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SCIENTIFIC OPINION

Update of the Scientific Opinion on the safety and efficacy of erythrosine in feed for cats, dogs, reptiles and ornamental fish[1]

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)[2,3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

Erythrosine is a sensory additive belonging to the functional group colourants, namely "substances that add or restore colour in feedingstuffs". Derived from a No Observed Adverse Effect Level determined in a 60-day rat study (30 mg erythrosine/kg body weight per day based on perturbation of thyroid function) and applying an uncertainty factor of 100, safe concentrations in complete feed are estimated to be 16 mg/kg for dogs, 13 mg/kg for cats and 59 mg/kg for ornamental fish. The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) is not in a position to derive a safe concentration of erythrosine in feed for reptiles. Regarding the iodine content of erythrosine (56 %) and the limitation in the analytical determination of erythrosine in feed, the use of erythrosine in complete feed for dogs, cats and reptiles would be limited to a maximum of 18 mg/kg and for ornamental fish would be limited to 35 mg/kg feed to comply with the currently authorised maximum content of total iodine in complete feed. Erythrosine has the potential to add red colour to feedingstuffs, which was demonstrated in complementary feed at a range of 50 to 500 mg/kg.

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KEY WORDS

erythrosine, colourant, iodine, thyroid function, safety, cats, dogs

[1] On request from the European Commission, Question No EFSA-Q-2013-00340, adopted on 8 September 2015.

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SUMMARY

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of erythrosine for cats, dogs, ornamental fish and reptiles. Erythrosine is a sensory additive belonging to the functional group colourants, namely "substances that add or restore colour in feedingstuffs".

Derived from a No Observed Adverse Effect Level determined in a 60-day rat study (30 mg erythrosine/kg body weight per day based on perturbation of thyroid function) and applying an uncertainty factor of 100, safe concentrations in complete feed are estimated to be 16 mg/kg for dogs, 13 mg/kg for cats and 59 mg/kg for ornamental fish. The FEEDAP Panel is not in a position to derive a safe concentration of erythrosine in feed for reptiles. Regarding the iodine content of erythrosine (56 %) and the limitation in the analytical determination of erythrosine in feed, the use of erythrosine in complete feed for dogs, cats and reptiles would be limited to a maximum of 18 mg/kg and for ornamental fish would be limited to 35 mg/kg feed to comply with the currently authorised maximum content of total iodine in complete feed.

Erythrosine has the potential to add red colour to feedingstuffs, which was demonstrated in complementary feed at a range of 50 to 500 mg/kg.

The mathematically derived maximum safe dietary concentration of erythrosine could be rounded to 15 mg/kg complete feed for dogs and cats and 60 mg/kg complete feed for ornamental fish.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Table 1: Description of the substance

Category of additive	Sensory additives
Functional group of additive	Colourant
Trade name	-
Description	Erythrosine
Target animal category	Cats, dogs, ornamental fish and reptiles
Applicant	Roha UK Ltd.
Type of request	Update opinion

Erythrosine is included in the European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003. It is authorised without a time limit in application of Article 9t (b) of Council Directive 70/524/EEC concerning additives (2004/C 50/01) for its use in feedingstuffs for cats, dogs and ornamental fish as a colourant additive. The additive is further authorised for all species or categories of animals with the exception of cats and dogs in animal feedingstuffs only in products processed from: (i) waste products of foodstuffs, (ii) other base substances, with the exception of cereals and manioc flour, denaturated by means of these agents or coloured during technical preparation to ensure the necessary identification during manufacture. No maximum levels of erythrosine in feeds and no MRLs are established in the EU.

Erythrosine is an approved food colourant in the EU and it is listed in Annex I of Directive 94/36/EC. It is authorised for use in cocktail and candied cherries up to 200 mg/kg, and bigarreaux cherries up to 150 mg/kg. Erythrosine is permitted to be used as a colour in cosmetics, pharmaceuticals and for use in pesticide formulation in the EU.

Erythrosine has previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1986 and 1990 (JECFA, 1986, 1990) and the EU Scientific Committee for Food (SCF) in 1989 (EC, 1989). The Scientific Committee on Consumer Safety (SCCS) published an opinion in 2010 on its use in toothpaste products (EC, 2010). EFSA Panel on food additives and nutrient sources added to food (ANS) has issued a scientific opinion on the re-evaluation of erythrosine as a food additive (EFSA ANS Panel, 2011). In 2011 EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) adopted an opinion on the safety and efficacy of erythrosine in feed for cats and dogs, ornamental fish and reptiles (EFSA FEEDAP Panel, 2011).

TERMS OF REFERENCE AS PROVIDED BY EUROPEAN COMMISSION

In 2011, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) adopted an opinion on the safety and efficacy of erythrosine in feed for cats and dogs, ornamental fish and reptiles (EFSA FEEDAP Panel, 2011).

Data not included in the original application was made available to the Commission. This information appears not to have been considered by the Panel, as it is not mentioned in the reference of the opinion and it is not publicly available.

These studies are: (i) an unpublished report on bioavailability in rats (Obrist et al., 1986). This report was used by the ANS Panel when re-evaluating erythrosine as food additive and addresses the metabolism of erythrosine and (ii) a toxicity study on ornamental fish at high dosage, higher doses that the EFSA recommended in its opinion.

The Commission has now received this supplementary information in the form of a dossier from the applicant company Roha UK Ltd, including the full reports and additional references to the mentioned studies.

The Commission, in order to give appropriate follow-up to the original application, asks the European Food Safety Authority to issue an updated opinion on the safety and efficacy of erythrosine on all the animal species as requested in the original application.

ASSESSMENT

1. Introduction

Erythrosine is a sensory additive belonging to the functional group colourants, namely "substances that add or restore colour in feedingstuffs". In November 2011, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) delivered an opinion on the safety and efficacy of erythrosine for cats, dogs, ornamental fish and reptiles (EFSA FEEDAP Panel, 2011). In that opinion, conclusions were made on the upper safe dietary erythrosine concentrations for target animals, which were derived from toxicological studies in rats and dogs, iodine tolerance data and maximum content of iodine in feed.[4] No conclusion could be made on efficacy owing to an absence of data.

The applicant provided new information to the European Commission (EC), referring to (i) the bioavailability of iodine from erythrosine in rats,[5] (ii) data supporting the safety of erythrosine for ornamental fish,[6] (iii) statements of feed producers on the history of safe use of erythrosine in feed for cats and reptiles,[7] (iv) visual demonstration of the colouring efficacy of erythrosine in feed for fish, cats and dogs[8] and (v) two publications on the effects of food colours on the growth of farmed fish.[9]

The EC requested that the European Food Safety Authority (EFSA) consider the supplementary information from the applicant and issue an updated opinion on the safety and efficacy of erythrosine on the same animal species considered in the original opinion.

In the new dossier, the applicant modified the proposed former maximum content of erythrosine in feed for target animals (500 mg/kg complete feed) to 1 000 mg/kg complete feed for dogs, 55 mg/kg for cats and 3 000 mg erythrosine/kg for ornamental fish and reptiles.

As the iodine content of erythrosine would legally limit its use in feed when respecting the maximum authorised content for total iodine, the FEEDAP Panel also considered analytical aspects for the determination of iodine.

Considering the intended lifetime use of erythrosine in target animals, in its re-assessment of erythrosine, the FEEDAP Panel revisited the interaction between erythrosine and thyroid physiology. When updating the opinion, the FEEDAP Panel considered, in particular, (i) the data submitted by the applicant, (ii) the impact of erythrosine on thyroid physiology and (iii) the newly identified upper safe levels of iodine for target animals (EFSA FEEDAP Panel, 2013). The FEEDAP Panel also considered the conclusions on the safety of erythrosine for humans (JECFA, 1986, 1990) and the scientific opinion on the re-evaluation of erythrosine as a food additive (EFSA ANS Panel, 2011).

2. Analytical aspects of the determination of iodine from erythrosine in feedingstuffs

At the time of the original evaluation (EFSA FEEDAP Panel, 2011), the applicant did not provide a method to analyse erythrosine in premixture, feedingstuffs and water. In the absence of such a method,

[4] Commission Regulation (EC) No 1459/2005 of 8 September 2005 amending the conditions for authorisation of a number of feed additives belonging to the group of trace elements. OJ L 233, 9.9.2005. p. 8.
[5] Technical dossier/Section III/Appendix 1.
[6] Technical dossier/Section III/Appendix 2.
[7] Technical dossier/Section III/Appendix 4.
[8] Technical dossier/Section IV.
[9] Technical dossier/Section IV/Annexes 1 and 2.

the European Union Reference Laboratories (EURL) could neither "evaluate nor recommend any method for official control to determine erythrosine in premixtures and feedingstuffs".[10]

Upon request, the applicant provided a report on the measurement of inorganic iodine, erythrosine-bound iodine and erythrosine.[11] A mixture of predominantly vegetable sources without supplemental iodine was chosen as a feed matrix. Erythrosine was supplemented at different concentrations (0, 100, 250 and 500 mg erythrosine/kg mixture). Measurements for iodine were performed using an in-house method that was claimed to be equivalent to the official method (EN15111:2007). No detailed study report was submitted, only certificates of analysis with a summary of the methodology used.

The feed samples containing erythrosine were digested/extracted by the highly alkaline reagent tetramethylammonium hydroxide at elevated temperature (95 °C) followed by quantification of iodine using inductively coupled plasma–mass spectrometry. The quantification of erythrosine was performed using an in-house method developed for routine determination of 14 synthetic colourants in different food matrices and based on methanolic ammonia extraction and high-performance liquid chromatography with diode array detection.

The quantification of erythrosine amounted to only 45 to 51 %, indicating limitations of the extraction procedure. The FEEDAP Panel concluded that there is presently no method available to determine the content of the additive in feedingstuffs at intended concentrations.

The recovery of iodine from erythrosine added at graded concentrations to a cereal-based feedingstuff for fish, not supplemented with another iodine source, was about 72 %. The recovery of iodine from iodide in samples, spiked with iodide in comparable concentrations to those expected to result from erythrosine supplementation, was about 97 %.

The iodine content of the feed can be considered indicative for erythrosine. However, iodine measurements performed with a method of analysis of iodine stated to be equivalent to the official European method neither differentiate between iodide and erythrosine as iodine sources nor allow a complete determination of the sum of iodine from both sources.

These findings confirm the assumption made in the former FEEDAP Panel opinion (EFSA FEEDAP Panel, 2011) that iodine from erythrosine would add to total iodine in feedingstuffs when determined by the official method. Consequently, any erythrosine supplementation of feed for target animals requires careful consideration of the total maximum authorised iodine content.

3. Safety

The relevant studies on the metabolic fate of erythrosine are reviewed and summarised in Appendix A. A short review of the thyroid function can be found in Appendix B. Appendix C contains a review of the relevant toxicological studies (a full review can be found in the opinion of the Panel on Food Additives and Nutrient Sources added to Food (EFSA ANS Panel, 2011)). The findings are briefly summarised in the text below, which does not contain references (see Appendices).

3.1. Absorption, distribution, metabolism and excretion

Orally administered erythrosine is absorbed in rats to a very limited extent and excreted essentially unchanged via faeces (84–97 % of the dose administered); biliary excretion (0.44–1.67 %) contributes to the faecal fraction. Urine is a minor excretory route (0.01–0.7 %); small amounts of the metabolites di- and tri-iodofluorescein were identified in the urine, indicating a limited de-iodination process. Erythrosine and its metabolites were identified in plasma, liver and kidney. The highest concentrations of erythrosine were found in the liver, indicating an extensive first-pass metabolism, including de-

[10] http://www.researchgate.net/publication/274510136_Evaluation_on_the_analytical_method_-_Erythrosine
[11] Technical dossier/Supplementary information February 2014 and July 2014.

iodination. Erythrosine was not detected in the thyroid, but low iodine residues from erythrosine were measured.

3.2. Thyroid function and interaction with erythrosine

The anatomy of the thyroid gland and hormonal regulation, metabolism and physiological function are similar in all vertebrates. The thyroid produces two hormones, tetraiodothyronine (T4, pre-hormone) and tri-iodothyronine (T3, the active hormone at cellular level). Larger amounts of T3 are produced in the liver by de-iodination of T4. In plasma, T4 is bound to transport proteins (thyroxine-binding globulin (TBG), thyroxine-binding pre-albumin and albumin). The affinity of thyroid hormones to these proteins varies considerably among species.

Orally administered erythrosine is capable of interfering with the hepatic metabolism of T4, having impacts on the homeostasis of thyroid hormones (decrease of plasma T3, increase in pituitary thyroxine-stimulating hormone (TSH)).

Because the level of iodine varies greatly with different food sources, animals have developed efficient mechanisms to adapt to the iodine supply from their food. The lower the iodine supply in the food, the more effort is taken to minimise the losses of this element by excretion. High plasma TBG levels ensure low urinary iodine losses.

3.3. Toxicology

An Acceptable Daily Intake (ADI) of 0–0.1 mg/kg body weight (bw) per day for erythrosine has been established by JECFA (1990) and the ANS Panel (EFSA ANS Panel, 2011) derived from a No Observed Adverse Effect Level (NOAEL) of 60 mg/person per day from a 14-day study with human volunteers, applying a safety factor of 10.

The lowest NOAEL in animal studies was identified in a 60-day rat study to be 0.06 % erythrosine in the diet (30 mg/kg bw per day for perturbation of thyroid function (increase in serum TSH, T4 and reverse (r)T3 and decrease in serum T3 at 0.25 % and 0.4 %). The sensitivity of thyroid functional parameters for indicating adverse effects of erythrosine were also observed in a 14-week study with pigs, in which the lowest dose tested (167 mg/kg feed) induced a decrease in serum T4.

Erythrosine is not genotoxic and has no effect on reproduction/development (EFSA ANS Panel, 2011). A two-year study in mice did not reveal tumorigenic effects. Erythrosine administered to rats in a chronic toxicity study increased significantly the combined number of thyroid adenomas and carcinomas in male rats at 4 % in the diet (18 out of 68 compared with 2 out of 68 in control males). A significant increase in the combined adenomas and carcinomas in male rats was also seen for erythrosine concentrations of 0.1, 0.5 and 1 %. A dose without effect was not identified in males. In female rats, a significant increase in tumours was found in only the 1 % erythrosine group. The tumorigenic effects in rats are most likely to be epigenetic and threshold related. It is suggested that interference of erythrosine with the metabolism of T4 and T3 may increase TSH levels in serum, producing hyper-stimulation of the thyroid, which may be associated with a tumorigenic effect.

3.4. Safety for the target animals

In its previous opinion on erythrosine, the EFSA FEEDAP Panel derived the dietary safe concentration in complete feed for dogs from a two-year dog toxicity study, assuming that 1 % erythrosine was the NOAEL. A re-assessment of this study (Hansen et al., 1973) revealed that (i) the critical endpoint that identified 2 % as an effect level was not investigated for the lower levels and (ii) no parameters of thyroid function were measured in any dose group. Thus, the dog study does not provide data that would allow the FEEDAP Panel to conclude on a safe dietary concentration in feed for dogs. Consequently, the FEEDAP Panel has to withdraw its former conclusion.

The applicant provided data[12] on a feeding study in fish with 10 colourants, including erythrosine, which was fed at a nominal concentration of 1 920 mg/kg[13] to duplicate groups of ornate tetra (*Hyphessobrycon bentosi*), firemouth cichlid (*Thorichthys meeki*) and red barb (*Puntius conchonius*) for 12 weeks. Mortality, weight gain, feed intake and feed conversion ratio were examined every three weeks. There were no adverse effects detected in any of the parameters measured. However, few details were provided on the experimental design and results: haematological parameters, blood biochemistry and histological observations were lacking. In the absence of haematology and clinical chemistry, this study is of limited value.

Toxicological or tolerance studies with erythrosine in cats and reptiles were not provided.

In the absence of any suitable study with target animals, a safe feed concentration can be estimated from data obtained in toxicological studies with laboratory animals such as rats. The lowest NOAEL observed in rats was 30 mg erythrosine/kg bw per day[14] based on perturbation of thyroid function (see section 3.3). By applying an uncertainty factor of 100 and the default values of the FEEDAP guidance for the preparation of dossiers for additives already authorised for use in food (EFSA FEEDAP Panel, 2012), the following safe concentrations in complete feed are derived: 16 mg/kg for dogs, 13 mg/kg for cats and 59 mg/kg for ornamental fish (Table 2). In the absence of default values for reptiles, the FEEDAP Panel is not in a position to derive a safe dietary concentration for these animals.

Table 2: Calculated maximum safe dietary levels of erythrosine in complete feeds for cats, dogs and ornamental fish

Species	Body weight (kg)	Safe intake (mg/day)	Feed intake (g dry matter/day)	Maximum safe dietary level	
				(mg/kg dry matter)	(mg/kg complete feed)
Cat	3	0.9	60	15.0	13.2
Dog	15	4.5	250	18.0	15.8
Ornamental fish	0.012	0.0036	0.054	66.7	58.7

When using erythrosine in feedingstuffs, its contribution to iodine supplementation should be considered. Taking into consideration the maximum currently authorised content for iodine in feedingstuffs[15] and the iodine content of erythrosine (56.5 % w/w), the maximum supplementation with erythrosine would be 35 mg/kg complete feed for ornamental fish and 18 mg/kg complete feed for the other target species,[16] but only when neglecting the background concentration of iodine in feed and assuming that no other source of iodine is added. These levels are in agreement with the maximum safe concentrations in feed of the target species, with the exception of ornamental fish.

4. Efficacy

Where the function requested for feed is the same as that used in food, no further demonstration of efficacy is be necessary (Regulation (EC) No 429/2008).[17] However, considering the wide variety of feedingstuffs used in complete and complementary feed for cats, dogs and ornamental fish and the uncertainty about the concentration of erythrosine that would result in a visible effect, a demonstration of a dose effect in a typical compound feedingstuff appeared necessary.

[12] Technical dossier/Section III/Appendix 2.
[13] From 3 000 mg of a commercial erythrosine preparation.
[14] The estimated dosage of 35.8 mg/kg bw per day is taken from the opinion of the ANS Panel (EFSA ANS Panel, 2011). Use of the conversion factor recommended later by EFSA guidelines (EFSA SC Panel, 2012) to convert the dietary concentration of 0.06 % to a dosage would give a value of 30 mg/kg bw per day.
[15] OJ L 233, 9.9.2005. p. 8 (maximum content in mg/kg of complete feedingstuffs with a moisture content of 12 %: equines, 4 mg/kg; dairy cow and laying hens, 5 mg/kg; fish, 20 mg/kg; other species or categories of animals, 10 mg/kg).
[16] Considering the newly proposed maximum contents for iodine in complete feedingstuffs (EFSA FEEDAP Panel, 2013), these values would be reduced to 7 mg erythrosine/kg for dogs and 9 mg erythrosine/kg for cats.
[17] 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.

Visual demonstration (for the owner) of the efficacy in a range of commercial feeds for pets and fish was provided. The darker the feed base is (e.g. from meat or fish meal) the more erythrosine is needed to obtain a visual red colour. Several examples provided for pet food (kibbles), in which only some kibbles are coloured red (others green and yellow), showed that the visual identification of red kibbles is easily possible without exceeding about 12 mg erythrosine/kg complete feed. Feed produced on the basis of brown components develop perceptible effects at about 50 to 100 mg erythrosine/kg feed, with easily identifiable effects at 500 mg/kg.

The applicant referred to the fish study mentioned above in section 3.4 (safety for the target animal) and to two other studies[18] in which the effect of erythrosine on the growth of several fish species was tested. However, these studies are not considered adequate to investigate the colouring effect of the additive.

Erythrosine has the potential to add red colour to feedingstuffs. The minimum effective dose depends on the colour of the feed matrix.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Derived from a NOAEL observed in a 60-day rat study (30 mg erythrosine/kg bw per day based on perturbation of thyroid function) and applying an uncertainty factor of 100, safe concentrations in complete feed are estimated to be 16 mg/kg for dogs, 13 mg/kg for cats and 59 mg/kg for ornamental fish. The FEEDAP Panel is not in a position to derive a safe concentration of erythrosine in feed for reptiles. Regarding the iodine content of erythrosine (56 %) and the limitation in the analytical determination of erythrosine in feed, the use of erythrosine in complete feed for dogs, cats and reptiles would be limited to a maximum of 18 mg/kg and for ornamental fish would be limited to 35 mg/kg feed to comply with the currently authorised maximum content of total iodine in complete feed.

Erythrosine has the potential to add red colour to feedingstuffs, which was demonstrated in complementary feed at a range of 50 to 500 mg/kg.

RECOMMENDATION

Efforts should be taken to develop a method to determine erythrosine as such in feedingstuffs.

The mathematically derived maximum safe dietary concentration of erythrosine could be rounded to 15 mg/kg complete feed for dogs and cats and 60 mg/kg complete feed for ornamental fish.

DOCUMENTATION PROVIDED TO EFSA

1. Erythrosine. May 2012. Submitted by Roha UK Ltd.
2. Erythrosine. Supplementary information. February 2014. Submitted by Roha UK Ltd.
3. Erythrosine. Supplementary information. July 2014. Submitted by Roha UK Ltd.
4. Erythrosine. Supplementary information. March 2015. Submitted by Roha UK Ltd.

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[18] Technical dossier/Section IV/Annexes 1 and 2.

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APPENDICES

Appendix A. Absorption, distribution, metabolism and excretion

In its assessment of erythrosine as a food additive, the EFSA Panel on Food Additives and Nutrient Sources added to Food (EFSA ANS Panel, 2011) concluded that: "only a small portion of erythrosine is absorbed. Erythrosine is excreted almost completely via faeces with unchanged iodine content."

The study of Obrist et al. (1989)[19] was not made available by the applicant for the former assessment of erythrosine as a feed additive (EFSA FEEDAP Panel, 2011), but is considered pivotal for the assessment of erythrosine metabolism. The metabolic fates of ^{14}C-ring-labelled and ^{125}I-labelled erythrosine were investigated in the rat. Animals (12 males and 12 females per group, aged seven weeks and weighing around 300 g) were put on a diet containing 0, 0.5 or 4 % unlabelled erythrosine for seven days, then on day 8 administered (via gavage) a single dose of 0.2 or 1 g of the radio-labelled test materials per kilogram body weight. The animals were then slaughtered sequentially over 120 hours after dosing. Blood, urine and faeces were collected during the experimental period and tissues and organs (thyroid gland, liver and kidney) were taken at slaughter (four animals per time point). Total radioactivity was measured and attempts were made to extract and identify erythrosine and related metabolites in the urine, faeces and organs. The main results were the following: (i) erythrosine was excreted essentially unchanged via faeces (84–97 % of the dose administered), urine being a minor excretory route (0.01–0.7 % and 0.08–0.98 % for ^{14}C- and ^{125}I-erythrosine, respectively); minor amounts of di- and tri-iodofluorescein were identified in the urine; (ii) low residual levels were measured in liver, kidney and blood, indicating a very limited absorption independent from sex, radiolabelling or amount of erythrosine in the diet; erythrosine and di- and tri-iodofluoresceins were identified in the plasma, liver and kidney; and (iii) ^{14}C-residues were not detectable in the thyroid, whereas low amounts of ^{125}I-residues were measured in the thyroid of both sexes (< 0.01 % of the highest administered dose). The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) notes that the pre-treatment with high doses of erythrosine might have resulted in an inhibition of the ^{125}I uptake by the thyroid (Marignan et al., 1965; Vought et al., 1972 (see below)).

In a study (Vought et al., 1972) already considered in the former FEEDAP opinion (EFSA FEEDAP Panel, 2011), but not assessed by the ANS Panel (EFSA ANS Panel, 2011), three separate experiments were performed on rats fed for three to five weeks a control diet (0.2 to 0.4 mg inorganic iodine/kg diet) or a diet based on an erythrosine-containing cereal (9.1 to 24.4 mg erythrosine/kg diet, corresponding to 5.2 to 13.9 mg iodine from erythrosine/kg diet). A single dose of ^{131}I was administered intraperitoneally at the end of each study and the distribution of ^{131}I and ^{127}I (non-radioactive) in thyroid, serum and urine was studied. The effect of erythrosine was to decrease the 24-hour ^{131}I uptake by the thyroid gland and increase the total thyroidal ^{127}I, suggesting a saturation of the thyroid with iodine from the diet made available from erythrosine. Total urinary ^{127}I was sharply increased (3.32 µg/24 hours versus 0.55 µg/24 hours for the control diet). In another experiment, ^{131}I-erythrosine (stability of the ^{131}I-labelling not demonstrated) was administered intraperitoneally to rats at doses of 0.625 and 2.125 mg/rat and one-quarter to one-third of the label was excreted in the iodide form in the urine. These experiments show that deiodinases are capable of abstracting iodine from organic-bound iodine in erythrosine at least to some extent.

In a rat study (Webb et al., 1962), a first trial consisted of the intravenous administration of erythrosine at a dose of 3 mg/kg body weight to six male animals (weighing 300–350 g), with urine and bile collected over a two-hour period. Erythrosine was determined by a fluorometric method. Biliary excretion was in the range of 50 to 58 % of the administered dose, whereas 0.8 to 1.8 % was recovered in the urine. No glucuronic acid conjugate(s) was found in either excreta. In a second trial, five rats were administered by gavage 500 mg erythrosine/kg bw and the faeces collected for five consecutive days. All administered erythrosine was recovered in faeces (101.9 %).

[19] Technical dossier/Section III/Appendix 1.

Daniel (1962) measured the biliary and faecal excretion of erythrosine in rats (weighing 200 g) administered 0.5 g erythrosine/kg bw via oral gavage. Only very limited information was given concerning the experimental protocol applied at the same time to 12 food colourants. Urine and faeces were collected separately over a three-day period. Bile was collected via a bile duct catheter (duration not given). Erythrosine was measured by a fluorometric method in the excreta and bile. No erythrosine was excreted in the urine, whereas about 72 % of the administered dose was recovered in the faeces (four animals) and 0.44 and 1.67 % was excreted in the bile (two animals). It must be noted that erythrosine and its metabolites di- and tri-iodofluorescein mentioned above (Vought et al., 1972) have similar absorption wavelengths and absorbance (Webb et al., 1962), which probably indicate comparable fluorometric characteristics; therefore, the biliary excretion measured should correspond to the sum of erythrosine and its metabolites. Moreover, although the bile collection time was not given, it can be expected that it was not sufficiently long to indicate the total amount of erythrosine and metabolites by this route.

Despite the uncertainties regarding the results from these studies with limitations in their protocols (e.g. short duration of bile collection) and the analytical method available, it can be assumed that, because the urinary and biliary excretion of erythrosine and metabolites (glucuronoconjugates and di- and tri-iodofluorescein) following oral administration is very limited, erythrosine is absorbed to a very limited extent. The extensive first-pass metabolism observed, including de-iodination, is supported by the fact that the liver contains the highest erythrosine concentrations compared with the thyroid and kidney.

Appendix B. Thyroid function

The anatomy of the thyroid gland and hormonal regulation, metabolism and physiological function are similar in all vertebrates (Stacia et al., 2009). Two thyroid hormones, tri-iodothyronine (T3) and tetraiodothyronine (T4) have been identified. The active hormone at cellular level is T3, which is mainly produced by 5′-de-iodination of T4 in the liver. T3 has multiple functions in the regulation of growth and development, energy regulation and blood pressure and in the function of other hormones. Thyroid hormones are the only functional molecules containing organic-bound iodine. Inorganic iodide from food is actively transported into the thyroidal cells, where the hormones are synthesised by a sequence of chemical reactions. After release from the thyroid gland, the hormones are tightly bound to transport proteins, in humans mainly to thyroxine-binding globulin (TBG) and, to a lesser extent, to thyroxine-binding prealbumin (TBPA) or albumin. The protein binding of T4 in humans is about 99.97 % and that of T3 is 99.5 %. This very tight protein binding reduces the excretion of the hormones via the kidneys and thus the loss of iodine. The blood concentration of unbound T3 mainly regulates the synthesis and release of thyroid hormones by a negative feedback mechanism via the secretion of the regulating hormones thyrotropin-releasing hormone and thyroxine-stimulating hormone (TSH) from the hypothalamus and pituitary gland. TSH has a stimulating effect on all thyroid functions and on the growth of the thyroid gland.

Because the level of iodine varies greatly with different food sources, animals have developed mechanisms to adapt to the iodine supply in their regular food. The lower the iodine supply in the food, the more effort is taken to minimise the loss of this element by excretion.

Although TBG is expressed by all mammalian species, its binding capacity for thyroid hormones is very different and may even vary within a single animal depending on development and thyroid status (Larsson et al., 1985; Vranckx et al., 1990). In humans, monkeys, cattle, sheep, horses and dogs, thyroxine is predominantly bound to TBG, whereas, in cats, rats, mice, birds, amphibians and reptiles, the binding is almost entirely to TBPA or albumin (Farer et al., 1962; Larsson et al., 1985; Schelling and von Vergleich, 2002). The limitation of the hormone binding to the less specific TBPA and albumin increases its turnover and loss of iodine by excretion and enhances the sensitivity of the thyroid to TSH (Döhler et al., 1979; Capen, 1997).

The production of thyroid hormones in fish has been reviewed by Eales and Brown (1993), and it resembles that in higher vertebrates.[20]

It was demonstrated in several studies with animals and humans that erythrosine is capable of interfering with the hepatic metabolism of thyroxine, having impacts on the homeostasis of the thyroid. Because of the multifunctionality of thyroid hormones and their importance for vital regulatory processes in all vertebrates, the FEEDAP Panel reviewed the existing literature with respect to the impact of erythrosine on thyroid physiology. The following description of selected studies on the effect of erythrosine on the thyroid function is adopted from the scientific opinion on erythrosine of the ANS Panel (EFSA ANS Panel, 2011).

Butterworth et al. (1976) treated four groups of Large White pigs (three males and three females/group weighing approximately 20 kg) with erythrosine in their diet at doses of 0, 167, 500 or 1 500 mg/kg per day for 14 weeks. The treated pigs exhibited decreased levels of serum thyroxine compared with controls. There were dose-related increases in the serum levels of protein-bound iodine, iodine not bound to protein and protein-bound erythrosine in animals of all treated groups. A dose-related increase in thyroid weight was noted, although the differences were statistically significant in only

[20] In teleost fish, the thyroid gland is mainly found in the subpharyngeal region. However, in several species, thyroid follicles are found in other tissues such as heart, head kidney and kidney, and these follicles are thought to work together with the subpharyngeal thyroid. In Mozambique tilapia (*Oreochromis mossambicus*), thyroid activity is restricted to the subpharyngeal region, whereas, in common carp (*Cyprinus carpio*), the functional endocrine thyroid is associated with renal tissues (Geven et al., 2007). The subpharyngeal follicles of carp constitute only 10 % of the total thyroid tissue, and these follicles neither accumulate iodide nor produce significant amounts of thyroid hormones.

female pigs at the higher dose levels (500 and 1 500 mg/kg per day) compared with the controls. None of the treated pigs revealed pathological changes of the thyroid.

Three groups of 160 male Sprague-Dawley rats were administered erythrosine at dose levels of 0.0, 0.25 or 4.0 % in the diet (equivalent to 0.0, 150 or 2 500 mg/kg bw per day) for 60 days. Physical observations and body weight and food consumption measurements were performed on all animals pre-test and at weekly intervals during the treatment period. Necropsy was performed with up to 20 animals per test group at days 0, 3, 7, 10, 14, 21, 30 and 60. Serum was prepared from blood samples taken from the abdominal aorta at each sacrifice interval and analysed by radioimmunoassay for TSH, T4, T3 and reverse T3 (rT3).[21] Thyroid and pituitary glands were weighed at each interval and organ/body weight ratios were calculated. Gross post-mortem examinations were conducted on the thyroid and pituitary glands only. Three rats receiving 4.0 % erythrosine in the diet died spontaneously during the second week of the study. The animals receiving 4 % erythrosine in the diet lost weight during the first week of the study and the mean body weights were significantly lower than control values throughout the study (13 % at week one and 17 % at week 8). Food consumption of the animals receiving 4.0 % erythrosine in the diet was significantly lower than the control value at week one, but after week two it was comparable. This probably reflected a palatability issue during the first two weeks. The absolute pituitary weights of males receiving 4 % erythrosine were statistically significantly lower than control values at days 7, 10, 14, 21 and 60. The differences were considered to reflect the body weight differences between the high-dose animals and the controls. The absolute thyroid/parathyroid weights of the rats at the 4 % level were generally lower than the control values, but the differences were slight and may be due to the body weight differences between these groups. The relative weights of these organs were significantly greater at day 21; otherwise relative weights were only slightly greater and not significant. Thyroid/parathyroid absolute and relative weights of the rats fed 0.25 % erythrosine were significantly lower at day 60, otherwise they were comparable to controls. Gross post-mortem examinations of thyroid and pituitaries did not show treatment-related changes (Kelly and Daly, 1988, as cited by EFSA ANS Panel, 2011).

The analysis of serum hormone levels in the rats of the study described above revealed the following: there was a change (slight increase) in serum TSH levels in the control rats during the 60-day experimental period. The baseline (day 0) TSH level was significantly lower than the levels on days 21, 30, and 60. In the 0.25 % group, serum TSH concentrations were significantly increased over baseline (day 0) at days 14, 21, 30 and 60. When compared to the TSH levels in control animals, a significant increase was observed at days 21, 30 and 60 in the 4.0 % group. In the 4.0 % group the TSH levels were significantly increased over the baseline (day 0) level and the corresponding control levels at all time points. When compared to the 0.25 % group the serum TSH levels in the high dose group were significantly greater at days 3, 7, 10, and 14. Serum T4 concentrations were increased over baseline and control values at days 10 and 14 in the 0.25 % group, while in the 4.0 % group the serum T4 concentrations were increased at all time points. Furthermore, the high dose animals had significantly greater T4 concentrations than the low dose animals at days 7, 10, 21, 30 and 60. Serum T3 concentrations in the low dose rats were comparable to the control values except for a decrease at day 30. In the high dose rats serum, T3 concentrations were significantly lower than baseline (day 0) and control values at all time points. In addition, serum T3 concentrations were decreased compared to those of the low dose animals on days 3, 10, 14, 21, 30, and 60. Serum rT3 concentrations were increased above baseline (day 0) in the low dose group at days 7, 10, 14, 21, 30 and 60; and increased above control values at days 10, 14 and 21. A marked increase in serum rT3 over controls and low dose animals was seen in the high dose group at all time points. The results indicate that the ingestion of a dietary concentration of 4 % erythrosine induces a rapid and sustained increase in serum TSH, T4, and rT3 and a comparable decrease in serum T3 concentrations, and that these changes are also induced, but are less pronounced, after administration at dose levels of 0.25 % erythrosine in the diet. These findings are consistent with an inhibition by erythrosine of the deiodination in the 5′-position of T4 and rT3, resulting in a decreased production of T3 from T4 and a decreased deiodination of rT3,

[21] Reverse tri-iodothyronine; 3,3′,5′-tri-iodothyronine.

respectively (Braverman and DeVito, 1988, as cited by EFSA ANS Panel, 2011). Because a NOAEL could not be observed, the experiment, was repeated with lower doses.

Three groups of 80 male Sprague-Dawley rats were administered erythrosine at dose levels of 0.0, 0.03, 0.06 and 4.0 % in the diet for a maximum of 60 days (corresponding to 0.0, 17.5, 35.8, and 2671.7 mg/kg bw/day, respectively). Control animals (100 males) received standard laboratory diets. Physical observations, body weight and food consumption measurements were performed on all animals pre-test and at weekly intervals during the study period. For the determination of baseline data, 20 control animals were bled for radioimmunoassays of TSH, T4, T3, and rT3 and sacrificed on test day 0, prior to the initiation of dosing. Additional necropsy intervals were staggered so that on days 7, 21, 30 and 60, an additional 20 animals per group at each interval were bled for radioimmunoassay samples. Brain, pituitary and thyroid were weighed and organ to body and organ to brain weight ratios were calculated for all animals. Gross post mortem examinations were performed on the thyroids, pituitary and brains of all animals. In the animals receiving 4 % erythrosine in the diet, a substantial loss of body weight and decreased food consumption during week one of the study, probably due to poor palatability of the diet, resulted in statistically significantly lower body weights of the animals throughout the study period. The absolute and relative thyroid:parathyroid weights of the animals receiving 4.0 % erythrosine were increased at days 21 and 30, and at day 60 (relative organ to body weight ratio). The absolute and relative (organ to brain weight ratio) pituitary weights of animals at the 4.0 % level were lower than control values at day seven. In the 0.03 % Erythrosine group, absolute and relative thyroid to parathyroid weights were greater than corresponding control values at days 21 and 30, but comparable to control values at days 7 and 60. Thus, no consistent and dose-related changes in organ weight, absolute or relative, were found at the lower doses. Gross post-mortem examination of the thyroid, pituitaries and brain did not reveal any treatment related effects (Kelly and Daly, 1989, as cited by EFSA ANS Panel, 2011).

The analysis of serum hormone levels in the rats of the study above revealed the following: in the 0.03 % and 0.06 % groups there were no significant changes in serum TSH, T4, T3, and rT3 concentrations during the 60-day treatment period. In the 4.0 % group, TSH concentrations were significantly greater than the corresponding control values at days 21, 30, and 60. A 41 % increase after seven days was not statistically significant compared to the control value. Serum TSH concentrations in the 4 % group were significantly greater than those of the 0.03 % group at days 21, 30, and 60, and the 0.06 % group at day 30. In the 4.0 % group, serum T4 concentrations were slightly elevated above controls during the treatment period. However, the increase was only statistically significant on day 30. In the high dose animals, serum T3 concentrations were significantly lower than controls at all time points. Serum rT3 concentrations were markedly increased in the high dose animals compared to controls or animals fed 0.03 % and 0.06 % erythrosine at all time points (Braverman and DeVito, 1989, as cited by EFSA ANS Panel, 2011). From this study, a NOAEL of 0.06 % of erythrosine in the diet (corresponding to 30 mg/kg bw) was derived by the FEEDAP Panel.

In a pivotal clinical study, Gardner et al. (1987, as cited by EFSA ANS Panel, 2011) examined the effects of Erythrosine on the thyroid function in 30 healthy male subjects. Ten men per group consumed capsules containing 20, 60, or 200 mg/day of erythrosine for 14 days. Serum T4, T3, reverse T3, T3-charcoal uptake, TSH, PBI, total iodine, and total urinary iodine excretion were measured on days 1, 8, and 15. Thyrotropin-releasing hormone (TRH) was measured on days 1 and 15. No significant changes in serum T3, T4, rT3 and T3 uptake were seen in any group. Mean basal serum TSH concentration increased from 1.7 on day 1 to 2.2 µU/ml on day 15 ($p < 0.05$) and the mean peak TSH increment after TRH increased from 6.3 to 10.5 µU/ml ($p < 0.05$) in the high-dose group. There were no significant changes in basal or peak TSH responses at the two lower dose levels. Significant dose-related increases in serum total iodide and PBI concentrations occurred in all three groups and significant dose-related increases in urinary iodide excretion occurred in the 60 and 200 mg/day dose groups. The dose-dependent increases in serum and urinary iodide levels indicated that some of the iodine in the Erythrosine molecule is bioavailable. The daily iodide load estimated from the mean 24-hour urinary excretion was approximately 0.5 % by weight of the daily dose of erythrosine. Given the presence of 0.25 % sodium iodide in the erythrosine, the authors attributed

about half of this iodide load (e.g. 1000 µg/day at the 200 mg/day dose level) to erythrosine deiodination. The authors suggested that these data indicated that the increase in TSH secretion was related to the effect of increased serum iodide rather than a direct effect of erythrosine on thyroid hormone secretion or peripheral metabolism. The authors state that the NOAEL in this clinical study for effects on basal and TRH-stimulated TSH secretion is considered 200 mg erythrosine per day (Gardner et al., 1987, as cited by EFSA ANS Panel, 2011). The ANS Panel considered erythrosine has a minimal effect in humans at a clinical oral dose of 200 mg daily over 14 days, while a dose of 60 mg daily was without effect.

In vitro metabolism of ^{125}I-labelled T4 was greatly altered in liver homogenates from rats fed 4.0 % erythrosine in the diet, with degradation of T4 decreasing to approximately 40 % of values in homogenates of control livers. There was an associated decrease of about 75 % in the generation of ^{125}I-iodide and an approximately 80 % decrease in the generation of ^{125}T3 from ^{125}T -T4. The percentage of degradation of T4 and generation of iodide and T3 in liver homogenates from rats fed 0.5 % erythrosine were similar to controls. *In vitro* metabolism of T4 was studied in pituitary glands from the control, 1, 2, and 4 % dose groups. Overall, ^{125}I-T4 degradation and generation of ^{125}I-iodide appeared higher in the two higher erythrosine dose groups than in controls, but none of the differences were statistically significant. The results were interpreted as indicating that the primary effect of high doses of erythrosine on thyroid hormones is inhibition of type I 5′-monodeiodination of T4 to T3. As a consequence, TSH secretory mechanisms were activated in the pituitary. The increases in serum rT3 levels were considered to arise from both increased availability of the T4 precursor and inhibition of metabolism of rT3 by 5′-monodeiodination (Ingbar et al., 1984, as cited by EFSA ANS Panel, 2011).

5′-Deiodinase is responsible for the conversion of the precursor hormone T4 into the active form T3. The inhibition of the enzyme therefore causes a decrease in T3 and an increase in T4 concentration in the serum. The enzyme 5′-deiodinase, which converts T4 to the inactive form rT3, seems to be less sensitive to inhibition by erythrosine. T4 is therefore mainly converted to rT3 (Ingbar et al., 1984). The inhibition of 5′-deiodinase also reduces the further degradation of rT3 to T2, which is another reason for the observed increase of the rT3 concentration in the serum. The reduction of T3 concentration in the serum causes a compensatory increase of the secretion of the thyroid-stimulating hormone TSH from the pituitary gland, followed by follicular cell hypertrophy and hyperplasia of the thyroid, which is responsible for the increased incidence of follicular cell tumours in male rats treated with erythrosine for two years (Borzelleca et al., 1987).

Appendix C. Toxicology

Groups of three male and three female Beagle dogs were fed diets containing 0, 0.5, 1.0 or 2.0 % erythrosine for two years (Hansen et al., 1973). Considering the occurrence of chronic thyroiditis in one male and one female of the 2.0 % group, the FEEDAP Panel (EFSA FEEDAP Panel, 2011) concluded that 1 % erythrosine in the diet could be regarded as the NOAEL. A re-assessment of this study revealed that (i) the critical endpoint that identified 2 % as an effect level was not investigated for the lower levels and (ii) no parameters of thyroid function were measured in any dose group. Thus, the dog study does not provide data that would allow the FEEDAP Panel to conclude on a safe dietary concentration in feed for dogs. Consequently, the FEEDAP Panel has to withdraw its former conclusion.

Charles River CD-1 mice (60 animals/sex/dose) were exposed to Erythrosine in the diet at dose levels of 0, 0.3, 1.0, or 3.0 % for 24 months, equivalent to on average 0, 424, 1474, or 4759 mg/kg bw per day for males and 0, 507, 1834, or 5779 mg/kg bw per day for females respectively. With the exception of significantly decreased body weights (throughout the entire study) of males and females at the 3.0 % dose level, other investigated parameters (mortality, food intake, haematology, gross pathology and histopathology) were not adversely affected by Erythrosine treatment at any dose level (Richter et al., 1981). These data have now been published in Borzelleca and Hallaghan (1987). There was a significant increase in lymphocytic lymphoma in male mice in the lowest dose group. However, since no dose–effect relationship was observed, this was considered to be of no toxicological significance (Borzelleca and Hallaghan, 1987, as cited by EFSA ANS Panel, 2011).

Charles River CD weanling rats (70 rats/sex/dose) were fed Erythrosine in the diet at levels of 0.1, 0.5, or 1.0%, corresponding to 49, 251, or 507 mg Erythrosine/kg bw per day for males and 61, 307, or 642 mg Erythrosine/kg bw per day for females for 30 months after *in utero* exposure. Two concurrent control groups (70 animals/sex/group) received no colour in the diet. There were no consistent significant compound-related effects during the *in utero* phase. In the main study, there were no consistent significant compound-related effects on the following: physical observation, behaviour, mortality, food consumption, haematology, clinical chemistry, urinalysis, or ophthalmological findings. Mean body weights of control and treated rats did not differ significantly during the exposure period. The gross pathological changes that were noted could not be attributed to treatment with Erythrosine. The incidence of non-neoplastic lesions was comparable between treated and control groups. There was a statistically significant increase in the incidence of benign thyroid tumours (follicular adenomas): 6/68 in the 1.0 % female test group versus 0/140 in the control group. The incidence of malignant tumours in rats of treated groups was comparable with that of the controls (Brewer et al., 1981, as cited by EFSA ANS Panel, 2011).

Two groups of Charles River CD weanling rats (70/sex/dose) were given Erythrosine in the diet at dose levels of 0 or 4.0 % for a period of approximately 29 months after *in utero* exposure. The average consumption of the Erythrosine was 2465 mg/kg bw per day for males and 3029 mg/kg bw per day for females. There were no consistent significant compound-related effects on the following; physical observations, behaviour, mortality, food consumption, haematology, clinical chemistry, urinalysis, or ophthalmological findings. Mean body weights of treated rats (both sexes) were slightly lower throughout the study than those of the control rats. These differences were statistically significant except at weeks 3–5 and 122 (males) and at weeks 0–4, 6 and 114 (females). The mean absolute and relative thyroid weights of treated males were more than twice those of the controls. Histopathological examination revealed that the incidence of thyroid hyperplasia (follicular and C-cell) was significantly increased in treated males. There was a statistically significant increase in the incidence of follicular adenoma of the thyroid in treated male rats (16/68) when compared with the controls (0/69). The incidence of malignant tumours, including thyroid C-cell and follicular carcinomas, was comparable among treated and control rats (Brewer et al., 1982, as cited by EFSA ANS Panel, 2011).

The results of the two long-term feeding studies in rats after *in utero* exposure to Erythrosine (Brewer et al., 1981; Brewer et al., 1982, as cited by EFSA ANS Panel, 2011) that were reviewed by JECFA at

the thirtieth meeting have now been published (Borzelleca et al., 1987, as cited by EFSA ANS Panel, 2011). In the statistical analyses thyroid follicular cell adenomas and carcinomas were treated as separate tumour classes. The authors' conclusion remains that Erythrosine at a level of 4 % in the diet for 128 weeks induces an increased incidence in thyroid follicular cell adenomas in male rats (15/69) compared to controls (1/69). The incidence of thyroid follicular cell carcinomas (3/69) was not statistically significantly different from the control value (2/69). In females at the 4 % level the incidence of thyroid follicular cell adenomas (5/69) or carcinomas (0/69) were not different than the controls (0/69 and 0/69, respectively). In female rats fed 0.1, 0.5, or 1 % Erythrosine in the diet, a numerical increase in adenomas was observed (1/67, 3/68 and 5/69, respectively compared to 1/139 for control females), but the increases were not statistically significant. The incidences of females with carcinomas were 0/67, 0/68 and 1/69 compared to 0/139. In the males, at the 0.1, 0.5 and 1.0 % Erythrosine dose levels the incidence of adenomas (0/67, 2/68 and 1/69 compared to 0/139) and carcinomas (3/67, 1/68, and 3/69 compared to 0/139) were not considered significantly different.