 1974). No details were given.

A.2.3.2 Rat studies

The report of JMPR (FAO, 1969) summarised an otherwise unavailable rat chronic toxicity/carcinogenicity study from 1959 that did not conform to current OECD Guidelines. Ethoxyquin was given at dietary concentrations of 0, 62, 125, 250, 500, 1000, 2000 or 4000 ppm to groups of 10 male and 10 female rats of an unspecified strain for up to 715 days. Unspecified numbers of the animals from each group were killed after 200, 400, 600 and 715 days of treatment. Occasional reductions in body weight gain were seen in the rats given 2000 ppm. Haemoglobin levels in blood were reported to be normal for both sexes of groups given 2000 or 4000 ppm in blood samples taken on Days 100 and 300 of treatment. Increased relative weights of liver and kidneys were found in males from the 250 ppm group and females from the 1000 ppm group that were killed on Day 200. Unspecified histopathological changes to the renal cortex were seen in males (but not females) of the 2000 and 4000 ppm groups that were killed on Day 200. All other organs appeared normal in the rats killed on Day 200. In the male rats (but not females) killed on Day 400, there was pyelonephrosis of the kidneys and unspecified lesions of the liver and thyroid, but the JMPR did not report which dose groups were affected. Similar lesions were seen in both sexes at all of the later killing times, with the males being the most severely affected. Various types of tumours were found in all groups, including controls, but did not appear to be treatment-related. The JMPR noted that 'no clearly defined lesions were present from feeding 62 ppm'. The JMPR (FAO, 1969) concluded that the NOAEL for this study was 125 ppm (equivalent to 6.25 mg/kg bw/day). It is noted that the reporting of the study is insufficiently clear to allow the FEEDAP to confirm this NOAEL. Furthermore, the unspecified but small number of animals treated for the full length of the study severely limits the reliability of the study for evaluating carcinogenicity.

The report of JMPR (FAO, 1998) summarised two otherwise unavailable rat toxicity studies from 1992. The studies were similar to one-another, using the same single dose level and the same strain of rat. In one of the studies, groups of 6 to 19 F344 rats of each sex received diets containing 0 or 5000 ppm of ethoxyquin (equivalent to 250 mg/kg bw/day) for either 18 months or for 24 weeks followed by 34 weeks on control diet. Unspecified numbers of animals were killed at 4, 12 or 14, 24 and 78 weeks of the study. Body weight gain and feed intake were reduced in all treated groups. Males had significant interstitial degeneration of the papilla of the kidneys from week 4 and it progressed to pyelonephritis of the cortex and hyperplasia of the epithelium of the renal pelvis by week 24. In females there was only slight interstitial degeneration of the papilla, which did not appear until week 14 and did not progress consistently. A brown pigment, which was shown by histochemistry to be lipofuscin, was detected in the proximal tubules of treated rats. The kidney lesions present after 24 months of treatment did not regress after 34 weeks on control diet. This study did not identify a NOAEL as renal lesions were found in both sexes at the only dose level used.

In the other 1992 toxicity study reported by JMPR (FAO, 1998), groups of 4 to 8 male F344 rats were fed from 3 or 8 weeks-of-age a diet containing 5000 ppm ethoxyquin (equivalent to 250 mg/kg bw/day) for 20, 26 or 30 weeks, whilst 8 females received the same dose of ethoxyquin for 30 weeks starting at 8 weeks-of-age. No mention was made of control groups. Body weight gain was reduced in all treated groups. Relative weights of kidneys were increased in both sexes. In all groups of treated males the urinary concentration of $alpha_{2u}$-globulin was decreased and of albumin was increased. Histochemistry with bromodeoxyuridine (BrdU) showed cell regeneration in the renal tubules of males killed at 30 weeks, but this was not seen in males killed at 20 weeks. Renal cortical changes (eosinic

cytoplasic inclusions in tubular epithelial cells and protein accumulation in the lamina of the tubules) were seen in groups of males exposed to ethoxyquin from 8 weeks-of–age. The kidneys of males dosed from 3 weeks-of-age also had the cortical changes but in addition had papillary necrosis, slight deposition of calcium and hyperplasia of the transitional epithelium of the renal pelvis. The kidneys of the treated females were histologically similar to the controls, except for having a high concentration of lipofuscin deposition. It was concluded that the pattern of renal changes caused in rats by ethoxyquin is dependent upon the sex of the subject and the age of initial exposure. An NOAEL could not be identified for this study as adverse effects were found at the only dose level used.

A series of experiments were performed on rats to investigate the effect of ethoxyquin in modifying the carcinogenicity to specific organs of various known carcinogens (Ito, et al., 1986a and 1986b; Fukushima, et al., 1987a and 1987b; Miyata, et al., 1985). These studies are described in more detail below. These experiments used control groups that had been given dietary ethoxyquin in the absence of the known carcinogen. The results for these groups are of relevance to the carcinogenicity testing of ethoxyquin. A group of 25 male F344 rats were given 0.8 % ethoxyquin (equivalent to 720 mg/kg bw per day) in their feed for 29 weeks, and developed no tumours in their liver or kidneys. Similarly, no tumours were found in the oesophagus or fore-stomach of 20 male F344 rats given 0.8 % ethoxyquin in their feed for 32 weeks. No tumours were found in the ear duct or mammary glands of 25 female Sprague-Dawley rats given 0.8 % ethoxyquin in their diet for 32 weeks. A group of 15 male F344 rats with unilateral urothelial ligation developed no bladder tumours when they were given 0.8 % ethoxyquin for 22 weeks. The results of these experiments give no evidence to suggest that ethoxyquin is carcinogenic to rats, It is noted that the group sizes in these studies were less than those recommended in OECD Guidelines, the studies were not performed in both sexes, and only a limited number of organs were investigated.

In a sequential study of chronic nephrotoxicity, groups of male and female F344 rats were fed diet containing 0 (control) or 0.5 % ethoxyquin and killed after exposure periods of 4 weeks, 12–14 weeks, 24 weeks, 58 weeks or 18 months (Hard and Neal, 1991). The average dosages received were 65.3 mg/kg bw per day for males and 70.6 mg/kg bw per day for females. Kidneys were taken for microscopic examination, which included conventional haematoxylin-eosin stained sections and histochemical staining of some sections for lipofuscin and for iron. The numbers of rats of each sex from each group that were killed at each time period varied between 6 and 20. Male rats developed interstitial degeneration of the renal papillary tip as early as 4 weeks, and this progressed to papillary necrosis by 24 weeks. The papillary necrosis was consistently accompanied by pyelonephritis affecting the cortex and by urothelial hyperplasia of the renal pelvis. Females were less affected by treatment than the males, developing papillary interstitial degeneration only after longer exposure, the lesion never progressing further. Accumulation of lipofuscin-related pigment was seen in the proximal tubules of females, but not in males. Spontaneous chronic progressive nephropathy (CPN) was exacerbated by ethoxyquin in males and to a lesser extent in females. Proximal tubule hyperplasia was most frequently observed in ethoxyquin-treated males at the later sampling times. In all cases, such proliferative lesions were associated either with pyelonephritis or with the most advanced stages of CPN. There was no evidence that ethoxyquin directly induced preneoplastic renal tubule hyperplasia.

In a subsequent study, groups of F344 rats were given ethoxyquin (90 % pure) in the diet at concentrations of 0, 0.01, 0.05, 0.1, 0.25 or 0.5 % for 3 or 6 months (Neal, et al., 2003). The group sizes were 5 males for the three-month part of the study and 8 males and 8 females for the 6-month part. At the end of the treatment periods, the rats were killed and their kidneys were removed for histological examination. Feed intake was similar for all groups; body weight was not recorded. None of the rats died prematurely. In females, there was only one animal affected: a high-dose female that had minimal interstitial degeneration of the kidney. In males at both 3 and 6 months, there was either interstitial degeneration of the renal papilla or frank necrosis in those given the highest dose level, but not in those given 0.25 % or lower concentrations. Fluorescence microscopy showed a 'very slight increase in lysosome distribution' in proximal tubules of 0.25 % and 0.5 % males at both 3 and 6 months, but there was no evidence of accumulation of hyaline droplets. The urinary concentration of albumin was increased in both sexes at the highest dose level. Following administration of ^{14}C-labelled ethoxyquin by the intraperitoneal or oral route, the radiolabel was associated with urinary albumin and not with α2u-globulin. Autoradiography of the kidneys showed no sex difference in the distribution or retention of radiolabel. Furthermore the profiles of faecal and urinary metabolites were also similar in

both sexes. The NOAEL for this study was 0.1 %, which is equivalent to 50 mg/kg bw per day (using EFSA default values for chronic exposure of rats).

In a study looking at the effects of ethoxyquin on AFB1-induced effects on the liver and kidneys in F344 rats, it was found that 0.5 % dietary ethoxyquin given for 23 weeks in the absence of AFB1 caused renal changes including glomerulonephrosis, tubular hyperplasia, and putative preneoplastic tubules (Manson, et al., 1987). A follow-up study was performed using groups of 4 to 8 male and female F344 rats of aged 3 or 8 weeks at the start of the study, which were fed 0.5 % ethoxyquin for 20 to 30 weeks (Manson, et al., 1992). There was little evidence of any renal toxicity in females of either age, with effects limited to increased relative kidney weight and accumulation of a brown pigment in the tubules. In male the additional effects seen included proteinuria and damage to the renal cortex, with extensive renal papillary necrosis being also seen in the younger males. Urinary levels of α2u-globulin were unaffected by treatment. The NOAEL for this study was 0.5 %, which is equivalent to 250 mg/kg bw per day, calculated using the default values described in the EFSA Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data (EFSA Scientific Committee, 2012).

Male Wistar rats were fed diet containing 0.1 % ethoxyquin (50 mg/kg bw per day) for 32 weeks and compared with untreated controls. Renal stones and calcification were observed from the renal papilla to the pelvis region in treated rats. Chemical element analysis of the stones showed phosphorus and calcium to be the primary constituants (text taken from US EPA, 2004, which cites Imazawa, et al., 1989).

Groups of 5 weanling Wistar rats were given either 0 (control) or 0.5 % ethoxyquin (equivalent to 250 mg ethoxyquin/kg bw per day) in their diet (Rudra, *et al.*, 1974). The rats given ethoxyquin ate less than untreated controls fed *ad libitum*, but when feed intake of controls was matched to that of rats fed ethoxyquin there was no difference in body weight gain over the first 280 days of the study. However, after this time the treated rats gained less body weight than controls. This was probably due to renal toxicity as most (4/5) of the ethoxyquin rats subsequently died of severe kidney failure.

Some old unpublished studies of ethoxyquin had been reviewed and summarised in a report of the US National Toxicology Program (US NTP, 1990). These studies were not conducted to current regulatory and scientific standards, but might provide some additional insight into the chronic toxicity of ethoxyquin. As the original reports are not available to the FEEDAP Panel, the US NTP summaries are reproduced here ad verbatim as reported by US EPA (2004).

'In a chronic oral feeding study, two trial experiments were conducted on weanling albino rats. In trial 1, 10 male and 10 female rats were continually fed a diet containing 0.0, 0.2, 0.4 % ethoxyquin in alfalfa. On day 155, males and females in the 0.4 % dose group weighed significantly less ($p<0.05$ and $p<0.01$, respectively) than control rats. Rats in the 0.2 % group also weighed less (not significant) than controls. In trial 2, groups of 10 male rats were placed on diets containing 0, 0.0062, 0.0125, 0.025, 0.05, 0.1 and 0.2 % ethoxyquin and groups of 10 females were fed diets containing 0, 0.05, 0.1 and 0.2 % ethoxyquin for 225 days. In addition, 5 females were fed diets containing 0.0125 and 0.025 % ethoxyquin. Half of the male animals in the 0, 0.2, 0.1, 0.05 and 0.025 % ethoxyquin and half of the females in the 0, 0.2, 0.1 % dose groups were sacrificed on day 225. A small indication of kidney lesions was seen in the 0.1 and 0.2 % ethoxyquin groups. Liver weights among females in the 0.2 % ethoxyquin group and males in the 0.1 % groups as well as liver weights in all higher dose groups were significantly greater ($p<0.01$) in the 0.2 % (trials 1 and 2) and 0.4 % (trial 1) dose groups versus controls. Upon visual inspection of kidneys of 2 male rats in the 0.4 % ethoxyquin group, stones in the renal pelvis were noted. Microscopic examination revealed well-developed chronic pyelonephritis among males in the high dose groups. Two kidneys had areas of calcification. The thyroid glands of male rats exposed to higher doses of ethoxyquin had evidence of hyperplasia. All male rats in the 0.2 % ethoxyquin group, 2/3 in the 0.1 % group and 2/5 in the 0.05 % group had small kidney scars [Western Utilization Research Branch, 1954]. As a supplement to the above study, the surviving rats (5–10 males and 5–10 females) were fed ethoxyquin in their diet at the same concentrations as the male rat diet described above. Mortality occurred at the 700th day due to infection. Pathological inspection revealed a pitted surface of the kidneys in male rats at the 0.2 % and 0.1 % ethoxyquin dose groups. The authors state that other observed gross changes bore no apparent relationship to ethoxyquin including respiratory tract infection, tumors, cystic ovaries and

infected uteri. In this study, the NOEL for microscopic pathological changes was calculated to be 13 and 15 mg/kg/day for male and female rats, respectively, at day 225.'

A.2.3.3 Dog studies

In the 1980s, there were several reports of adverse effects of dietary ethoxyquin in dogs. The symptoms observed by dog owners and veterinarians were liver, kidney, thyroid and reproductive dysfunction, teratogenic and carcinogenic effects, allergic reactions, and a host of skin and hair abnormalities (Błaszczyk, et al., 2013).

A 1-year toxicity study was performed in groups of four beagle dogs of each sex given ethoxyquin in the diets providing 0, 0.9, 1.8, 2.7, or 3.6 mg ethoxyquin/kg bw per day. Ophthalmoscopy was performed pre-treatment and just before killing for autopsy. Blood and urine samples were taken pre-treatment, after 3 and 6 months of treatment and prior to autopsy. Autopsies were performed on all animals at the end of the treatment period. Histopathology of a full selection of organs was performed only on animals in control and the highest dose groups. In addition, all gross lesions and adrenals were examined microscopically in all dogs, and the liver was examined in all male dogs in the 2.7 mg/kg bw per day group and in one male from the 0.9 mg/kg bw per day group. The treatment had no effects on mortality, body weight, food intake, clinical signs, ophthalmoscopy, haematology, blood biochemistry, urinalysis, organ weights or gross pathology. A trace amount of an anisotropic pigment was detected in liver Kupffer cells of one male dog treated with 3.6 mg/kg bw per day, but there was no associated clinical chemistry or histopathology and the finding was not regarded by the author as an adverse effect. No other treatment-related microscopic changes were detected. The NOEL for this study was the highest dose level, 3.6 mg/kg bw per day for females and 2.7 mg/kg bw per day for males.

The original report of the following study was not available to the FEEDAP Panel, the US NTP summary is reproduced here *ad verbatim* as reported by US EPA, 2004:

'In a one year chronic oral administration study supported by Monsanto, 7 male and 7 female mongrel dogs were divided into 4 groups (mixed male and female) and put on the following dose regime: no test compound (group 1; 1 male, 1 female); 10 mg/kg ethoxyquin (group 2; 2 males, 1 female); 50 mg/kg ethoxyquin (group 3; 1 male, 2 females); and 100 mg/kg (group 4; 1 male 2 females).All doses were administered orally in capsule form, five days per week, with the exception of group 3 , which received one dose per day for the first 6 weeks, and 2 doses per day thereafter. Group 4 administration was discontinued after 6 weeks due to toxicity. Group 4 dogs were sacrificed at week 9 and replaced by group 5 (1 male, 1 female), which received 3 mg/kg ethoxyquin. Gross examination revealed abdominal tenderness in one dog in groups 2, 3, and 5, within 5 weeks. Anorexia occurred in 2 dogs in group 4 and 1 dog in group 5. Depression and soft feces were also observed in one group 5 dog. Dark brown livers were seen in groups 2 (associated with evidence of intestinal parasitic infection), 3 and 4. Urine was also discoloured in groups 3 and 4, with occasional appearance of globules. Group 3 dogs developed irritation of the large and small intestine. On dog in group 5 had an enlarged liver. Bromsulphalein liver function was decreased in groups 2, 3, and 4 as early as the seventh week. All dogs in group4 and one in group 3 had erythrocyte sedimentation. Upon microscopic examination, liver and kidney changes were seen in all groups. Kidney swelling with fatty accumulation was seen in groups 2, 3, and 5. Granulation of cells in collecting tubules was also seen in groups 2 and 3. Liver degeneration and fatty metamorphosis of the liver was seen in groups 2, 3, and 5. Groups 3 and 4 exhibited marked retention of exogenous pigment in the liver. One dog in group 3 had massive fibrosis. However, there was no evidence of permanent damage or destruction of tissue in the test animals in which liver and kidney changes were evident, suggesting that the histological changes observed may be. The NOAEL in this study for microscopic pathological changes was 28 mg/kg/day at day 84.'

In a study that was reported in 1955, dogs received ethoxyquin capsules (Santoquin®) for 58 weeks. Groups of three dogs (one or two of each sex) were administered 0, 10, 50 or 100 mg/kg bw ethoxyquin daily (as Santoquin®), in two divided doses, on 5 days per week. After 6 weeks of treatment, the dogs given 100 mg/kg bw had to be euthanised as one dog was anaemic, and the other two were anorexic and losing weight. They were replaced by another group which was given 3 mg/kg bw. Blood and urine were sampled throughout the study. All dogs were autopsied at the end of the study and selected organs (liver, kidneys, small intestine, adrenals and thyroids) were examined microscopically. Reduced food intake and reduced body weight gain were seen in at least

one animal from each of the groups treated with ethoxyquin. The results of bromsulphthalein retention tests showed reduced liver function in the dogs of all treated groups. BUN levels were normal and similar to controls in all groups.

Haematology showed that in the animals groups given 50 or 100 mg/kg bw RBC count, haemoglobin level and haematocrit were reduced. On microscopic examination the RBCs of affected animals showed anisocytosis, poikilocytosis, macrocytosis, hypochromia and polychromatophilia. All groups of animals had high eosinophil counts (typical of parasitic infections). The urine of dogs given 50 or 100 mg/kg was coloured dark brown and contained large amounts of protein. One of the dogs given 3 mg/kg also had highly proteinaceous dark-coloured urine, and this urine also contained occult blood. Gross examination of the organs of controls showed no abnormalities. One of the dogs given 3 mg/kg bw had an enlarged liver of normal colour, but animals in this group appeared otherwise normal at post-mortem. In the group given 10, 50 or 100 mg/kg bw, the livers and associated lymph nodes appeared dark coloured but other organs appeared normal.

Intestinal parasites were found in several of the dogs. All groups of treated dogs had increased organ weights: liver, spleen, kidneys and heart. Microscopic examination of the liver of one of the control dogs showed abscesses that were thought to be due to a parasitic infestation. The dogs in the 3 mg/kg bw group which had an enlarged liver also exhibited areas of ulceration in the small intestine, granulomatous infiltration of the liver and fatty change in the hepatocytes and the renal tubular epithelial cells, effects that were reported to be due to histoplasmosis (infection by *Histoplasma capsulatum*). The tissues of the other dogs in the 3 mg/kg bw group appeared normal. Pigmentation of the hepatocytes and Kupffer cells of the liver and damage to the epithelial cells of renal tubules were found in dogs given 10 mg/kg bw or more. At 50 mg/kg bw, fatty change was seen in hepatocytes, and at 100 mg/kg bw there was also hepatic fibrosis. In can be concluded from this study that chronic exposure to oral ethoxyquin caused renal and hepatic toxicity. Although adverse effects were seen at all dose levels, the presence of various infections in the dogs makes it difficult to tell whether these effects were all entirely due to exposure to ethoxyquin. It is not possible to identify a reliable NOAEL from this study.

A 5-year toxicological study (started in 1959 and reported in 1966) was performed in beagle dogs. Ethoxyquin was fed to groups of seven male and seven female dogs at dietary concentrations of 0 or 300 ppm (w/w) for up to 5 years. No record was kept of food consumption, and body weights were measured only at the start of the study and at termination. Blood and urine were collected for analysis at termination. Gross pathological and histopathological examinations were performed on all animals. One control dog died prematurely after 4 year 9 months as a result of a lung infection. No treatment-related effects were observed on body weight, haematology, urine analysis, limited clinical chemistry end points (serum AST, BUN and bromosulphthalein retention time), absolute and relative organ weights, gross pathology or histopathology. The NOAEL for the study was the only dose tested: 300 ppm (equivalent to 15 mg/kg bw/day).

No effects on carcinogenesis were reported for any of the dog studies, although none of the studies were designed to investigate cancer end points.

The Environmental Protection Agency of the USA (EPA) set a chronic reference dose (chronic RfD) for dietary exposure of all human populations to ethoxyquin of 0.02 mg/kg bw/day (Jacquith, 2004). This was set by applying a safety factor of 100 to a LOAEL of 4 mg/kg bw/day for elevated serum enzymes and microscopic changes to the liver, including cytoplasmic vacuolation and minimal necrosis in hepatocytes, in a 90-day dog study.

A.2.4. Studies of effects modifying the carcinogenicity and/or toxicity of other substances

A.2.4.1 Rat studies

In an experiment to investigate the effects of antioxidants in promoting cancers of the oesophagus and forestomach, a group of 20 male F344 rats were pretreated with N,N-dibutylnitrosamine (DBN) and treated with ethoxyquin and were compared with a groups of 21 rats given DBN alone and 20 rats given ethoxyquin alone (Fukushima et al., 1987b). Pretreatment DBN was administered in drinking water at a concentration of 0.05 % for 4 weeks. Ethoxyquin was given by diet at a concentration of 0.8 % for 32 weeks. No carcinomas were found in the oesophagus or forestomach of any animals in any the three groups. The group given ethoxyquin alone also developed no papillomas

of the forestomach and oesophagus and no preneoplastic hyperplasia of the oesophagus. Six rats with oesophageal papillomas were found in the group given DBN plus ethoxyquin, compared with none of those given DBN alone. The prevalence of oesophageal preneoplastic hyperplasia and forestomach papillomas was similar in the group given DBN and ethoxyquin and the group given DBN alone. It is concluded that ethoxyquin enhanced the production of oesophageal papillomas in male F344 rats.

Takahashi et al. (1979) investigated the effects of ethoxyquin on stomach tumours caused by treatment of Wistar rats with N-methyl-N′-nitrosoguanidine (MNNG). Groups of 20 males were fed a high-salt diet (10 % NaCl) throughout the experiment and were given 100 mg/L MNNG in their drinking water for 8 weeks. One group was then given 1 % ethoxyquin in the diet for 32 weeks, whereas a control group was given the basal diet without supplementation. All rats were killed at the end of this treatment period and the development of gastroduodenal tumours was determined. The treatment with ethoxyquin plus MNNG increased the number of tumours in the glandular stomach (compared with the controls given MNNG alone), but not in the forestomach. In addition, nephrocalcinosis was seen in the rats given ethoxyquin plus MNNG but not in those given MNNG alone. It is concluded that ethoxyquin enhanced the production of tumours of the glandular stomach in male Wistar rats.

development of colon cancer, a group of 12 male F344 rats was pretreated with four weekly subcutaneous doses of 20 mg/kg bw 1,2-dimethylhydrazine (DMH) then fed for 36 weeks a diet containing 0.8 % ethoxyquin (Ito et al. 1986a, b). The number of tumours (adenomas plus adenocarcinomas) found in the descending colon (11 tumours) was significantly greater than in a control group (three tumours) that had been pretreated with DMH. No tumours were found in the transverse colon, and the number of tumours in the ascending colon and caecum (one and none respectively) was lower than in controls (one and three), although the difference was not significantly different. It is concluded that dietary ethoxyquin enhanced the carcinogenicity of DMH in the descending colon, but had no clear effect in other regions of the colon and caecum.

In an experiment to investigate the effects of antioxidants in promoting cancers of the mammary and ear duct, animals pretreated with 7,12-dimethylbenz[a]anthracene (DMBA) and treated with ethoxyquin were compared with others given either DMBA or ethoxyquin alone (Ito et al., 1986a, b). Groups of 25 female Sprague–Dawley rats were used. Pretreatment with DMBA involved administration of a single dose of 25 mg/kg bw by stomach tube. Ethoxyquin was given in the diet at a concentration of 0.8 % for 33 weeks. No tumours of the mammary or ear duct were found in rats given ethoxyquin alone. The numbers of mammary carcinomas and mammary fibroadenomas were significantly lower in the group given DMBA plus ethoxyquin than in the group given DMBA alone. There were also fewer carcinomas and adenomas of the ear duct (not significant) in the group given DMBA plus ethoxyquin. It is concluded that dietary ethoxyquin inhibited the carcinogenesis of DMBA in the mammary gland.

In an experiment to investigate the effects of antioxidants in promoting cancers of the urinary bladder, a group of 23 male F344 rats were pretreated orally with N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) and then treated with ethoxyquin and were compared with a group of 24 rats given BBN alone (Ito et al., 1986a). Pretreatment BBN was administered in drinking water at a concentration of 0.05 % for 4 weeks. Ethoxyquin was given in the diet at a concentration of 0.8 % for 32 weeks. Unilateral urothelial ligation was performed on all rats The prevalence of pre-neoplastic hyperplasia (papillary or nodular hyperplasia) and of papilloma of the bladder, and the average number of lesions per animal, were significantly higher in the group of rats given BBN plus ethoxyquin than in the group given BBN alone. There was no significant modifying effect of ethoxyquin on the prevalence or number of malignant tumours in the bladder. This result suggests that, although 0.8 % dietary ethoxyquin enhanced the effect of BBN in producing pre-neoplastic hyperplasia and benign papillomas in the bladder, it did not enhance the bladder cancer initiated by BBN.

In a similar experiment (Miyata et al., 1985), groups of male F344 rats were given 0.05 % BBN in drinking water for 2 weeks followed by 22 weeks of dietary exposure to a series of chemicals under test. One such chemical was ethoxyquin, which was given at a dietary concentration of 0.8 %. Unilateral urothelial ligation was performed on all rats at the end of week 3. The prevalence of bladder tumours was significantly higher in a group of 14 rats given BBN plus ethoxyquin (5 %) than in a group of 44 rats given only BBN (3 %). No bladder tumours were found in 15 rats given ethoxyquin

without BBN pretreatment. These results suggest that ethoxyquin increased the carcinogenicity of BBN to the rat bladder.

In a follow-up experiment (Ito et al., 1986b; Fukushima et al., 1987a) looking into promoting effects on the bladder, multiple dose levels of ethoxyquin were used. Groups of 13 to 16 male F344 rats were given dietary concentrations of 0.125, 0.25 or 0.5 % ethoxyquin after pretreatment with BBN. A similar protocol to the earlier experiment was followed. The only bladder cancer seen in the experiment was in one of the rats given BBN alone. Treatment with BBN plus ethoxyquin had no significant effects on the prevalences of bladder papillomas or of preneoplastic hyperplasia in any of the dose groups compared with the prevalence in control animals given BBN alone. This result indicates that up to 0.5 % ethoxyquin in the diet has no effect on the production by BBN of lesions in the rat bladder.

In an experiment to investigate the effects of antioxidants in promoting cancers of the liver and kidneys, animals pretreated with N-ethyl-N-hydroxyethylnitrosamine (EHEN) and treated with ethoxyquin were compared with others given either EHEN or ethoxyquin alone (It, et al., 1986a, b; Tsuda et al., 1984). Groups of 23 to 27 male F344 rats were used. Pretreatment EHEN was administered in drinking water at a concentration of 0.1 % for 2 weeks. Ethoxyquin was given in the diet at a concentration of 0.8 % for 29 weeks. No tumours of the liver or kidneys were found in rats given ethoxyquin alone. The prevalence of renal adenomas and the number of renal adenomas per rat were significantly higher in the rats given EHEN plus ethoxyquin than in those given EHEN alone. In contrast, the prevalence of hepatocellular carcinomas was significantly lower, and the number and area of γGT-positive foci in the liver were reduced. There was no significant effect on the prevalence of hyperplastic liver nodules or of renal adenocarcinomas. It is concluded that dietary ethoxyquin enhanced the carcinogenicity of EHEN in the kidney but inhibited its carcinogenicity in the liver.

Masui et al. (1986) investigated the effects of several substances, including ethoxyquin, on the hepatocarcinogenicity of diethylnitrosamine (DEN) in rats. Groups of 17 rats were given an intraperitoneal injection of 200 mg DEN/kg bw and then 2 weeks later were fed a diet containing 0.02 % 2-acetylaminofluorene (AF) for 6 weeks. All rats were subjected to a partial hepatectomy at the end of week 3 of the experiment. Experimental diets were fed to these rats from week 12 to week 36 and those given 0.8 % ethoxyquin were compared with controls given only basal diet. All rats were killed 2 weeks after the cessation of treatment with the test diets. Significantly fewer rats with hepatocellular carcinomas (trabecular carcinomas plus adenocarcinomas) were found in the group given ethoxyquin (8/17, 57 %) than in controls (15/17, 88 %), indicating that ethoxyquin suppressed the development of liver cancer induced by DEN/AF treatment.

Ito et al. (1986a) investigated the effects of ethoxyquin on the liver activity of the placental form of glutathione-S-transferase (GST-P), an enzyme thought to be involved in the inhibition by antioxidants of chemical carcinogenesis. Groups of 19 to 22 male F344 rats were pretreated with 200 mg/kg bw per day DEN in their diet for 2 weeks. This was followed by treatment with 0.125, 0.25 or 0.5 % ethoxyquin in the diet for 6 weeks. All rats were subjected to partial hepatectomy 3 weeks after the last dose of DEN. There was a significant decrease in the number of GST-P-positive foci in the livers of rats in the mid-dose group, but not those given the high dose or low dose of ethoxyquin. The area of GST-P-positive foci was not affected by any of the treatments with ethoxyquin. More extensive results from this experiment were reported by Thamavit et al. (1985). These showed that there was a significant dose-related decrease in the number of foci of induced γGT and in the amount of liver tissue affected by these foci. These results suggest that ethoxyquin might be protective against the hepatocarcinogenicity of DEN. Histological examination of the livers from rats from this study showed biliary toxicity (bile duct hyperplasia without cholangiofibrosis or oval cell hyperplasia) but no evidence of hepatocellular toxicity (Ward et al., 1989).

The effects of dietary administration of ethoxyquin on aflatoxin B1 (AFB1) metabolism, DNA adduct formation and removal, and hepatic tumorigenesis were examined in male F344 rats (Kensler et al., 1986). Rats were fed a diet containing 0.4 % ethoxyquin for 1 week, then gavaged with 250 mg of AFB1 per kg bw five times per week for the next 2 weeks. Finally, the control diet was reinstated 1 week after cessation of dosing. At 4 months, focal areas of hepatocellular alteration were identified and quantitated by staining sections of liver for γGT. Treatment with ethoxyquin reduced by > 95 % both area and volume of liver occupied by γGT-positive foci. Utilising the same multiple dosing protocol, patterns of covalent modifications of DNA by AFB1 were determined. Ethoxyquin produced a

dramatic reduction in the binding of AFB1 to hepatic DNA: 18-fold initially and 3-fold at the end of the dosing period. Although binding was detectable at 3 and 4 months post dosing, no effect of ethoxyquin was observed, suggesting that these persistent adducts are not of primary relevance to AFB1 carcinogenesis. Analysis of nucleic acid bases by HPLC revealed no qualitative differences in adduct species between treatment groups. The inhibitory effect of ethoxyquin on AFB1 binding to DNA and tumorigenesis appears related to induction of detoxifying enzymes. Rats fed 0.4 % ethoxyquin for 7 days showed a 5-fold increase in hepatic cytosolic GST-specific activities. Multiple molecular forms of GST were induced, and concomitant elevations in mRNA levels coding for the synthesis of GST subunits were observed. Correspondingly, biliary elimination of AFB1–glutathione conjugate was increased 4.5-fold in animals on the ethoxyquin diet during the first 2 hours following oral administration of 250 µg of AFB1 per kg bw. These results suggest that induction by ethoxyquin of enzymes important to AFB1 detoxification, such as GST, can lead to enhanced carcinogen elimination, as well as to reduced AFB1–DNA adduct formation and subsequent expression of preneoplastic lesions and, ultimately, reduced neoplasia.

Power et al. (1987) (cited in US EPA, 2004) found that ethoxyquin at 0.5 % in the diet induced γGT in periportal and mid-zonal regions of liver lobules during AFB1-induced carcinogenesis.

Manson et al. (1987) investigated the effects of dietary ethoxyquin on the liver and kidneys of male F344 rats in the presence and absence of AFB1. The diets contained 50 % peanut meal, with the AFB1-containing diet being made up with contaminated meal to achieve a concentration of 1 % AFB1 and the AFB1-free diet being made up with uncontaminated meal. Ethoxyquin was added to diets at 0.5 %. Groups of 8 to 10 rats were subjected to four different treatment schedules. Group 1 (negative controls) was not given any AFB1 or ethoxyquin; Group 2 was given AFB1-free diet for 4 weeks, then given an intraperitoneal injection of 0.25 mg/kg bw AFB1 followed by AFB1 in the diet; Group 3 was treated similarly to Group 2 except that the diet contained ethoxyquin throughout the experimental period; and Group 4 was given ethoxyquin in AFB1-free diet. All rats were killed at the end of the 23-week experimental period. Livers and kidneys were taken for routine histological examination and examination using special staining techniques including staining for γGT, iron and lipofuscin, and immunohistochemistry for GST-P. The results showed that dietary ethoxyquin completely prevented the formation by AFB1 of preneoplastic liver nodules, as judged by morphological changes or by the biochemical markers γGT, GST-P and J1 (an uncharacterised membrane marker). Although the liver was protected, ethoxyquin alone caused severe damage to the kidneys, including glomerulonephrosis (the authors commented that this appeared to show that ethoxyquin accelerated the ageing process), tubular hyperplasia and putative preneoplastic tubules (the authors suggested this may be indicative of a carcinogenic effect if a longer dosing period were to be employed).

Ethoxyquin inhibited the liver carcinogenicity of aflatoxin B1 in F344 rats in a dose-related manner (Cabral and Neal, 1983, cited in US EPA, 2004). The review by the US EPA (2004) reported that 'a dose of 0.05 % reduced the incidence of neoplastic nodules and liver cell tumours and a dose of 0.5 % caused a further inhibition of aflatoxin carcinogenicity. There was a high incidence of altered foci, neoplastic nodules and liver cell tumours in rats fed aflatoxin only. Addition of ethoxyquin to a diet containing aflatoxin completely inhibited liver tumours. When ethoxyquin was administered after aflatoxin, there was considerable reduction in the incidence of altered foci and neoplastic nodules in the liver. The most effective inhibition is obtained when these agents are given simultaneously. No liver tumours were found in rats fed ethoxyquin alone or in untreated controls.'

Male Wistar rats were treated with 100 ppm N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) in their drinking water and 10 % sodium chloride in their diet for 8 weeks. Subsequently, they were fed a dietary supplement of 0.1 % ethoxyquin for 32 weeks. An additional group received ethoxyquin treatment alone. Calcification and calculi were seen in the papilla and pelvis regions of the kidneys in all ethoxyquin-treated groups. Chemical element analysis of the renal calculi showed that the main constituents were phosphorus and calcium. On ultrastructural examination the microvilli on the surface of the renal papilla cells appeared degenerated or were completely absent in the ethoxyquin-treated groups. The prevalence and severity of the lesion were greatest in the group that was pretreated with MNNG and sodium chloride (Imazawa, et al., 1989, cited in US EPA, 2004).

Hirose et al. (1986) (as cited by US EPA, 2004) studied the induction of forestomach lesions in rats by antioxidants. One group of male F344 rats was fed a diet containing 0.25 % ethoxyquin for 2 weeks. During the second week they were also given 1 % BHA in their diet. Control rats received neither

antioxidant in their diet. All rats were killed at the end of the 2-week treatment period. Livers and kidneys were weighed and the stomach was examined microscopically. Pronounced epithelial damage including hyperkeratosis and ulcer formation was seen in the forestomachs of rats treated with ethoxyquin plus BHA. It was stated that ethoxyquin increased the mild hyperplasia caused when BHA was administered on its own. Ethoxyquin inhibited the carcinogenicity of DMBA in the forestomach of the mouse and also inhibited mammary tumour formation produced by oral administration of DMBA to female Sprague–Dawley rats (Wattenberg, 1972, as cited by US EPA, 2004). Furthermore, the pretreatment of female rats with ethoxyquin 1 hour before the administration of 30 mg DMBA prevented the adrenal necrosis that was seen in rats not given the pretreatment with ethoxyquin.

The feeding of 0.75 % ethoxyquin in the diet for 4 months had no effect on the formation of lung tumours in male mice injected with urethane (Witschi, 1981, cited in US EPA, 2004).

A.2.5. Reproduction toxicity

A.2.5.1 Rat studies

A two-generation reproduction study was performed on ethoxyquin using Sprague–Dawley rats. Only an abstract of the study was available (Delaney et al, 1995). Groups of 20 parental generation (F_0) rats of each sex were given 0, 75, 150 or 300 mg ethoxyquin/kg bw/day by oral gavage in maize oil, starting 1 week prior to breeding. It is assumed that subsequent generations were fed the same doses. The F_0 generation produced two litters: the F_{1a} litters, born in the 16 weeks following the initial breeding, were killed aged 1 day for examination; the second litter (F_{1b}) was reared by the F_0 dams until weaning. Selected F_{1b} weanlings were kept until the age of about 80 days, when they were mated to produce F_2-generation litter. The F_{1b} parents were then killed for examination. Reduced body weights were seen in F_0- and F_1-generation males in high-dose group. Dose-related increases in the weights of liver and kidneys were found in the F_0 and F_1 animals of all dose groups. Adverse effects on reproduction were seen in the F_0 generation at the high dose level, as evidence by decreased numbers of litters, decreased numbers of live pups per litter and prolonged gestation. At subsequent cross-over matings to determine which sex was most affected, pups born to treated females weighed less than control pups, whereas pups born to treated males were of similar weight to the control pups. No effects of ethoxyquin were found in F_1 breeding pairs. The authors concluded that adverse effects on reproduction were seen at 300 mg/kg bw/day but not at 150 mg/kg bw/day or less, but a NOAEL could not be identified as there were effects on the weights of liver and kidneys at all doses tested (down to 75 mg/kg bw/day).

The reports of JMPR (FAO, 1969, 1998) summarised a two-generation rat study from 1956 to 1959. Groups of rats (strain and group size not specified) were fed a diet that was slightly deficient in tocopherol with additions of 0, 250, 500 or 1000 mg ethoxyquin/kg diet for 40 days prior to mating. The offspring (F_1 generation) of the first litter produced by the 0, 250 and 500 mg/kg groups were then mated to produce the next (F_2) generation. It was not explained why the 1000 mg/kg group was not used to produce another generation. There were no effects on fertility, litter size or survival of offspring. It was concluded that the NOAEL for this study was 500 mg/kg (equivalent to 45 mg/kg bw/day, using the EFSA default value of 0.09 for sub-chronic exposure).

The reports of JMPR (FAO, 1969 and 1998) summarised a non-standard single-generation rat study from 1956. Groups of eight or nine female rats (strain not given) were fed diets containing 0, 125, 375 or 1125 mg ethoxyquin/kg diet on the day they were mated. The treatments had no effect on the length of gestation, but concentrations of 375 mg/kg or more caused reduced litter sizes, and 1125 mg/kg caused increased numbers of stillbirths and reduced survival to weaning. Separate groups of pregnant rats were given up to 1125 mg ethoxyquin/kg in their diet on days 1 to 10 of gestation with no effects on litter size, number of stillbirths, survival to weaning or pup weight at weaning. It was concluded that the NOAEL for this non-standard study of reproduction was 125 mg/kg diet (equivalent to 11 mg/kg bw/day).

A developmental toxicity study was performed in Sprague–Dawley rats (unpublished study, summarised in US EPA, 2004, and DAR (Germany, 2007)) The study was GLP compliant. Groups of 21 to 25 mated females were given daily gavage doses of 0, 50, 150 or 350 mg ethoxyquin/kg bw in maize oil on days 6 to 19 of gestation. All animals were killed for examination on day 20 of gestation. There was no unscheduled mortality in the mated females. In the 150 and 350 mg/kg bw dose groups, there was a reduction in body weight on the first three days of dosing (and also on gestation

days 12 to 16 in the highest dose group). Many of the animals in the mid- and high-dose groups also had yellow- or brown-stained and matted fur. Feed intake and body weight gain were significantly lower in the mid-dose and high-dose groups than in concurrent controls. There were no treatment-related changes to numbers of corpora lutea, implantation sites, early and late resorptions sites, viable fetuses and dead fetuses. In addition, there were no effects on the number or type of external malformations, soft tissue malformations or skeletal malformations. It was concluded that oral doses of up to 350 mg ethoxyquin/kg bw/day did not cause any developmental toxicity in rats, although doses of 150 mg/kg bw/day or more were maternally toxic (NOAEL = 50 mg/kg bw/day).

A rat developmental toxicity study was performed on the product Santoquin, which contains 67 % ethoxyquin (Khera et al., 1979, cited in US EPA, 2004). Groups of 20 pregnant Wistar rats were given Santoquin by oral gavage at dosages of 0, 125, 250 or 500 mg/kg bw/day in corn oil on gestation days 6 to 15. Dams were killed on day 22. The treatments had no effects on pregnancy parameters: pregnancy rate, sex ratio, fetal weight, and mean numbers of corpora lutea, live fetuses per dam, implantations, resorptions, runts and dead fetuses. There was an increased incidence of 'anomalous fetuses' at the mid-dose but not at the highest dose. This suggests that the effect was not treatment-related and the NOAEL for the study was 500 mg Santoquin/kg bw/day (335 mg ethoxyquin/kg bw/day).

In a study evaluating the effects of ethoxyquin and several other antioxidants on fetal resorption, Telford, et al. (1962) (cited in US EPA, 2004) administered ethoxyquin in the diet at 57 mg/kg bw/day to pregnant rats on gestation days 1 to 22. The fetal absorption rate in the ethoxyquin-treated rats was lower than in controls.

A.2.5.2 Rabbit studies

Experiments were performed to investigate whether ethoxyquin caused abortion and pup mortality in New Zealand White rabbits (Isenstein, 1970). In the first experiment, groups of 16 to 20 female rabbits were given either ethoxyquin-free feed, commercial feed containing 0.0025 % ethoxyquin or feed containing 0.005 % ethoxyquin. These diets were fed from 10 days prior to mating until 2 weeks after parturition. The males used to service the females were given the same diets from at least 10 days prior to mating. The rabbits were kept in unheated housing. In a second experiment in heated housing, some of the rabbits from the first experiment were randomly reallocated to new groups of 11 to 16 females which were given ethoxyquin-free diet, commercial diet (0.0025 % ethoxyquin) or diet containing 0.01 % ethoxyquin. The protocol of the second experiment was otherwise the same as that for the first experiment. Gestation periods, litter sizes and numbers of still-births were not affected by the presence of ethoxyquin in the diet. No abortions occurred in either experiment and no physically abnormal fetuses were found. In the first experiment, many newborn or young rabbits died as a result of cold weather, but mortality did not seem to be affected by the amount of ethoxyquin in the diet. In the second experiment, there was a dose-related decrease in mortality, with the mortality at the highest dietary level being about a third of that in the ethoxyquin-free group.

A.2.5.3 Dog studies

A report was provided of a two-generation reproduction toxicity study in beagle dogs.[25] The study was GLP compliant. Groups of 5 male and 10 female F_0-generation dogs were fed diets to which 0, 180, or 360 ppm of ethoxyquin was added, giving average concentrations (by analysis) of 0, 102 and 224 (equal to approximately 0, 2.5 and 6 mg ethoxyquin/kg bw/day) for 82 days before mating. The same diets were fed to groups of 8 male and 13 female F_1-generation dogs that had been randomly selected from the pups born to each dose group of the F_0-generation parents. The F_1-generation dogs were mated at age 10 to 30 months (after the females' second oestrus) to produce the F_2-generation pups. Semen samples were taken around the time of mating. Blood samples were taken before treatment and at the end of the F_0 phase, at weeks 0, 23, 36, 49 and 62 of the F_1 growth phase, and at termination of the F_1 mating phase. Ophthalmoscopy was performed at the beginning and end of the F_1 growth and mating phases. All F_1 adults or pups and F_2 pups that showed clinical signs, plus any dogs that died prematurely, were autopsied and selected organs were examined microscopically. In addition, histopathological examination of a limited selection of organs was performed on F_1 adults from the lowest dose group. The results of the study showed that semen quality, reproductive performance and outcome were not affected by the treatment with ethoxyquin. However, at both

[25] Technical dossier /SectionIII/Annex III_2_2_5_1_4

concentrations of ethyoxyquin there was an increased prevalence of some clinical signs in dogs of the F_0 and F_1 generations (including excessive lacrimation and dehydration) which were commonly seen in untreated dogs of the breeding colony. In females at both dose levels, there was deposition of a dark-brown pigment (identified as protoporphyrin IX) in periportal hepatocytes and Kupffer cells without any pathological changes to the liver. The intensity of the pigmentation was greater at the higher dose. There were changes to some blood biochemistry parameters in F_1 generation dogs. There were decreases in total protein and albumin in serum of females at both dose levels but not at all time points. Serum ALT activity was consistently raised in the high-dose females from week 23 to week 62 of life and was raised in males in the high-dose group in weeks 36 and 62 only. Serum alkaline phosphatase (AP) was raised in animals of both sexes, and at both dose levels, in week 10, but only occasionally at the highest dose level thereafter. Elevated AP was also reported in females of the F_0 generation in the high-dose group. Haematology, urinalysis and ophthalmoscopy results were not affected by treatment with ethoxyquin. It was not possible to identify a NOEL for this study as pigment deposition in the liver was seen at all doses tested. The toxicological relevance of this finding is unclear. Otherwise there was no clear indication of toxicity at doses of 2.5 mg/kg bw/day or less.

A.2.6. Genotoxicity studies including mutagenicity

The orginal reports of the the following studies were not available. However, they are extensively described in DAR (Germany, 2007).

In a bacterial reverse mutation assay compliant with the OECD Guideline 471 (Mecchi, 2004), ethoxyquin (purity 98.93 %) was tested for its ability to induce reverse mutations either in the presence or in the absence of mammalian microsomal in Salmonella typhimurium (strains TA98, TA100, TA1535 and TA1537) and in *Escherichia coli* strain WP2uvrA, up to 5 000 µg/plate in the presence and absence of S9 mix obtained from Aroclor-induced rat livers. The test article did not cause an increase in the mean number of revertants per plate with any of the tester strains either in the presence or absence of metabolic activation (S9). The positive control substances resulted in the expected increase in mutant frequencies. Ethoxyquin (purity 98.6 %) was assayed for mutagenic potential in the mouse lymphoma L5178Y cell line, scoring for forward mutations at the thymidine kinase locus, in the absence and in the presence of S9 mix from Aroclor 1254-induced livers of adult rats, according to OECD Guideline 476 (Riach, 2005). The substance was tested up to cytotoxic concentrations (25 and 4.4 µg/mL in the absence and in the presence of S9 mix, respectively) after an exposure period of 4 hours, in two independent experiments. All experiments (with and without S9 mix) included at least one concentration that tested significant at $P < 0.05$ and had a highly significant ($P < 0.001$) linear trend in mutant fraction with dose level of ethoxyquin. The analysis of the colony size indicated that the mutagenicity of Ethoxyquin is more closely associated with large-scale chromosomal damage, than with small scale damage or point mutations.

In an *in vitro* clastogenicity test, (Murli, 2004), ethoxyquin (purity 98.93 %) was analysed for the ability to induce chromosomal aberrations in cultured Chinese hamster ovary (CHO) cells, in the presence or absence of an exogenous metabolic activation system, in compliance with OECD Guideline 473. The target cells were treated up to cytotoxic concentrations (30.0 and 25.0 µg/mL without and with metabolic activation, respectively). Treatment lasted ~20 hours without metabolic activation and 3 hours with metabolic activation. A significant increase in cells with chromosomal aberrations was observed both with and without metabolic activation. At the upper concentrations polyploidy was observed in all the experimental conditions. In the presence of metabolic activation, polyploidy was at least in part due to cytotoxicity, as indicated by the observed endoreduplication. The induction of polyploidy, particularly without endoreduplication, could indicate an interaction with the mitotic spindle proteins and,, therefore, potential aneugenicity. In an in vivo micronucleous test (Erexson, 2004), ethoxyquin (purity 98.93 %) was tested for *in vivo* clastogenic activity and/or disruption of the mitotic apparatus by detecting micronuclei in polychromatic erythrocytes (PCE) in Crl:CD-1®(ICR)BR mouse bone marrow, in compliance with OECD Guideline 474. The substance was administered by oral gavage up to the maximum tolerated dose of 1500 mg/kg bw, determined by a previous dose range-finding assay. At least 2 000 polychromatic erythrocytes (PCEs) were analysed for the frequency of micronuclei. Cytotoxicity was assessed by scoring the number of PCEs and normochromatic erythrocytes (NCEs) in at least the first 500 total erythrocytes from each animal. Ethoxyquin did not induce statistically significant increases in micronucleated PCEs at any test article dose examined, whereas a positive control substance, cyclophosphamide, resulted in the expected increase in the

percentage of micronucleated polychromatic erythrocytes. No statistically significant decrease was observed in the PCE/NCE ratio was observed at any dose of ethoxyquin.

Unscheduled DNA synthesis (UDS) was assessed in the hepatocytes of male Sprague-Dawley (CD) rats following treatment with ethoxyquin (purity > 99 %), in compliance with OECD Guideline 486 (Pritchard, 2006). Two oral administrations of the test item were given with an interval of 14 hours: 2 x 225 and 2 x 750 mg/kg bw, that was considered the maximum tolerated dose. Clear signs of toxicity occurred at both dose levels. Rats were sacrificed 2 hours after the second administration. Hepatocytes were isolated by enzymatic dissociation after exposure of the animals to the test substance. Four animals from the treatment and vehicle control groups and two animals from the positive control group were assessed. The isolated hepatocytes were allowed to attach to glass coverslips and were cultured *in vitro* with tritiated thymidine for 4 hours to 'radiolabel' DNA undergoing repair replication, followed by 20 hours' culture in the presence of unlabelled thymidine; the cells were then fixed on slides and processed for autoradiography. One hundred cells per animal (50 × 2 independent cultures) were analysed. Ethoxyquin did not cause any statistically significant increases in the net nuclear grain count at any dose level compared to vehicle control values. The positive control substance gave the expected response.

Besides the DAR (Germany, 2007), the following published studies were considered by the FEEDAP Panel:

An *in vitro* chromosome aberration test in human lymphocytes was employed to investigate the potential in vitro clastogenicity of ethoxyquin (Błaszczyk et al., 2003). Human lymphocytes from three healthy donors were incubated with 0.01, 0.025, 0.05, 0.1, 0.25 or 0.5 mM ethoxyquin, both with and without metabolic activation (S9 mix from Aroclor 1254-induced rats). Three alternative treatment schedules were used: 3 hours treatment without S9 mix; 24 hours treatment without S9 mix; 2 hours treatment with S9 mix. Dose-dependent increases in chromosome aberrations frequency was reported in all experimental conditions. Pooling together chromosome aberrations and gaps, these increases were statistically significant from 0.05 mM in the 24-hour treatment without S9 mix and from 0.025 mM in the 2-hour treatment with S9 mix. However, it should be noted that, according to the OECD Guideline 473, gaps should be recorded separately and not included in the total aberration frequency.

An *in vitro* alkaline single cell gel electrophoresis assay (comet assay) was used to measure DNA damage induced by ethoxyquin in human lymphocytes, with and without metabolic activation (S9 mix from Aroclor 1254-induced rats) (Błaszczyk et al., 2006). The cells were treated with six concentrations of the test item, ranging from 1 to 250 µM. A statistically significant and dose dependent induction of DNA damage was observed at all the analysed concentrations, with a lower effect in the presence of S9 mix. It should be noted that the in vitro comet assay is not a validated test and is not currently used for risk assessment, also because of the uncertainties on the nature of the molecular events responsible for the observed effect.

Appendix B – Summary of the studies on metabolic fate and toxicological profile of *p*-phenetidine

B.1. Metabolic fate

The main metabolic pathways of *p*-phenetidine are described in Figure 4. Knowledge of the kinetics of *p*-phenetidine in laboratory and in target species is limited. Most of the few published studies were performed many decades ago using analytical techniques of limited sensitivity and specificity. In addition, most of the available information is concerned with *p*-phenetidine not as a chemical but rather as a metabolite of phenacetin, i.e. its N-acetylated analogue, a widely used analgesic and antipyretic drug developed as a prodrug of acetaminophen (APAP), which was generated via a CYP-mediated O-de-ethylation. Phenacetin has been withdrawn from the market because it was reported to cause severe adverse effects such as methaemoglobin formation and renal failure in a number of patients (Jensen and Hollow, 1991); recently, it has been classified as a Group I carcinogen by IARC (2012) because of remarkable incidence of renal pelvic and other urothelial tumours in patients consuming large amounts of phenacetin-containing analgesics.

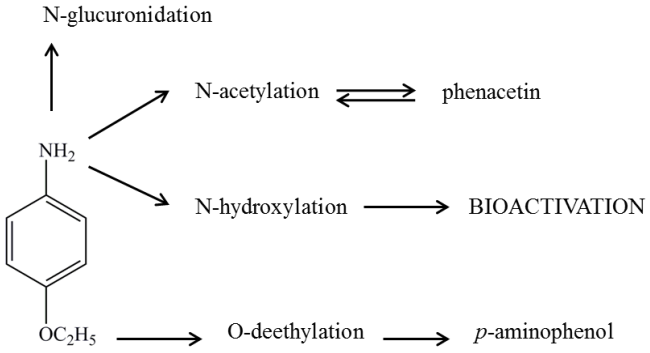


Figure 4: Main metabolic pathways of *p*-phenetidine

No data on *p*-phenetidine absorption and distribution following either oral or inhalatory exposure could be found (Pauluhn and Mohr, 2001). In mammalian species, the metabolic fate involves both the ethoxylic group (-O-C2H5) and the amino group (Figure 4). The former undergoes a CYP-mediated O-de-ethylation to *p*-aminophenol, which is then conjugated, yielding mainly glucuronide derivatives, which may be found in the urine. *p*-Aminophenol has been reported to act as a nephrotoxicant in F344 rats (Newton et al., 1982) and is thought to play a significant role in the generation of methaemoglobinaemia in canine and feline erythrocytes (McConkey et al., 2009).

As for many other arylamines, the metabolic reactions involving the -NH_2 group are of great importance in dictating the activation/detoxification of *p*-phenetidine. The formation of N-glucuronides has been reported in orally dosed rabbits (Smith and Williams, 1949). In addition, in common with other arylamines, *p*-phenetidine may undergo N-acetylation by cytosolic N-acetyltransferases (NAT) yielding the N-acetylated derivative phenacetin, which has been detected in urine from orally exposed rabbits (Smith and Williams, 1949). Although this may be considered a detoxification reaction, it should be noted that a number of tissue carboxylesterases are able to de-acetylate (hydrolyze) phenacetin back to *p*-phenetidine (Watanabe et al., 2010).

The conjugative pathways may compete with the N-oxidation pathway, which generates an hydroxylamino-derivative (N-hydroxyphenetidine, also reported as *p*-hydroxyaminophenetol) (Uehleke, 1971), which may be further oxidized to a *p*-nitroso -derivative (see below) and is generally accepted as the major bioactivation route for *p*-phenetidine. Male Sprague–Dawley rats treated once with about 12, 19 or 39 mg N-hydroxyphenetidine/kg bw by the intraperitoneal route exhibited a rapid onset of methaemoglobinaemia reaching peak levels (nearly 60 to 70 % of total haemoglobin) as soon as 30 minutes after dosing (Kiese, 1974 as cited by Jensen ad Jollow, 1971).N-hydroxyphenetidine has also been shown to play a predominant role in the onset of haemolysis and methaemoglobinaemia in human patients as a consequence of overdosage and chronic abuse of phenacetin (Jensen and

Jollow, 1991). No traces of N-hydroxyphenetidine could be detected in the blood of treated rats, in agreement with the findings of other authors, suggesting that in humans, rats, and dogs this compound is only a very minor urinary metabolite of both phenacetin and *p*-phenetidine (Kiese, 1974 as cited by Jensen ad Jollow, 1971). A further oxidation step to *p*-nitroso derivatives (e.g. *p*-nitrosophenetol) has been reported in dogs intavenously injected with 12 mg phenetidine/kg bw (Baader et al., 1960). In rats and rabbits, the generation of 2-hydroxy-phenetedine has been also documented; this metabolite is mainly excreted as the sulphate derivative in urine (Smith and Williams, 1949; Dubach and Raaflaub, 1969, as cited by IARC, 2012).

Prostaglandin synthetase (PGS) and other peroxidases are also responsible for *p*-phenetidine oxidation. Such enzymes are particularly expressed in extrahepatic tissues, including renal medulla and urinary bladder, and in many instances are involved in the formation of reactive intermediates and/or products with (geno)toxic properties. The PGS-mediated oxidation of *p*-phenetidine by human and rabbit kidney microsomes generates several reactive metabolites which have been shown to bind covalently to proteins and to be inactivated by GSH (Larsson et al., 1985). The PGS-mediated oxidation of *p*-phenetidine resulting from the hydrolysis of phenacetin is thought to be the key step in the nephrotoxicity of the latter (Larsson et al., 1985)

Peroxidase-catalysed metabolism of *p*-phenetidine also results in the generation of DNA-binding species that have been reported to induce DNA single-strand breaks in human fibroblasts (Andersson et al., 1982).

Based on the metabolic pathways described above, conditions known to depress the rate of glucuronidation and/or N-acetylation are expected to act as predisposing factors predisposing to *p*-phenetidine toxicity, in that a larger amount of the compound would be available for CYP- and/or PGS-mediated oxidation yielding (geno)toxic and reactive metabolites. This may be the case for cats and dogs, inasmuch as cats only express NAT1, while dogs lack both NAT genes (Trepanier et al., 1997, 1998). In this respect, evidence has been provided supporting the ability of dogs to hydroxylate several arylamines (e.g. 2-aminonaphthalene, 4-acetylaminobiphenyl) and the view that the N-hydroxylation pathway of such compounds is a key step in the bioactivation process leading for instance to bladder cancer in that species (Poirier et al., 1963). In addition, cats are long known to be more sensitive than other species to phenol derivatives, including the *p*-phenetidine metabolite *p*-aminophenol, owing to their inability to glucuronidate it as the result of the mentioned lack of UGT 1A6 and 1A9 enzymes (Court , 2013); the unconjugated *p*-aminophenol is therefore free to promote methaemoglobin formation in feline erythrocytes (McConkey et al., 2009).The metabolic fate of phenetidine has been established in the rabbit (Smith and Williams, 1949). O-de-ethylation gave rise to *p*-aminophenol, which was acetylated to *p*-acetamidophenol (acetaminophen, paracetamol) and excreted as glucuronide. Another pathway was direct hydroxylation to 2-hydroxy-4-ethoxy-aniline followed by sulphation. About 2 % of *p*-phenetidine administered orally to rabbits was excreted unchanged in the urine, while 5–6 % was metabolised to acetylated *p*-phenetidine, 30 % to ethereal sulphates, of which 8 to 9 % was 2-hydroxy-4-ethoxy-aniline sulphate, and 58 % was metabolised to *p*-toluidine in glucuronidated form. Three percent of the dose appeared as free diazotizable amino group and the *p*-acetamidophenylglucuronide was also identified.

B.2. Toxicological profile

B.2.1 Genotoxicity studies including mutagenicity

In a bacterial reverse mutation study on 300 chemicals, *p*-phenetidine was tested in *Salmonella typhimurium* TA98 and TA100 (pre-incubation assay). The substance was positive in TA 100 with metabolic activation, while, equivocal results were reported in the other conditions (Zeiger et al. 1988).

In a study that aimed to characterise the mutagenicity of the analgesic bucetin, its metabolite *p*-phenetidine was found to be positive in a bacterial reverse mutation assay in Salmonella typhimurium TA 100 (pre-incubation test) (Nohmi 1985). The metabolic activation system contained S9 fraction prepared from the liver of PCB-induced Golden Syrian hamster, instead of the usual rat liver S9.

A bacterial reverse mutation assay was conducted in *Salmonella typhimurium* TA97, TA98, TA100 and TA102 (pre-incubation assay) (unpublished study commissioned by the Japanese Ministry of Health and Welfare, undated). The test item was tested at concentrations up to 5 mg/plate with and without

metabolic activation (S9 from the liver of rats pre-treated with phenobarbital/5,6-benzoflavone). A slight increase in revertant colonies was observed only in the TA100 and TA102 strains, and only in the presence of S9-mix; however, this increase was less than the double of the control value and, therefore, the result was considered biologically irrelevant.

The test substance was also found to be positive for mutagenic effects in Chinese hamster CHL cells, with and without metabolic activation, in an unpublished report of the Japanese Ministry of Health, and Welfare, mentioned by OECD (1994); however, the original report was not available to the FEEDAP Panel.

A micronucleus test after oral administration (gavage) was performed in male and female Crj: BDF1 mice to evaluate the *in vivo* cytogenetic effect of *p*-phenetidine (unpublished study commissioned by the Japanese Ministry of Health and Welfare, undated). The maximum tolerated doses (MTDs) were established by a dose range-finding test and were 600 mg/kg bw in males and 1 000 mg/kg in females. In a preliminary micronucleus test, without either negative or positive controls, the MTDs were administered to five animal per group and bone marrow smears were prepared at 24, 48 and 72 hours after administration. At the 24 hours sampling time, the frequency of micronuclei appeared clearly higher than at 48 and 72 hours, both in males and in females; however, this result could not be considered definitive evidence of genotoxicity owing to the absence of negative controls. On the basis of this preliminary experiment, only a sampling time of 24 hours was applied in the subsequent experiment. In the main experiment, doses of 150, 300 and 600 mg/kg bw were administered to males and doses of 250, 500 and 1 000 mg/kg bw were administered to females. In males, no statistically significant increase in the frequency of micronuclei compared with the solvent control group was observed in any administration group. In females, the frequency of micronuclei was significantly increased in the 1 000 mg/kg group. No suppression of bone marrow cell proliferation was observed in either males or females in any administration group. This study apparently indicates a differential response between males and females. However, it should be noted that in the dose-finding experiment the MTD for males was set at 600 mg/kg bw on the basis of a single observation at 800 mg/kg bw (one dead animal out of five) and that females were positive only at 1 000 mg/kg bw.

Appendix C – Summary of the toxicological studies with ethoxyquin quinone imine

C.1. Genotoxicity studies including mutagenicity

EQI was tested in the *Salmonella typhimurium* reverse mutation assay with four histidine-requiring strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and in the *Escherichia coli* reverse mutation assay with a tryptophan-requiring strain of *Escherichia coli* (WP2uvrA) following the most recent test guideline, OECD 471.[26] The test was performed in two independent experiments in the presence and absence of S9-mix (rat liver S9-mix induced by Aroclor 1254). An additional experiment was performed with the strains TA1537 and WP2uvrA.

Based on the results of a toxicity dose range-finding experiment, EQI was tested in the first mutation assay at a concentration range of 5.4 to 1600 µg/plate in the absence and presence of S9-mix (TA1535, TA1537 and TA98). Evidence of toxicity was observed in all three strains at doses of 512 µg/plate and above. No strain produced a biologically significant increase in the number of revertants.

In the second experiment, treatments of all the tester strains were performed in the absence and presence of S9-mix using a concentration range of 86 to 1568 µg/plate. Toxicity was observed in all tester strains except in strain WP2uvrA. In the tester strain TA1537 in the absence of S9-mix, the test substance induced an increase up to 5.1-fold in the number of colonies compared with the solvent control. However, this increase was observed at only two toxic concentrations (878 and 1568 µg/plate). This increase could not be repeated in two repeat experiments and therefore was not considered biologically relevant. The authors of the study suggest that this increase could arise from the histidine released by dead bacteria at the high toxicity level and available to surviving cells. All other bacterial strains showed negative responses over the entire dose range.

The possible clastogenicity and aneugenicity of EQI were tested in an *in vitro* micronucleus assay in cultured peripheral human lymphocytes.[27] Two independent experiments in the presence and absence of a metabolic activation system (phenobarbital and ß-naphthoflavone induced rat liver S9-mix) were performed in compliance with OECD Guideline 487.

The EQI (purity 99.4 %) was dissolved in dimethyl sulphoxide. In the first experiment the test item was tested up to 14 and 12 µg/ml for a 3 hours exposure time with a 27 hours harvest time in the absence and presence of S9-fraction, respectively.

In the second experiment the test item was tested at concentrations up to 11 µg/mL in the absence of S9-mix. The exposure time harvest time were both 24 hours. In both experiments severe toxicity was observed at the highest dose level.

EQI induced a dose dependent, statistically significant increase in the number of micronuclei both in the absence and in the presence of S9-mix, in both in mono- and binucleated cells, in two independent experiments.

The number of mono- and binucleated cells with micronuclei found in the solvent control cultures was within the laboratory historical control data range. Two known clastogens used as positive control chemicals, mitomycin C and cyclophosphamide, produced statistically significant increases in micronuclei only in the binucleated cells while the aneugenic compound colchicine induced the same effect only in mononucleated cells.

[26] Supplementary Information July 2015/Annex 05
[27] Supplementary Information July 2015/Annex 06

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

In the current application authorisation is sought for ethoxyquin, under Article 10(2) for the category/functional group 1(b) 'technological additives'/'antioxidants', according to the classification system of article 6 of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the feed additive for all animal species and categories. The feed additive is intended to be mixed in premixtures or added directly to complete feedingstuffs. The Applicant proposed a maximum level of 150 mg/kg for ethoxyquin alone or for the sum of ethoxyquin with butyl hydroxy anisole (BHA, E320) and/or butylated hydroxytoluene (BHT, E321); for dogs, ethoxyquin shall not exceed 100 mg/kg. Furthermore the Applicant suggests Maximum Residue Limits (MRLs) in target tissues (muscle, liver, kidney, fat, milk, eggs). As these MRLs are not set up by Commission Regulation (EC) No 37/2010, the correspondent methods of analysis have to be evaluated by the EURL.

For the determination of ethoxyquin in the feed additive, the Applicant submitted the titrimetric method described in the 'Ethoxyquin monograph' of the Food Chemical Codex (FCC). Even though no performance characteristics are provided, the EURL recommends for official control the internationally recognised FCC method based on titration to determine ethoxyquin in the feed additive. For the determination of ethoxyquin in premixtures, the Applicant submitted a single laboratory validated and further verified multi-analyte method based on Reversed Phase High Performance Liquid Chromatography coupled with UltraViolet (RP-HPLC-UV) or Diode-Array Detector (DAD). For the determination of ethoxyquin in feedingstuffs, the Applicant submitted the ring trial validated RP-HPLC method coupled with Fluorescence Detection (RP-HPLC-FD) published by the Association of Official Analytical Chemists (AOAC 996.13 – 'Ethoxyquin in feeds'). Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified RP-HPLC-UV/DAD method to determine ethoxyquin in premixtures and the ring trial validated RP-HPLC-FD method, AOAC 996.13, for the determination of ethoxyquin in feedingstuffs. For the determination of ethoxyquin in target tissues the Applicant proposed a single laboratory and further verified RP-HPLC-FD method based on a procedure published in the peer-reviewed journal AOAC international. Based on the performance characteristics presented, the EURL recommends for official control the RP- HPLC-FD method or any equivalent other analytical methods complying with the requirements set by Commission Decision 2002/657/EC to enforce the MRLs for ethoxyquin 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.