European Food Safety Authority

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SCIENTIFIC OPINION

Scientific Opinion on the safety and efficacy of synthetic astaxanthin as feed additive for salmon and trout, other fish, ornamental fish, crustaceans and ornamental birds[1*]

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

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

Astaxanthin is a pigmenting carotenoid occurring naturally in plankton, crustaceans and fish. The astaxanthin under assessment is of synthetic origin. The FEEDAP Panel considers synthetic astaxanthin safe for salmonids up to 100 mg/kg complete diet. The conclusion on the safety of astaxanthin for salmonids can be extrapolated to other fish and ornamental fish at the same dose. Dietary concentrations of up to 100 mg astaxanthin/kg feed are safe for crustaceans. The FEEDAP Panel could not conclude on the safety of astaxanthin for ornamental birds. Based on a $BMDL_{10}$ of 3.4 mg/kg bw per day (calculated for liver hypertrophy in female rat in a carcinogenicity study) and applying an uncertainty factor of 100, it is possible to set an ADI of 0.034 mg ATX/kg bw (equivalent to 2.0 mg ATX per 60 kg person per day). The use of astaxanthin up to the maximum permitted dietary level for salmon and trout is of no concern for the safety of the consumer. As some formulations of astaxanthin may be dusty, and in the absence of data on inhalation toxicity, it is prudent to regard astaxanthin-containing additives as being potentially hazardous by inhalation. In the absence of any information on irritancy to skin or eyes or on skin sensitisation, astaxanthin-containing additives should be regarded as hazardous by exposure to skin or eyes. The FEEDAP Panel considers that the use of synthetic astaxanthin (100 mg astaxanthin/kg fish feed) does not pose a significant additional risk to the environment compared with natural astaxanthin. Astaxanthin is efficacious in colouring the flesh of salmonids and the epidermis of crustaceans. Astaxanthin is efficacious in pigmenting the flesh of food-producing fish other than salmonids and the skin of ornamental fish. No conclusion can be made on the efficacy of oral astaxanthin in pigmenting the plumage of ornamental birds.

KEY WORDS

sensory additive, astaxanthin, salmonids, ornamental fish, other fish, crustaceans, ornamental birds

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[1] On request from European Commission, Question No EFSA-Q-2011-01070, adopted on 20 May 2014.

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[3] Acknowledgement: The Panel wishes to thank the members of the Working Group on Colouring Agents, including Georges Bories, Jürgen Gropp and Anne-Katrine Lundebye, for the for the preparatory work on this scientific opinion.

* This scientific opinion has been edited following the adoption of the decision of the Commission on confidentiality claims submitted by the applicant, in accordance with Article 8(6) and Article 18 of Regulation (EC) No 1831/2003. The modified sections are indicated in the text.

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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 astaxanthin as feed additive for salmon and trout, other fish, ornamental fish, crustaceans and ornamental birds.

Astaxanthin is a pigmenting carotenoid occurring naturally in plankton, crustaceans and fish. Astaxanthin under application is a synthetic product characterised by a defined proportion of enantiomers of 25 % 3S,3′S, 50 % 3R,3′S and 25 % 3R,3′R.

The FEEDAP Panel considers synthetic astaxanthin safe for salmonids at concentrations of up to 100 mg/kg complete diet. The conclusion on the safety of astaxanthin for salmonids can be extrapolated to other fish and ornamental fish at the same dose. Dietary concentrations up to 100 mg astaxanthin/kg feed are safe for crustaceans. The FEEDAP Panel could not conclude on the safety of astaxanthin for ornamental birds.

Based on a $BMDL_{10}$ of 3.4 mg/kg bw per day (calculated for liver hypertrophy in female rat in a carcinogenicity study) and applying an uncertainty factor of 100, it is possible to set an ADI of 0.034 mg ATX/kg bw (equivalent to 2.0 mg ATX per 60 kg person per day). The use of astaxanthin up to the maximum permitted dietary level for salmon and trout is of no concern for the safety of the consumer.

As some formulations of astaxanthin may be dusty, and in the absence of data on inhalation toxicity, it is prudent to regard astaxanthin-containing additives as being potentially hazardous by inhalation. In the absence of any information on irritancy to skin or eyes or on skin sensitisation, astaxanthin-containing additives should be regarded as hazardous by exposure to skin or eyes.

The FEEDAP Panel considers that the use of synthetic ATX (100 mg astaxanthin/kg fish feed) does not pose a significant additional risk to the environment compared with natural astaxanth in.

Astaxanthin is efficacious in colouring the flesh of salmonids and the epidermis of crustaceans. Astaxanthin is efficacious in pigmenting the flesh of food-producing fish other than salmonids and the skin of ornamental fish. No conclusion can be made on the efficacy of oral astaxanthin in pigmenting the plumage of ornamental birds.

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BACKGROUND

Regulation (EC) No 1831/2003[4] establishes the rules governing the EU authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7. 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 time limit or pursuant to Directive 82/471/EEC.

The European Commission received a request from the CARAC EEIG Carotenoids Authorisation Consortium[5] for authorisation of the product astaxanthin, to be used as a feed additive for salmon and trout, other fish, ornamental fish, crustaceans, ornamental birds (category: sensory additives; functional group: (a) Colourants: (ii) Substances which, when fed to animals, add colours to food of animal origin; (a) Colourants: (iii) substances which favourably affect the colour of ornamental fish or birds) under the conditions mentioned in Table 1.

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 4(1) (authorisation of a feed additive or new use of a feed additive) and Article 10(2) (re-evaluation of an authorised feed additive). EFSA received directly from the applicant the technical dossier in support of this application.[6] 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 6 April 2010.

The synthetic astaxanthin (E161j) is authorised without a time limit under Council Directive 70/524/EEC as a sensory additive for salmon, trout and ornamental fish.[7] Astaxanthin-rich *Phaffia rhodozyma* (ATCC 74219) (E161z) is authorised without a time limit for use in salmon and trout.[8] Astaxanthin dimethyldisuccinate is authorised for use in salmon and trout until 21.05.2018.[9] Red carotenoid-rich *Paracoccus carotinifaciens* is authorised for use in salmon and trout until 18.08.2018.[10]

The Scientific Committee on Animal Nutrition (SCAN) issued several opinions on specific questions on the efficacy and the safety of synthetic (EC, 1989) and biosynthetic (*Phaffia rhodozyma* (ATCC 74219)) (EC, 2002; EC, 2003) astaxanthin. The FEEDAP Panel has adopted a number of opinions on astaxanthin. One opinion was on the environmental impact of astaxanthin-rich *Phaffia rhodozyma* (ATCC 74219) (EFSA, 2004); another one dealt with the safety of astaxanthin in animal nutrition (EFSA, 2005a); in a third opinion, the safety and efficacy of an astaxanthin-rich *Phaffia rhodozyma* (ATCC SD-5340) product were assessed (EFSA, 2005b); the last two opinions were on the safety and

[4] 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.

[5] On 13/03/2013, EFSA was informed by the applicant that CARAC 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), including the following companies: BASF SE, Carotenoid Technologies SA, Europe-Asia Import Export GmbH, Feed Additives technologies SARL and Sunvit GmbH. Avenue Louise, 130A, Box 1, 1050 Brussels, Belgium.

[6] EFSA Dossier reference: FAD-2009-0054.

[7] Council Directive of 23 November 1970 concerning additives in feeding-stuffs. OJ L 270, 14.12.70, p. 1.

[8] Commission Regulation (EC) No 1288/2004 of 14 July 2004 concerning the permanent authorisation of certain additives and provisional authorisation of a new use of an additive already authorised in feedingstuffs. OJ L 243, 15.7.2004, p. 10

[9] Commission Regulation (EC) No 393/2008 of 30 April 2008 concerning the authorisation of astaxanthin dimethyldisuccinate as a feed additive. OJ L 117, 1.5.2008, p. 20.

[10] Commission Regulation (EC) No 721/2008 of 25 July 2008 concerning the authoriation of a preparation of red carotenoid-rich bacterium *Paracoccus carotinifaciens* as feed additives. OJ L 193, 26.7.2008, p. 23.

efficacy of astaxanthin dimethyldisuccinate (EFSA, 2007a) and of a red carotenoid-rich bacterium *Paracoccus carotinifaciens* (EFSA, 2007b) as feed additives for salmon and trout. In 2010, the FEEDAP Panel adopted a scientific opinion on modification of the terms of authorisation of *Paracoccus carotinifaciens* (EFSA FEEDAP Panel, 2010). In 2014, the FEEDAP Panel has adopted an opinion on the safety and efficacy of astaxanthin for salmonids and ornamental fish (EFSA FEEDAP Panel, 2014).

TERMS OF REFERENCE

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 astaxanthin, when used under the conditions described in Table 1.

Table 1: Description and conditions of use of the additive as proposed by the applicant[11]

Additive	Astaxanthin
Registration number/EC No/No (if appropriate)	**E161j**
Category of additive	2. Sensory additives
Functional group(s) of additive	a. Colourants
Sub-classification	(ii) Substances which, when fed to animals, add colours to food of animal origin (iii) Substances which favourably affect the colour of ornamental fish or birds

Description			
Composition, description	Chemical formula	Purity criteria (if appropriate)	Method of analysis (if appropriate)
Astaxanthin	$C_{40}H_{52}O_4$	Assay (expressed as astaxanthin): min 96 % of total colouring matter Carotenoids other than astaxanthin: max 5 % of total coluring matter.	Spectrophotometry; absorbance maximum in Methylene chloride between 485 and 489nm HPLC

Trade name (if appropriate)	Not appropriate
Name of the holder of authorisation (if appropriate)	Not appropriate

Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period (if appropriate)
		mg/kg of complete feedingstuffs, supplementary feed (based on end feed) and in water*		
Salmon and trout	-	-	100	Not appropriate
Pets: • Ornamental fish and birds	-	-	100	Not appropriate
Minor species • Crustaceans • Other fish	-	-	100	Not appropriate

[11] During the course of the assessment the applicant modified the proposal for the maximum content for the target species, ornamental fish and birds.

Other provisions and additional requirements for the labelling	
Chickens for fattening	Not appropriate
Chickens reared for laying	Not applicable
Laying hens	Not applicable
Salmon and trout	The mixture of Astaxanthin with Canthaxanthin is allowed provided that the total concentration of the mixture does not exceed 100 mg/kg in complete feedingstuff.
Pets: • Dogs • Cats • ornamental fish and birds	-Ornamental fish are treated in the same way as salmon and trout. -Not applicable to dogs and cats.
Other birds such as ducks, geese, quails, pheasants	Not applicable
Specific conditions or restrictions for handling (if appropriate)	Not applicable
Post market monitoring (if appropriate)	Not applicable
Specific conditions for use in complementary feedingstuffs or water (if appropriate)	-Can only be placed on the market in form of a stabilised form. -These formulations of Astaxanthin are aimed to be incorporated into feed.

Maximum Residue Limit (MRL) (if appropriate)			
Marker residue	Species or category of animal	Target tissue(s) or food products	Maximum content in tissues
-	-	-	-

ASSESSMENT

This opinion is based on data provided by a consortium of companies involved in the production/distribution of astaxanthin (ATX). It should be recognised that these data cover only a fraction of existing additives containing ATX. The composition of the additives is not the subject of the application. The FEEDAP Panel has sought to use the data provided together with data from other sources to deliver an opinion.

The application contains data from four sources of ATX obtained by chemical synthesis.

1. Introduction

Synthetic astaxanthin (E161j) is authorised without a time limit under Council Directive 70/524/EEC as a colourant for salmon and trout from six months of age onwards and for ornamental fish. The applicant is seeking the re-evaluation/authorisation of astaxanthin as a sensory additive, functional groups "colourants (ii) Substances which, when fed to animals, add colours to food of animal origin" and "colourants (iii) Substances which favourably affect the colour of ornamental fish or birds", for use in salmon and trout, ornamental fish and birds, crustaceans and other fish.

The dossier contains information relating to four sources of ATX obtained from chemical synthesis.

Owing to its high susceptibility to oxidation, ATX requires stabilisation, which in turn necessitates a suitable product formulation. Consequently, several formulated products (additives) with different physico-chemical properties are also described. Moreover, the content of ATX in the additive may vary.

The applicant proposes a maximum content of ATX of 100 mg/kg complete feedingstuff for salmon, trout, other fish and crustaceans. The same maximum content is proposed for ornamental fish and ornamental birds.

2. Characterisation

2.1. Characterisation of the active substance[12]

Astaxanthin (International Union of Pure and Applied Chemistry (IUPAC) name 6-hydroxy-3-[(1E,3E,5E,7E,9E,11E,13E,15E,17E)-18-(4-hydroxy-2,6,6-trimethyl-3-oxo-1-cyclohexenyl)-3,7,12,16-tetramethyloctadeca-1,3,5,7,9,11,13,15,17-nonaenyl]-2,4,4-trimethyl-1-cyclohex-2-enone; chemical formula $C_{40}H_{52}O_4$; molecular weight 596.8; Chemical Abstracts Service (CAS) number 7542-45-2; European Inventory of Existing Commercial chemical Substances (EINECS) number 207-451-4) is a violet-brown to violet-red hydrophobic crystalline powder. The structural formula is showed in Figure 1. The enantiomeric composition of synthetic ATX is 25 % 3S,3′S, 50 % 3R, 3′S and 25 % 3R,3′R. The synthetic ATX under assessment contains by specification not less than 96 % ATX and not more than 5 % other carotenoids.

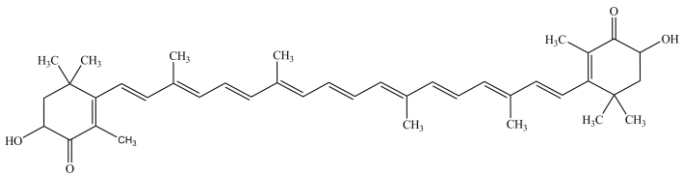

Figure 1: Structural formula of astaxanthin

The analysis of 19 samples (from four different companies) showed conformity with the specification, with average concentrations of ATX of 98.0 % and of other carotenoids of 2.8 %.[13] The carotenoids

[12] This section has been edited following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003.

other than ATX are described in the dossier. Additional data for two products indicate that the specification for ATX content is not consistently met (ATX 95.4 % and 94.3 %).[14]

In five batches of one product the proportion of the geometrical isomer all-E and 13Z was 94 and 1 %, respectively. Different proportions of geometrical isomers were reported in two products (one batch each), namely 79 and 75 % for all-E and13 and 15 % for 13Z.[15] In a fourth product (three batches), the proportion of all-E ATX was 75 % and of all Z-isomers was 22 %.[16] Influences of temperature and solvent (Yuan and Chen, 1999), acid (Mortensen and Skibsted, 2000) and ions (Zhao et al., 2005) on geometrical isomerisation of ATX have been identified. The chemical synthesis described (see section 2.3) involves a thermal isomerisation step to control the amount of the all-*trans* isomer.

Data on residual solvent concentrations in the active substance (five batches) have been provided by one company, with concentrations ranging from 2 035 to 2 865 mg/kg for ethanol and from 255 to 458 mg/kg for dichloromethane.[17] Data on residual solvents in the final formulated additive (nine batches, five different products) were provided.[18,19,20,21] One additive (three batches) contained 10 mg methanol/kg, all other potential residual solvents (butylenoxide, acetone, dichloromethane, diethylketone, ethylacetate, technical heptane, n-hexane, i-butanol, i-propanol, methanol, n-propylacetate, n-octane, t-butanol, methyl-tertbutylether) were < 10 mg/kg. Four other additives (six batches) showed concentrations of dichloromethane between 21 and 58 mg/kg. Ethanol was found in two additives (four batches) at concentrations between 110 and 500 mg/kg, and the toluene in one product (one batch) was below the limit of detection (LOD = 89 mg/kg). All values comply with the International Cooperation on Harmonisation of Technical requirements for Registration of Veterinary Medicinal Products (VICH)[22] thresholds.

Data on the triphenylphosphine oxide (TPPO, a structurally unrelated reaction by-product) content of an ATX-containing additive (three batches) were provided one producer and amounted to 170, 197 and 78 mg/kg.[23] The values are, in two cases, above the threshold of 100 mg/kg introduced by the Joint WHO/FAO Committee on Food Additives (JECFA) for lycopene and zeaxanthin which was used by the FEEDAP Panel in the assessment of carotenoids (EFSA, 2007a). Another producer provided a statement that no triphenylphosphine was used in the manufacturing process, thus indicating that no TPPO can be formed.[24]

Data on sulphated ash concentrations in the active substance were submitted in the dossier.

Heavy metals (expressed as lead) were specified by two companies. In a later submission, additional data were given for three batches from one company[25] (lead, 2 mg/kg; arsenic, < 2 mg/kg; mercury and cadmium, not detected (LOD not given)). All values complied with the company's specifications (lead ≤ 5 mg/kg, arsenic ≤ 3 mg/kg, mercury and cadmium ≤ 1 mg/kg).

2.2. Manufacturing

Astaxanthin is chemically synthesised using two main routes fully described in the literature, the C_{15} + C_{10} + C_{15} (Wittig reaction) and the C_{19} + C_2 + C_{19} (Grignard reaction) condensation strategies (Isler,

[13] Technical dossier/Section II/Annexes 2.1.3.a–d.
[14] Supplementary information/February 2013/Annexes Qi.
[15] Supplementary information/February 2013/Annexes Qi.
[16] Supplementary information/February 2013/Annexes Qi.
[17] Technical dossier/Section II/Annex 2.1.3.e.
[18] Supplemetary information/December 2010/Annex IIa.
[19] Supplemetary information/December 2010/Annex IIb.
[20] Supplemetary information/December 2010/Annex IIc.
[21] Supplemetary information/December 2010/Annex IId.
[22] http://www.vichsec.org/en/GL18(R)_ST7.pdf
[23] Supplementary information/February 2013/Annex Qiii
[24] Supplementary information/February 2013/Annex Qiii.
[25] Supplemetary information December 2010/Annex IId.

1979). The Witting reaction involves the C_{15} hydroxyphosphonium activated compound and a double Wittig condensation process with the synthon C_{10} dialdehyde, giving rise to TPPO as a by-product. The final product is crystallised in organic solvents. In this process, in addition to the desired (all-E)-configured carotenoid, certain amounts of mono- and (di-Z)-stereoisomers are produced. These mixtures of isomers are as a rule thermally isomerised, for example by heating for several hours in heptane or ethanol, to form the desired (all-E)-configured products. In doing so, the poorly soluble (all-E)-isomer crystallises out and is thus removed from the isomerisation equilibrium (Hansgeorg, 2002).

The applicant also provided a description of the production process for the stabilised additives.[26]

2.3. Characterisation of the additive(s)[27]

Six solid preparations contained 10 % active ingredient (ATX) and a seventh preparation 8 %. Some preparations contain ATX beadlets, others crystallised ATX, and some were spray dried. Information on the formulations were provided in the dossier. Oily preparations, which were originally included in the application, were withdrawn during the course of the assessment.[28]

Particle size distribution was available for the seven products (three batches each) from four different companies. Five additives contained less than 1 % particles (w/w) smaller than 50 µm. The other two products (from the same company) showed a range of 3–4.4 % particles (w/w) smaller than 45 µm.[29]

At the request of the FEEDAP Panel, the applicant submitted additional data on one of the additives (10 % ATX) showing the highest proportion of particles < 50 µm diameter. Based on the Stauber–Heubach method, the dusting potential was 0.295 g/m^3. The amount of active substance in the dust was further analysed and found to be 0.14 % ATX. Ten per cent of the particles in the dust were of respirable size (diameter ≤ 10 µm).[30]

2.4. Stability and homogeneity

Owing to its high susceptibility to oxidation, ATX requires stabilisation, which in turn necessitates a suitable product formulation. Stability and homogeneity of ATX was described with examples of formulated products of the applicant.

2.4.1. Shelf-life of the additive

The applicant submitted data related to the shelf-life of two formulated solid products (ATX 10 %, three batches each) at 25 °C. After 12 months' storage, analytical data indicated an ATX loss of 3 to 5 % of the initial content.[31] Data on ATX loss after 24 and 36 months' storage were submitted for three batches of one additive, showing losses of about 5 and 7 %, respectively.[32] Storage under accelerated conditions (40 °C) resulted in loss of about 8 % after six months.[33]

2.4.2. Stability in premixtures and feedingstuffs

The applicant provided data on the stability of ATX (one additive, three batches) in an unspecified premixture (500 mg ATX/kg) for fish feed.[34] The ATX content in the premixtures decreased with storage time (by 2 %, 6 % and 12 % after 4, 8 and 12 months, respectively) at 25 °C and 60 % relative humidity (RH).

[26] Technical dossier/Section II/Annex 2.3.2.a, b and d.
[27] This section has been edited following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003.
[28] Supplementary information/February 2013.
[29] Technical dossier/Section II/Annexes 2.1.3.f and 2.1.3.g.
[30] Supplemetary information December 210/Annex v.a and Annex v.b/Supplementary information/February 2013.
[31] Technical dossier/Section II/Annex 2.4.1.a.
[32] Technical dossier/Section II/Annex 2.4.1.b.
[33] Technical dossier/Section II/Annex 2.4.1.a.
[34] Technical dossier/Section II/ Annex 2.4.1.c.

The stability of three different batches of one additive in a trace element premixture (900 mg/kg) for broiler feed was studied at 25 °C/60 % RH and at 30 °C/70 % RH.[35] ATX was stable under both conditions for a period of 30 weeks (maximum loss 7 % of the initial value).

The applicant provided results on the stability of three different batches of an extruded fish diet supplemented with 100 mg ATX from one additive.[36] A cold water-dispersible formulation was sprayed onto the extrudate. Stability was studied at 5 °C/~70 % RH and 15 °C/~50 % RH. About 97 % of the initial concentration was found after 16 weeks' storage at 5 °C, the corresponding figure at 15 °C being 90 %.

The FEEDAP Panel notes that no stability data were provided for ATX when it was incorporated into feedingstuffs by mixing or during feed processing (pelleting/expansion/extrusion).

2.4.3. Homogeneity

The applicant provided a statistical method (Jansen, 1992) to calculate the capacity of ATX to be homogeneously distributed in feed. The calculations resulted in a coefficient of variation (CV) of 1.1 % for the concentration of ATX in compound feed for salmon. However, this method was developed to test the working accuracy of mixing equipment and is not accepted by the FEEDAP Panel as a valid method for assessing the capacity of the additive to distribute homogeneously in feedingstuffs.

2.5. Conditions of use

The additive is intended to be used in feed for salmon and trout, other fish and crustaceans at up to a maximum content of 100 mg/kg complete feedingstuffs. In addition, it is added to feeds for ornamental fish and ornamental birds, to colour their skin or feathers, at a maximum concentration of 100 mg/kg of complete feedingstuff.

The applicant requests that the current provison, i.e. that a mixture of ATX and canthaxanthin is permitted in additives provided the total concentration of ATX and canthaxanthin from all sources does not exceed 100 mg/kg in complete feedingstuff, is maintained.

2.6. Evaluation of the analytical methods by the European Union Reference Laboratory (EURL)

EFSA has verified the EURL report as it relates to the methods used for the control of astaxanthin in animal feed. The Executive Summary of the EURL report can be found in Appendix A.

3. Safety

3.1. Safety for the target species

3.1.1. Salmonids

As reported in a previous FEEDAP opinion (EFSA, 2005a), no adverse effects of ATX up to 200 mg/kg feed have been recorded for Atlantic salmon throughout a whole production cycle (Torrissen et al., 1995) and in rainbow trout for six weeks (Choubert and Storebakken, 1989).

To further confirm the safety of ATX in salmonids, the applicant submitted a tolerance study in rainbow trout (*Oncorhynchus mykiss*) with ATX-dimethylsuccinate and including also ATX at the maximum use level only.[37] Taking into account that ATX-dimethyldisuccinate is hydrolysed to free

[35] Supplementary information February 2013/Annex Qiv.
[36] Supplementary information February 2013/Annex Qvii.
[37] Supplementary information/October 2013. EFSA received a letter from DSM Nutritional Products Ltd allowing the sharing of the study.

ATX and succinate before or during intestinal absorption and that there were no differences in the availability of the different forms (EFSA, 2007a), the study is taken as relevant for the assessment of the tolerance of ATX.

An eight-week feeding trial was conducted with 180 rainbow trout of 122 g mean initial body weight. Water temperature varied from 14.6 to 16.0 °C during the trial. Water quality was regularly monitored and kept below the critical levels of ammonia and nitrites. Two control groups were used, a negative control receiving unsupplemented feed (C-0) and a positive control (C-100) receiving feed containing 100 mg synthetic ATX/kg feed. Two experimental groups received feed containing 100 mg (E-100) and 1 000 mg (E-1000) ATX equivalents (from ATX-dimethyldisuccinate). Each diet was fed to triplicate tanks (3 × 15 fish). Daily feeding rate decreased during the trial from 1.8 % initially to 1.3–1.4 % body weight at the end of the trial.

The extruded diets contained 40.6 % crude protein and 27.0 % total lipids (both analysed). Dietary ATX and ATX-dimethyldisuccinate were determined at the start and at the end of the trial. The initial levels confirmed the intended values (93, 95 and 908 mg/kg for the groups C-100, E-100, and E-1000, respectively). The ATX losses by the end of the eight-week feeding period amounted to 11, 11 and 19 % for the groups C-100, E-100 and E-1000, respectively.

Survival, body weight, body weight gain, feed conversion ratio and specific growth rate were measured and computed for each replicate. At the end of the feeding period, macroscopic observation was performed on 10 individual fish per replicate tank (a total of 30 fish per treatment). Each fish was examined externally and then dissected for observation of viscera, liver, gall bladder, spleen and muscle. Body weight and length, liver weight, condition factor and liver somatic index were recorded for each sampled fish.

The initial body weight of the fish (122 g) was more than doubled during the course of the study (348 g), which is compliant with the requirements of Regulation (EC) No 429/2008.[38] The administration of ATX-dimethyldisuccinate at levels of 100 and 1 000 mg ATX equivalents/kg did not result in any modification of performance compared with both control groups (no fish died, average specific growth rate (four groups) 1.83 % bw per day, feed to gain ratio 0.81). In all groups, fish were healthy and in good nutritional condition; during necropsy, no gross pathological alterations were observed. The Panel considers that this study, notwithstanding some limitations (lack of haematology and blood chemistry), provides reassurance of the safety of ATX in rainbow trout.

3.1.2. Crustaceans

Yamada et al. (1990) fed prawns (*Penaeus japonicus*) diets supplemented with 0, 50, 100, 200 or 400 mg ATX/kg diet for eight weeks. No negative effects observed on weight gain, survival, daily feed intake, per cent gain or feed to gain ratio were observed in the prawns fed 400 mg/kg compared with the control or other treatment groups.

Niu et al. (2009) examined the effect of ATX supplementation (0, 100, 200 or 400 mg/kg feed) for 30 days on growth, survival and stress tolerance of postlarval shrimp (*Litopenaeus vannamei*). Survival, weight gain and final body weight were significantly higher in the groups fed diets supplemented with 100, 200 or 400 mg/kg than in the control group.

Chen and Shiau (2005) fed kuruma prawn (*Marsupenaeus japonicus*) diets containing 0, 50 or 100 mg/kg ATX for nine weeks. The body weight of prawns tripled during the study (from approximately 0.4 to approximately 1.2 g). The survival rate of prawns treated with ATX was significantly higher than survival in the unsupplemented group (55/53 vs. 37 % $P < 0.05$). No

[38] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implemetation of Regulation 1831/2003 of the European Parliament and 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.

differences in final body weight or on weight gain were observed, although it was numerically higher in ATX-fed prawns (312/342 vs. 281 %).

In the study by Zhang et al. (2013), pacific white shrimp (*Litopenaeus vannamei*, 1 050 individuals in 35 tanks, i.e. 30 shrimp per tank) with an initial mean weight of 1.0 g were fed either a control diet or a diet containing 25, 50, 75, 100, 125 or 150 mg ATX/kg for 56 days. After 56 days of culture, weight gain, specific growth rate and total antioxidant status were higher ($P < 0.05$) and superoxide dismutase and catalase activity were lower ($P < 0.05$) in shrimp fed 125 and 150 mg ATX/kg than in shrimp fed the control diet.

The FEEDAP Panel concludes that ATX is tolerated in crustaceans at up to 400 mg/kg feed. It is therefore concluded that dietary concentrations up to 100 mg ATX/kg feed are safe for crustaceans.

3.1.3. Ornamental birds

No data on the tolerance of ornamental birds to ATX were initially provided by the applicant. Since data on a major poultry species are also unavailable, the applicant was requested to perform a literature search and to provide relevant data which would allow the FEEDAP Panel to conclude on the safety of 100 mg ATX/kg complete feed, the highest proposed dietary concentration. In total, 20 publications were provided, of which only the most relevant ones are described below.[39]

It should be noted that ATX does not occur in the natural habitat of land-living birds. However, several publications describe the occurrence and importance of ATX for the brilliant colour of the plumage of birds. ATX is the dominant carotenoid in the plumage of *Carduelinae* (Stradi et al., 1997) and of wild and captive bullfinch (*Pyrrhula pyrrhula*) (Stradi et al., 2001). In red-winged blackbirds (*Agelaius phoeniceus*) use canthaxanthin and ATX for red colouring of the epaulettes (McGraw et al., 2004). McGraw et al. (2005) noted that ATX is not consumed but derived from other carotenoids in the American goldfinch (*Carduelis tristis*). In addition, McGraw and Hardy (2006) reported on the occurrence of ATX in the plumage of ornamental birds. Egeland et al. (1993) reported a study on capercaillie (*Tetrao urogallus*) with zeaxanthin and lutein and found that zeaxanthin is a precursor of ATX.

Sea birds may consume ATX from their prey. ATX is the major carotenoid in tissues of white storks (*Ciconia ciconia*) fed with red swamp crayfish (Negro and Garrido Fernandez, 2000). In frigate birds (*Fregata minor*), ATX accounts for 85 % of plasma carotenoids (Juola et al., 2008). These studies indicate their natural exposure, but fail to show any quantitative relation.

Toomey and McGraw (2011) reported ATX accumulation in the retina of the house finch (*Carpodacus mexicanus*) after giving 35 mg ATX/L water for drinking for eight weeks. Toomey and McGraw (2010) described the same study but concluded that retinal ATX deposition is independent of dietary ATX and they suggested a specific metabolism of ATX and accumulation. The FEEDAP Panel noted that, to eliminate the potential adverse effect indicated in these studies (compromised visual discrimination) as a consequence of oral administration of 35 mg ATX/L (dose below the proposed maximum feed concentration), further study would be needed.

In summary, the FEEDAP Panel is not in a position to conclude on the safety of ATX at the proposed maximum level of 100 mg/kg complete feed for ornamental birds.

3.1.4. Conclusion on the safety for the target species

The tolerance study indicated that ATX-dimethyldisuccinate was well tolerated by rainbow trout at a dietary level of 908 mg ATX equivalents/kg complete diet. Taking into account the former assessments of ATX (EFSA, 2005a) and ATX-dimethyldisuccinate (EFSA, 2007a), the FEEDAP Panel considers ATX safe for the target species up to the currently authorised maximum dietary

[39] Supplementary information/October 2013.

content for trout and salmon (100 mg ATX/kg complete feed). This conclusion is extrapolated to other fin-fish and ornamental fish at the same dose.

Thus, studies have shown that ATX is tolerated in crustaceans up to 400 mg/kg feed. It is therefore concluded that dietary concentrations up to 100 mg ATX/kg feed are safe for crustaceans.

The FEEDAP Panel could not conclude on the safety of ATX for ornamental birds.

3.2. Safety for the consumer

3.2.1. Absorption, distribution, metabolism, excretion (ADME) and residue studies

3.2.1.1. ADME studies

The metabolism of ATX in fish is described in the opinion of the FEEDAP Panel on the use of ATX in animal nutrition (EFSA, 2005a), based on published literature. The applicant refers to the main papers supporting the conclusions drawn in this opinion, which can be summarised as follows:

i. ATX apparent absorption in fish varies from 20 to 95 %, with most values lying between 50 and 70 %; absorption is determined by several factors, such as fish species, dietary lipid levels and ATX stereochemistry. The geometrical isomer all-E is absorbed more efficiently than the Z isomers, while no difference is observed for the optical isomers, e.g. 3S,3′S, 3R,3′R or 3R,3′S *meso* enantiomers.

ii. [15,15′-3H]-ATX is metabolised in fish mainly through reductive pathways. A double-step reduction at the 4 and 4′-oxo groups initiates a metabolic process leading to idoxanthin and/then to adonixanthin and finally zeaxanthin. No oxidation occurs in fish such as salmonids and therefore the conversion of zeaxanthin to ATX does not occur. ATX has been shown to be a vitamin A precursor for fish, which implies the cleavage of the polyene chain at C15,C15′.

iii. After ATX repeated administration, the pigment deposited in the flesh of trout and Chinook salmon (*Oncorhynchus tshawytscha*) is predominantly ATX (about 95 %); in Arctic charr (*Salvelinus alpinus*), idoxanthin is also deposited in the flesh (20–35 %), the corresponding figures for skin being 85 % ATX and 10 % idoxanthin, both esterified.

3.2.1.2. Residue studies

No new data were supplied by the applicant, which made reference to the FEEDAP Panel opinion (EFSA, 2005a) which concluded that:

i. A dose-related increase in ATX in the flesh of trout and salmon was observed with graded ATX levels in the diet. Since absorption capacity is limited, a plateau is reached in Atlantic salmon at about 10 mg ATX/kg flesh and in trout at a higher level of about 20–25 mg ATX/kg flesh.

ii. The composition of carotenoids deposited in the flesh reflects that of the dietary prey organisms or added carotenoids in terms of ATX stereoisomers; all-E isomers are deposited mainly in flesh, whereas Z isomers are preferentially stored in the liver and kidney.

3.2.2. Toxicological studies

Information was lacking on the isomers ratio of the ATX used in the various toxicological studies. In the absence of this information it is assumed that the isomers are of similar toxicity.

3.2.2.1. Genotoxicity studies including mutagenicity

Crystalline ATX was tested in a reverse mutation assay in *Salmonella* Typhimurium strains TA1535, TA1537, TA98 and TA100 and in *Escherichia coli* strain WP2uvrA.[40] Two independent experiments were performed in the presence and absence of S9-mix (from rat liver induced by a combination of phenobarbital and β-naphthoflavone) according to the OECD Guideline 471. The highest concentration tested (1000 μg/plate) produced precipitation but no cytotoxicity. No increase in the number of revertant colonies was reported under any experimental condition, while the positive controls gave the expected response.

An *in vitro* micronucleus assay[41] was performed with crystalline ATX in cultured peripheral human lymphocytes in the presence and absence of a metabolic activation system (phenobarbital and β-naphthoflavone induced rat liver S9-mix) in compliance with OECD Guideline 487. In a first assay, the compound was tested at concentrations of up to 100 μg/mL with an exposure time of 3 hours and a harvest time of 27 hours in the presence and absence of S9-mix. No statistically significant increase in the number of mono- and binucleated cells with micronuclei was reported. In a second assay, crystalline ATX was tested at concentrations of up to 333 μg/mL with an exposure time of 24 hours and a harvest time of 24 hours in the absence of S9-mix. The test item induced a non-concentration-dependent and statistically non-significant increase in micronuclei in binucleated cells and a significant increase in micronuclei in mononucleated cells. However, the increase observed in mononucleated cells was within the acceptability range of the test. Since the number of micronucleated cells in the second cytogenetic assay was relatively high in all groups (including the solvent control), the experiment was repeated to verify the results. In the repeat experiment, no increased number of micronucleated cells was observed at ATX concentrations up to 100 μg/mL. In all cases the highest concentration was determined by the solubility and produced a reduction of 7–9 % in the cytokinesis-block proliferation index. The positive controls performed as expected.

ATX (all-trans-ATX/rac-ATX with a purity of 96.6 %) was assessed for its potential clastogenic activity *in vitro* as measured by evaluating metaphase chromosomes of human peripheral blood lymphocytes, with and without metabolic activation with S9-mix (fraction from rats previously treated with Aroclor 1254).[42] The study was performed in accordance with to OECD Guideline 473 (rev. 1983). Isolated human lymphocytes were cultivated in the presence of the mitogen phytohaemagglutinin for 24 hours and then exposed to the test item, dissolved in dimethylsulphoxide. A concentration of 120 μg ATX per mL was tested as highest concentration because at this concentration there were still enough well-spread metaphases for evaluation. However, upon dosing, a precipitate formed at all concentrations, owing to the insolubility of ATX at this high concentration. Exposure to 6, 30, 60 and 120 μg/mL did not induce genetic damage in metaphase chromosomes of cultured human lymphocytes, either in the absence or in the presence of S-9 mix. Positive and negative controls gave expected results in these assays. Neither ATX nor its metabolites formed under these conditions were clastogenic at concentrations of up to 120 μg/mL. However, the poor solubility in water of the test article limits the biological significance of the results.

Conclusions on genotoxicity

ATX was negative in a bacterial reverse mutation assay, in an *in vitro* micronucleus test and in an *in vitro* cytogenetic assay. Therefore, the substance is not considered genotoxic.

3.2.2.2. Carcinogenicity study

Groups of 50 rats of each sex were fed a beadlet formulation containing 8 % ATX at dietary levels equivalent to dosages of 0 (untreated control), 0 (placebo control), 40, 200 or 1 000 mg ATX/kg bw

[40] Supplementary information February 2013/Annex Qix.
[41] Supplemetary information/February 2013/Annex Qix.
[42] Supplementary information/February 2013. EFSA received a letter from DSM Nutritional Products Ltd allowing the sharing of the study..

per day for two years. Satellite groups of 10 rats of each sex were treated for only one year, followed by an untreated recovery period of one year. Survival in the groups treated for two years was 76 to 88 % in males and 56 to 82 % in females. Feed consumption was unaffected by ATX exposure. Body weight gain of all animals with the beadlet formulation (with or without ATX) was reduced compared with the untreated controls. Body weight gain of females given ATX (significant at 200 and 1 000 mg/kg bw per day) was lower than in controls, and there was some recovery of body weight in the satellite groups during the recovery phase. There were no treatment-related adverse effects on clinical signs. Haematology showed minor changes in some red blood cell parameters in the groups given 200 or 1 000 mg/kg bw per day for two years: reduced erythrocyte count and packed cell volume and increased MCH and MCHC. Some effects were seen on blood biochemistry parameters in the female groups given 1 000 mg/kg bw/day and only rarely on those given 200 mg/kg bw, including increased plasma levels of cholesterol, bilirubin, alkaline phosphatase, alanine transaminase (ALT) and aspartate aminotransferase (AST). No relevant haematological or biochemical changes were observed in the recover animals after the second year of the study without treatment. A few organ weight variations (e.g. of the heart, brain or spleen) in the placebo- or ATX-treated groups were considered to be due to the lower body weights of treated groups than of the untreated controls. After the two-year treatment period, the treatment-related non-neopolastic changes were confined to the liver. Histopathological findings are summarised in Table 2. In female rats, increased incidences of hepatocellular vacuolation, hepatocellular hypertrophy and multinuclear hepatocytes at all dietary levels of ATX were observed, and there was also a significant increase in the incidence of hepatocellular adenomas at 200 and 1 000 mg ATX/kg bw. The number of females with hepatocellular adenomas in the negative control, placebo control and low-dose, mid-dose and high-dose groups given ATX for two years was 2, 1, 5, 9 and 14, respectively. The increased incidences of hepatocellular adenomas in females were statistically significant at 200 and 1 000 mg/kg bw per day. In males, there were increased incidences of centrilobular vacuolation of hepatocytes at 200 and 1 000 mg/kg bw/day dose levels. No increased incidence of malignant tumours was observed and, apart from the liver adenomas in females, there was no increased incidence of benign tumours.

Table 2: Histopathological findings in the liver of rats (carcinogenicity study). Figures given are numbers of animals out of 50 rats per treatment and sex (49 in the placebo group)

Astaxanthin intended (mg/kg bw)	**0 (control)**		**0 (Placebo)**		**40**		**200**		**1 000**	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Carcinoma	1	1	1	0	0	0	1	0	3	2
Adenoma	3	2	7	1	3	5	5	9	3	14
Yellow-brown pigmentation hepatocytes	0	11	0	13	0	40	1	34	0	34
Yellow-brown pigmentation macrophages	1	13	1	12	1	46	1	49	3	49
Hepatocellular hypertrophy	0	1	1	3	1	21	1	37	2	37
Inflammatory foci	6	5	8	7	1	7	1	17	5	17
Vacuolation [(a)]	8	11	7	5	5	8	9	16	15	32

(a): Periportal, diffuse and centrilobular.

Multinucleated hepatocytes were observed in about 13 control female rats and in 23, 29 and 41 rats treated with 40, 250 and 1 000 mg ATX/kg bw, respectively. In males, only one animal in each of the groups with 40 and 1 000 mg ATX/Kg bw per day showed multinucleated hepatocytes. The increased incidence of multinucleated hepatocytes in females can be considered a response to increased hepatic cell injury and cell deaths as observed by increased single-cell necrosis at the ATX top dose and inflammatory foci at the intermediate and top doses (see Table 2).

The results of the satellite group (rats treated for 53 weeks followed by a 51-week treatment-free recovery period) showed no treatment-related adverse effects.

As histopathological changes were seen in the livers of female rats at all tested doses of ATX (40 mg/kg bw per day or more), it was not possible to identify a NOAEL for this study.

The benchmark dose (BMD) approach was applied (EFSA, 2009) to analyse the incidence of liver adenoma and liver hypertrophy in female rats (placebo control and three treated groups, Appendix B). It must be noted that the design of the study (three ATX doses only, ATX dosing) was not optimal for the BMD approach.

For liver adenomas (Appendix B2), four quantal models and four continuous models were accepted considering their fitness by the log-likelihood. The lowest $BMDL_{10}$ values identified by the quantal models were in the range 1.1 to 3.5. However, the uncertainty of this estimate is very high, and the ratio of BMD/BMDL ranges from 17.0 to 56.5. Therefore, none of the $BMDL_{10}$ values could be retained. The continuous models H4 and E4 resulted in a $BMDL_{10}$ of 15.7 and 22.1, respectively. The estimates of the continuous models resulted in a less conservative $BMDL_{10}$ and a lower level of uncertainty than the quantal models. However, the ratio of BMD/BMDL (4.1 to 5.2) is still above the currently accepted maximum value of 2 (Muri et al., 2009).

For liver hypertrophy (Appendix B3), two quantal equations are accepted by the log-likelihood, but are not considered acceptable because the BMD/BMDL ratio is about 300. The same criteria led to the exclusion of the continuous model H3 (BMD/BMDL of 1 000). Two estimates remained, both of which were from the continuous models (E4 and H4). Model E4, with a $BMDL_{10}$ of 10.0, shows the lowest uncertainty (BMD/BMDL ratio of 1.4), whereas model H4 results in the most conservative $BMDL_{10}$ of 3.4 with a somewhat higher uncertainty (BMD/BMDL ratio of 2.0).

3.2.2.3. Reproduction toxicity including developmental toxicity

A good laboratory practice (GLP)-compliant study was performed in Fü-Albino strain rats using rac-ATX.[43] Groups of 32 males and 32 females were given oral gavage doses of 0, 25, 100 or 400 mg/kg bw per day. Males were dosed 70 days prior to and during the mating period; females were dosed 14 days prior to mating and during mating, gestation and lactation. There was no effect on parental mortality or clinical signs, but there was reduced body weight gain among males in the top-dose group. The treatment had no effect on mating performance, mating success or numbers of corpora lutea, implantation sites, viable fetuses, intrauterine deaths, resorptions and pups born alive. At the highest dose, there was an increased proportion of F1 pups dying during the lactation period, with the increase being marginally greater than the historical control value. No treatment-related effects were found upon visceral examination of F1 pups. There were no treatment-related effects on the physical and functional development or learning and memory (water-E-maze) of the F1 pups. Upon mating the F1 rats, there was no effect on pregnancy rate or numbers of implantations and resorptions. It is concluded that, apart from a marginal increase in pup mortality, no adverse effects on reproduction occurred in this study. The NOAEL is 100 mg/kg bw per day, based on reduced body weight gain in males and increased pup mortality during the lactation period at 400 mg/kg bw per day.

Groups of 20 pregnant rabbits were given oral gavage doses of crystalline ATX suspended in rapeseed oil at dosages of 0, 100, 200 and 400 mg ATX/kg bw per day from day 7 to day 19 of gestation.[44] There was no effect on maternal body weight gain or signs of maternal toxicity. Reproductive and litter parameters were unaffected by the treatments. In the high-dose group, the incidence of resorptions (38 %) was higher than in the other groups, but the incidence was not statistically different from that in the controls (33 %). There was no evidence of any embryotoxicity, fetotoxicity or teratogenicity at any dose. The NOAEL for this study is the highest dose level: 400 mg/kg bw per day.

[43] Supplementary information February 2013. EFSA received a letter from DSM Nutritional Products Ltd allowing the sharing of the study.

[44] Supplementary information February 2013. EFSA received a letter from DSM Nutritional Products Ltd allowing the sharing of the study.

3.2.2.4. Conclusion

In the two-year rat study, hepatocellular hypertrophy appeared to be dose related in females from 40 mg ATX/kg bw onwards (6, 42, 74 and 74 % in the placebo, 40, 200 and 1 000 mg ATX/kg bw group, respectively). An increase in the incidence of hepatocellular adenomas was observed in female rats at concentrations of 40 mg ATX/kg bw onwards, but not in male rats. In this study there was a numerically higher incidence of hepatocellular carcinoma in both sexes in the top-dose group (n = 5) than in the placebo control group (n = 1). Potential carcinogenicity of the ATX preparation cannot be fully excluded. Considering the absence of genotoxicity of ATX, it is likely that a threshold for the identified tumorigenicity exists, which in principle allows the setting of a NOAEL.

Since a NOAEL could not be derived from the carcinogenicity study with rats, the $BMDL_{10}$ was taken instead. The risk of the occurrence of liver adenomas in female rats could not be satisfactorily estimated by the BMD approach, since the models acceptable by a comparison of log-likelihood showed a high uncertainty. The $BMDL_{10}$ estimates for the model with the lowest uncertainty were between 15.7 and 22.1 mg ATX/kg bw per day. $BMDL_{10}$ estimates with an acceptable uncertainty were obtained for hepatocellular hypertrophy by two continuous models, the higher $BMDL_{10}$ (10.0 mg ATX/kg bw per day) showing the lowest level of uncertainty; a somewhat higher uncertainty is connected with a more conservative $BMDL_{10}$ (3.4 mg ATX/kg bw per day).

3.2.3. Assessment of consumer safety

3.2.3.1. Proposal for an Acceptable Daily Intake

Taking into account (1) the wide range of $BMDL_{10}$ estimates obtained by different model equations for two different endpoints, (2) the fact that preparations containing about 8 % ATX were used as test items instead of pure ATX and (3) the associated uncertainties, the data are regarded as sufficient to derive an ADI.

The ADI is 0.034 mg ATX/kg bw per day (corresponding to 2.0 mg ATX per day for a 60 kg person), derived from the lowest $BMDL_{10}$ calculated for hepatocellular hypertrophy in female rats, applying a safety factor of 100.

3.2.3.2. Consumer exposure

The bioavailability of natural ATX from wild Pacific *Oncorhynchus* spp. and farmed Atlantic salmon (*Salmo salar*) was compared by Rüfer et al. (2008) in a double-blind protocol performed in 28 healthy non-smoking men aged 29–40 years. During the intervention, subjects were told to consume 250 g wild or farmed salmon (containing 5 mg ATX/g salmon flesh) daily as part of their main meal (lunch or dinner) for four weeks. The method of preparation of the salmon was left to the participants' choice. On days 3, 6, 10 and 14 the ATX concentration in plasma was significantly greater after ingestion of farmed salmon. The authors discussed the different lipid contents of the two salmon sources (6.5 % in wild salmon, 17.3 % in farmed salmon) and their potentially different fatty acid profiles (not analysed) as reasons for the findings. They considered the lipid content of the least fatty fish (wild salmon) as already too high to influence carotenoid absorption. The authors continued: "*It is noteworthy that after 28 days of oral intake of wild and cultured salmon, respectively, no significant differences between the plasma ATX concentrations are observable*". It is likely that a certain duration of exposure is required until a steady state of absorption, metabolism, distribution and/or elimination is reached. The composition of the diet was not reported, so the role of dietary lipids could not be considered. The ATX isomer pattern in human plasma was similar to that of the ingested salmon, which reflects the source of ATX in the feed.

In a previous assessment (EFSA, 2005a) it was stated that ATX occurs also in wild fish (salmonids) and its concentration in flesh does not essentially differ from that of farmed fish. The FEEDAP Panel concluded that the study by Rüfer et al. (2008) confirms the previous statement of the FEEDAP Panel. The substitution of wild catch by farmed fish would not alter the quantitative chronic exposure of consumers to ATX.

The consumer exposure calculated using the conservative food basket set by Regulation (EC) No 429/2008 (300 g flesh/person per day) would lead to a daily intake of 3 mg ATX from salmon consumption and 7.5 mg ATX from trout consumption. Considering the proportion of salmon to trout in the daily food basket derived from European production figures (EFSA, 2005a; i.e. 2:1), the resulting exposure from salmonid consumption would be 4.5 mg ATX/person per day.

The EFSA Comprehensive Food Database (EFSA, 2011) allows a refined estimation of the 95th percentile fish consumption based on fish consumers only. This figure is 125 g per day and amounts to, applying the same proportion for salmon and trout as above, 83 g salmon flesh and 42 g trout flesh. The corresponding daily ATX exposure would be about 1.9 mg ATX, which is 94 % of the ADI.

Setting maximum residue limits (MRLs) for ATX in flesh is not necessary because the concentration in the flesh is limited, reaching a plateau at around the maximum dose proposed for use (see section 3.2.1.1), and consumer exposure is unlikely to exceed the ADI.

3.2.4. Conclusions on consumer safety

The use of ATX in the nutrition of salmonids up to the maximum permitted dietary level for salmon and trout is of no concern for the safety of the consumer.

3.3. Safety for the user

Some formulations were dusty, containing fine particles that can reach all parts of the respiratory tract when inhaled. No information was provided on respiratory toxicity so it is prudent to regard ATX-containing additives as being potentially hazardous by inhalation. In the absence of any information on irritancy to skin or eyes or on skin sensitisation, ATX-containing additives should be regarded as hazardous by exposure to skin or eyes.

3.4. Safety for the environment

Synthetic ATX differs from natural ATX by the proportion of stereo-isomers only. According to EFSA guidance on environmental risk assessment (EFSA, 2008) the use of natural additives is permitted when the application of 100 mg ATX/kg fish feed does not result in a substantial increase in the concentration in the environment. The term environment in this instance refers to the farming environment since the FEEDAP Panel evaluates the effects of additives and not the effects of farming on the environment.

The amount of synthetic ATX in salmon faeces can be calculated using the formula in EFSA guidance on environmental risk assessment (EFSA, 2008) as follows: $PC_{faeces} = 100 \times 15.1 = 1510$ mg/kg carbon in faeces. In sea farms, salmon are grown packed together in a confined space. The high density of salmon in the cages results in a high deposition of salmon faeces on the seabed sediment. To assess the risk to benthic organisms in this sediment, the Predicted Environmental Concentration in sediment ($PEC_{sediment}$) was calculated according to EFSA guidance on environmental risk assessment (EFSA, 2008). This calculation assumes that 100 % of the compound precipitates on the sediment. The calculation does not use substance-dependent parameters but is dependent only on the dose. The resulting concentration in the sediment is 21 198 µg/kg dry matter, which is much higher than the trigger value of 10 µg/kg dry matter. A similar dose of natural astaxanthin can be assumed to lead to a similar concentration of astaxanthin in the sediment.

Considering the above results and the expectation that a considerable percentage of ATX is excreted via faeces, a Phase II environmental risk assessment for marine sediment under fish farms would be required.

Astaxanthin in the environment is synthesised by algae. Algae contain up to 3 400 mg/kg carbon (Snoeijs, 2014). In unfiltered sea water, natural astaxanthin is present in the range of 0.37–36 ng/L (Snoeijs, 2014). Shrimp can contain natural astaxanthin in the range of 50–165 mg/kg total dry weight (Wipavee et al., 2012). Natural astaxanthin accumulates in wild salmon via the food chain.

In aquaculture operations, involving the use of sea cages, benthic organisms are considered to be most at risk. For salmon in cages to develop a red colour similar to wild salmon, they must receive a similar dose of ATX. If it would be possible to give salmon in cages the same feed as wild salmon the natural astaxanthin in the sediment would be the same.

Therefore, the FEEDAP Panel considers that the use of synthetic ATX (100 mg ATX/kg fish feed) does not pose a significant additional risk to the environment compared with natural astaxanthin.

4. Efficacy

The characteristic red/pink colour of salmon flesh is perceived by the consumer as one of the most important quality criteria. The market value of ornamental fish, such as koi carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and ornamental birds is highly dependent on skin or feather pigmentation.

Carotenoid deposition in skin, flesh and shell results in colourisation of these tissues. Therefore, both endpoints, tissue colour and pigment concentration in the tissue, are considered equally suitable indicators of colouring efficacy.

Pigment concentration is usually measured either by high-performance liquid chromatography (HPLC) or near-infrared spectroscopy (NIR) to quantify the ATX concentration in the fish fillet. Pigmentation efficiency of the product is assessed by the coloration of salmonid fillets, which is typically measured either visually using the DSM SalmoFan or by light reflectance colorimetry.

4.1. Salmonid

The applicant referred to an EFSA opinion (EFSA, 2005a) and a report from the Scientific Committee of Animal Nutrition (SCAN) (EC, 1989) to support the long-term efficacy of ATX in salmonids. One publication (Torrissen et al., 1995), describing a study on Atlantic salmon (*Salmo salar*) already cited in EFSA opinion (EFSA, 2005a), is reported in more detail.

4.1.1. Atlantic salmon

Eight experimental diets containing 10, 20, 40, 60, 80, 100, 150 or 200 mg ATX/kg feed (analytically confirmed) were fed to an initial 260 fish per sea cage, each for 21, 18, 15, 12, 9, 6 and 3 months (Torrissen et al., 1995). A control stock (19 500 salmon) received unsupplemented basal diet (1.5 mg ATX/kg). Additional fish, individually labelled, were transferred from the control stock to the main experimental groups every third month. At the time of each transfer, 10 fish from each dose and time group were sampled and the ATX concentration in flesh determined. Average fish weight at the beginning of the study was 115 g, and at termination was 3.2 kg. Feeding ATX at up to a dietary concentration of 60 mg/kg led to a dose-dependent increase in ATX in muscle tissue, no further increase being observed with higher levels. After 21 months of feeding, 9.5 mg ATX/kg muscle tissue was not exceeded. Multilinear regression showed that dietary ATX concentration was the most powerful factor influencing ATX deposition in flesh, followed by feeding time, final weight of the fish and the amount of lipids in flesh. However, ATX in flesh is bound to actomyosin and increased fat in muscle would dilute flesh ATX.

The same group (Torrisen and Christensen, 1995) described in a different publication an earlier experiment. Five extruded diets containing 0 (analysed 2), 12.5 (analysed 15), 25 (analysed 25), 37.5 (analysed 40) and 50 (analysed 55) mg ATX/kg were each fed for seven months to 250 fish per sea cage. The initial mean weight of the fish (pre-feeding with commercial diets) was 720 g and the final mean body weight was 2.7 kg. The initial ATX concentration in muscle was 1.9 mg ATX/kg. Ten fish per group were sampled every month during the experimental period for carotenoid analyses. By the end of the feeding period, the ATX concentration in the flesh of the control salmon was reduced to 1 mg/kg. After the seven-month feeding period there was a significant correlation between the ATX concentration in the feed and the muscle tissue (fillet), with the highest observed level in the group fed 50 mg ATX/kg diet (approximately 5.5 mg ATX/kg flesh as a mean). Average ATX retention was

calculated to be 27 % for the control group, and a decrease was observed with increasing dietary ATX down to < 10 % at 55 mg ATX/kg diet.

4.1.2. Rainbow trout

Torrisen and Christensen (1995) fed two experimental diets, a control (analysed 4 mg ATX/kg) and an experimental (intended 40 mg ATX/kg, analysed 32 mg ATX/kg), for three months to rainbow trout (*Oncorhynchus mykiss*) of approximately 250 g body weight (range 120–395 g). Group size was two replicates (circular glass fibre tanks with 40 trout each) for the control and four replicates for the experimental group. Water temperature was about 9 °C. Twenty fish were sampled for carotenoid analyses at start and 20 fish per tank at the end of the study. Final body weight was 810 g per fish. The initial ATX concentration in muscle tissue of the control group remained unchanged during the experimental period (3.6 mg ATX/kg) resulting in an ATX retention of 50 %. In the experimental group, the final concentration was 11.7 mg ATX/kg flesh, resulting in an ATX retention of about 21 %.

4.2. Other fish

Tejera et al. (2007) fed red porgy (*Pagrus pagrus*) (starting weight 5 g) a basal diet or a diet supplemented with 25 or 50 mg/kg ATX (analysed values 27.1 and 68.1 mg/kg) (3 replicates of 50 fish per treatment) for four months. Total ATX concentration in skin of fish at four months was significantly increased by ATX supplementation from 2.8 mg/kg in the control group to 29.1 and 31.6 mg/kg in the groups supplemented with 25 and 50 mg/kg respectively ($P < 0.05$). The effect on skin coloration was evidenced by pictures showing that fish from the control group were pale silver while those from the ATX-treated groups had a pink-reddish coloration.

4.3. Crustaceans

Yamada et al. (1990) fed prawns (*Penaeus japonicus*) with diets supplemented with 0, 50, 100, 200 or 400 mg/kg for eight weeks. Viscera-free body ATX ester concentrations after 8 weeks were significantly and dose-dependently increased in prawns fed 50, 100 or 200 mg/kg feed compared with the control group. There was no significant difference in ATX ester concentrations in the prawns fed 200 mg/kg compared with those fed 400 mg/kg feed. The other studies submitted by the applicant supported the pigmentation efficacy of ATX in prawns when supplemented at 50 or 100 mg ATX/kg diet (Nègre-Sadargues et al., 1993) and in black tiger prawn larvae (*Penaeus monodon*) when given ATX as constituents of natural feed (Pan and Chien, 2003). The study by Niu et al. (2009) showed increased survival of postlarval shrimp (*Litopenaeus vannamei*) when fed for 30 days with diets supplemented with 200 or 400 mg ATX/kg compared with a control group. Considering the effect of ATX on both growth performance and survival of postlarval shrimp, the authors recommend an ATX supplementation level of between 100 and 200 mg/kg of diet.

Chen and Shiau (2005) fed kuruma prawn (*Marsupenaeus japonicus*) diets supplemented with 0, 50 or 100 mg/kg ATX for nine weeks. ATX deposition in flesh and shell was significantly higher in both treated groups than in the control group, with no significant differences between ATX supplementation levels (flesh: 55 vs. 157 and 199 mg/kg DM; shell: 122, 472 and 610 mg/kg DM for 0, 50 and 100 mg/kg feed, respectively).

Three experimental diets containing β-carotene from carrots, synthetic canthaxanthin and ATX were fed to hermit crabs (*Clibanarius erythropus*) for two complete moulting cycles (Castillo and Nègre-Sadargues, 1995). The feed concentration of each pigment was 200 mg/kg. The crabs had been previously depigmented by feeding a carotenoid-free diet for three consecutive moulting cycles. At the end of the study, the pigmented pattern of animals receiving dietary β-carotene during two moulting cycles was not fundamentally different to that of individuals lacking carotenoids. In addition, the abdominal region of animals in the canthaxanthin and ATX groups appeared ‘coloured’ at the end of the second moulting cycle. The carotenoid content of the epidermis increased from 156 mg/kg dry weight to 1 184 mg/kg in the ATX group.

In the study of Zhang et al. (2013) (see section 3.1.2), the astaxanthin content of the shell of Pacific white shrimp (*Litopenaeus vannamei*) fed ATX levels of 25 to 150 mg/kg was significantly higher than that of a control group. No significantly increased differences in ATX contents were observed at levels above 50 mg/kg.

4.4. Ornamental fish

The effect of ATX (0, 25, 50, 75 or 100 mg/kg feed) on skin pigmentation in goldfish (*Carassius auratus*) was studied for four weeks in triplicate groups of 30 fish (initial body weight ~ 10 g) per tank (Paripatananout et al., 1999). Survival of fish was significantly lower in the control group than in the ATX-treated group. The pigmentation scores and chromatophore counts are given in Table 3. Dietary ATX at levels of 25 and 50 mg/kg improved significantly skin pigmentation of goldfish. Higher doses (75 and 100 mg ATX/kg) did not exert an additional effect.

Table 3: Effect of dietary astaxanthin on pigmentation in goldfish (four-week data)

	ATX (mg/kg feed)				
	0	**25**	**50**	**75**	**100**
Pigmentation score (1)	3.4[a]	5.4[b]	6.5[c]	6.1[c]	6.6[c]
Chromatophores (cells/field) (2)	3.0[a]	4.7[b]	5.4[c]	5.7[c]	6.0[c]

(1): By use of a colour chart rating from 0 (yellow) to 9 (red).
(2): Chromatophores were counted using a light microscope at three locations per microscope slide.
[a,b,c] Values within one row with different superscript are different ($P < 0.05$).

The effect of ATX on skin pigmentation in goldfish was also examined by Gouveia and Rema (2005).Duplicate groups of fish with a mean initial weight of 7.4 g were fed diets containing 45, 80 or 120 mg ATX/kg for five weeks. Adequate skin pigmentation (measured as total carotenoids) was achieved at the lowest concentration tested, and there was no significant difference in skin pigmentation among the ATX groups.

Pan and Chien (2009) examined the effects of dietary supplementation of ATX on pigmentation in red devil fish (*Cichlasoma citrenellum*). Groups of five fish (mean initial weight 8.8 g) were fed diets containing 0, 80 or 160 mg ATX/kg (analytically confirmed) for eight weeks in triplicate tanks. Final body weight was approximately 40 g. The fish fed ATX-containing diets had higher ATX levels in skin, muscle and fins than fish in the control group, but the ATX concentrations in liver, intestine and gonads were similar in both control and supplemented groups. Fish fed 160 mg ATX/kg had significantly higher ATX levels in skin than fish fed 80 mg ATX/kg.

Baron et al. (2008) studied the effect of synthetic ATX on the pigmentation of male flame-red dwarf gourami (*Colisa lalia*). There were three replicates of 10 fish per treatment. Fish were fed either an unsupplemented diet or the same diet supplemented with 100 mg/kg ATX (not analytically confirmed) for 12 weeks. Skin coloration was measured by means of light reflectance. Fish fed ATX-containing diets had a higher body redness value (a) and a lower lightness (L) than the control after 10 weeks of feeding. The effect on caudal redness was evident after eight weeks' supplementation.

The effect of dietary supplementation of ATX on pigmentation of characins (*Hyphessobrycon callistus*) was examined by Wang et al. (2006). Groups of 30 fish (mean initial weight 0.41 g) were fed diets containing 0, 10, 20 or 40 mg ATX/kg (analytically confirmed) for eight weeks in triplicate tanks. Total body ATX responded significantly to increasing levels of dietary ATX. However, this study was not considered further because skin pigmentation was not measured.

4.5. Ornamental birds

Three studies (Inouye et al., 2001; McGraw et al. 2004; McGraw and Hardy, 2006) reported the occurrence of ATX in the plumage of ornamental birds. No relationship to oral intake was described. Two of the articles noted endogenous synthesis of ATX from other carotenoids.

4.6. Conclusions on efficacy

Astaxanthin is efficacious in colouring the flesh of salmonids and the epidermis of crustaceans. ATX is efficacious in pigmenting the flesh of food-producing fish other than salmonids and the skin of ornamental fish.

In the absence of data, no conclusion can be made on the efficacy of oral ATX in pigmenting the plumage of ornamental birds.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

The FEEDAP Panel considers synthetic astaxanthin safe for salmonids at concentrations of up to 100 mg/kg complete diet. The conclusion on the safety of astaxanthin for salmonids can be extrapolated to other fish and ornamental fish at the same dose. Dietary concentrations up to 100 mg astaxanthin/kg feed are safe for crustaceans. The FEEDAP Panel could not conclude on the safety of astaxanthin for ornamental birds.

Based on a $BMDL_{10}$ of 3.4 mg/kg bw per day (calculated for liver hypertrophy in female rat in a carcinogenicity study) and applying an uncertainty factor of 100, it is possible to set an ADI of 0.034 mg ATX/kg bw (equivalent to 2.0 mg ATX per 60 kg person per day). The use of astaxanthin up to the maximum permitted dietary level for salmon and trout is of no concern for the safety of the consumer.

As some formulations of astaxanthin may be dusty and in the absence of data on inhalation toxicity, it is prudent to regard astaxanthin-containing additives as being potentially hazardous by inhalation. In the absence of any information on irritancy to skin or eyes or on skin sensitisation, astaxanthin-containing additives should be regarded as hazardous by exposure to skin or eyes.

The FEEDAP Panel considers that the use of synthetic ATX (100 mg ATX/kg fish feed) does not pose a significant additional risk to the environment compared with natural astaxanthin.

Astaxanthin is efficacious in colouring the flesh of salmonids and the epidermis of crustaceans. Astaxanthin is efficacious in pigmenting the flesh of food-producing fish other than salmonids and the skin of ornamental fish. No conclusion can be made on the efficacy of oral astaxanthin in pigmenting the plumage of ornamental birds.

RECOMMENDATIONS

Considering the sensitivity of ATX to heat, light and oxygen, it is recommended that "ATX stabilised" rather than the generic additive be included in the feed additive register.

The "chemical composition" of ATX should be described as enantiomeric composition: 25 % 3S,3′S, 50 % 3R, 3′S and 25 % 3R,3′R, which characterises the synthetic product.

The sum of specified substances should be 100 %.

Residues of organic solvents in "ATX stabilised" should follow the limits set in the VICH guideline.

A specification for TPPO (maximum 100 mg/kg additive) should be set and monitored.

DOCUMENTATION PROVIDED TO EFSA

1. Astaxanthin (a sensory additive in feed for salmon and trout, other fish, ornamental fish, crustaceans, ornamental birds) dossier. December 2009. Submitted by CARAC EEIG Carotenoids Authorisation Consortium.

2. Astaxanthin (a sensory additive in feed for salmon and trout, other fish, ornamental fish, crustaceans, ornamental birds) dossier. Supplementary information. December 2010. Submitted by CARAC EEIG Carotenoids Authorisation Consortium.

3. Astaxanthin (a sensory additive in feed for salmon and trout, other fish, ornamental fish, crustaceans, ornamental birds) dossier. Supplementary information. February 2013. Submitted by CARAC EEIG Carotenoids Authorisation Consortium.

4. Astaxanthin (a sensory additive in feed for salmon and trout, other fish, ornamental fish, crustaceans, ornamental birds) dossier. Supplementary information. October 2013. Submitted by CARAC EEIG Carotenoids Authorisation Consortium.

5. Evaluation report of the European Union Laboratory for Feed Additives on the methods(s) of analysis for astaxanthin.

6. Comments from Member States received through the ScienceNet.

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APPENDICES

APPENDIX A

Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for astaxanthin[45]

Astaxanthin is a *feed additive* for which authorisation is sought under the category "sensory additives", functional group 2(a) "colourants", sub-classification (ii) "substances which, when fed to animals, add colours to food of animal origin", (iii) "substances which favourably affect the colour of ornamental fish or birds" according to Annex I of Regulation (EC) No 1831/2003. In the current application submitted according to Article 4(1) (new use in water) and Article 10(2) (re-evaluation of additives already authorised under Directive 70/524/EC) of Regulation (EC) No 1831/2003, authorisation is requested for salmon and trout, ornamental fish and birds, crustaceans and other fish.

The active ingredient and the additive for registration is *Astaxanthin*, produced by a synthetic process and marketed in a stabilised form, e.g. with a spray-dried coating material (i.e. carbohydrates, protein).

The applicant proposed a maximum *Astaxanthin* concentration of 100 mg/kg *feedingstuffs* for salmon and trout and for minor species (crustaceans and other fish). However, no maximum content was proposed for pets (ornamental fish and birds). No minimum contents were proposed by the applicant.

The *Astaxanthin* concentration in *premixtures* and in *feedingstuffs* corresponds to the sum of geometrical *Astaxanthin* isomers detected, namely (1) all-E *Astaxanthin*, (2) 9Z *Astaxanthin*, (3) 13Z *Astaxanthin* and (4) other non-identified Z isomer(s). Here the E/Z-isomers notation is used instead of the terms trans/cis.

Furthermore, the presence of *Canthaxanthin* with *Astaxanthin* in completed *feedingstuffs* is allowed for salmon and trout with a maximum concentration of the sum of both substances of 100 mg/kg.

For the determination of the purity of the *crystalline Astaxanthin (feed additive)*, the applicant proposed a spectrophotometric method measuring at 486–487 nm, and a Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) using a visible detector with a wavelength measuring at 474 nm. The CRL considers the two methods submitted by the applicant suitable for intended purposes.

For the determination of *Astaxanthin* in the *premixtures* and *feedingstuffs*, the applicant proposed a ring-trial validated chromatographic method, based on Normal-Phase High-Performance Liquid Chromatography (NP-HPLC) using a visible detector at 470 nm. The following performance characteristics were reported:

For *premixtures* containing Astaxanthin at 4500 mg/kg:

- a relative standard deviation for *reproducibility* (RSD_R) of 10.2%.

For *feedingstuffs*, in the concentration ranging of Astaxanthin from 20 to 80 mg/kg:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 1.5 to 3.2%,
- a relative standard deviation for reproducibility (RSDR) ranging from 3.5 to 12.6 %
- a recovery rate (RRec) ranging from 98 to 102 %

[45] The full report is available on the EURL website: http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/FinRep-FAD-2009-0054.pdf.

- a limit of detection (LOD) and quantification (LOQ) of 0.005 and 0.01 mg/kg *feedingstuffs, respectively.*

Based on the acceptable performance characteristics presented, the CRL recommends for official control - in the frame of this authorisation - the ring-trial validated methods submitted by the applicant for the determination of *Astaxanthin* in *premixtures* and *feedingstuffs.*

Upon request of CRL, the applicant submitted experimental data proving that the ring-trial validated spectrophotometry method for the determination of *Astaxanthin* in the powdery or water dispersible formulations is also applicable for the determination of *Astaxanthin* in *water*. The target values were ranging from 30 to 100 mg/kg of *Astaxanthin* in drinking water. The following performance characteristics were reported: - RSD_r ranging from 0.45 to 1.10%; - RSD_R ranging from 1.0 to 3.3%; - R_{Rec} = 99.9%.

Based on the acceptable performance characteristics presented, the CRL recommends for official control - in the frame of this authorisation - the ring-trial validated methods submitted by the applicant for the determination of *Astaxanthin* in *water*.

For the determination of *Canthaxanthin* in *feedingstuffs,* the applicant submitted, upon request from the CRL, a single laboratory validated method, similar to the chromatographic method for *Astaxanthin* in *premixtures* and *feedingstuffs,* with slight modification of chromatographic conditions. The following performance characteristics for *feedingstuffs,* in the concentration ranging from 5 to 1000 mg/kg, were reported: - RSD_r ranging from 1 to 7%; - R_{Rec} ranging from 101 to 103%; and – LOD = 0.003 mg/kg.

Based on the acceptable performance characteristics presented, the CRL recommends for official control - in the frame of this authorisation - the ring-trial validated methods submitted by the applicant for the determination of *Canthaxanthin* in *feedingstuffs*.

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.

APPENDIX B

Application of benchmark dose (BMD) analysis to selected endpoints from a two-year carcinogenicity study in rats

The benchmark dose modelling has been used to estimate the benchmark dose (BMD) and the lower benchmark dose (BMDL) for astaxanthin in a two-year carcinogenicity study for the following endpoints: hepatocellular adenomas and liver hypertrophy.

APPENDIX B1

Material and methods

According to the EFSA Scientific Committee opinion on the use of the benchmark dose approach in risk assessment (2009), two softwares are recommended: the BMD software developed by the US EPA (www.epa.gov/ncea), or the PROAST software developed by RIVM (www.rivm.nl/proast). With the current dataset, both softwares (PROAST version 40.7 and BMDS version 2.4) were run and similar results were obtained.

PROAST consists of five nested models (No 1–5) with increasing complexity. The results obtained with PROAST version 40.7 are reported here.

The dose–response models fitted are those listed in the EFSA guidance document (EFSA, 2009) as the recommended models for use in the BMD approach.[46]

[46] In epidemiology, additional models, e.g. y = a + bx, are also used.

Model	Number of model parameters	Model expression response (y) as function of dose (x)	Constraints
Continuous data			
Exponential family			
Model 1 [(a)]	1	$y = a$	$a > 0$
Model 2	2	$y = a \exp(bx)$	$a > 0$
Model 3	3	$y = a \exp(bx^d)$	$a > 0, d > 1$
Model 4	3	$y = a [c – (c-1)\exp(– bx)]$	$a > 0, b > 0, c > 0$
Model 5	4	$y = a [c – (c-1)\exp(– bx^d)]$	$a > 0, b > 0, c > 0, d > 1$
Hill family			
Model 2	2	$y = a [1 – x/(b + x)]$	$a > 0$
Model 3	3	$y = a [1 – x^d/(b^d + x^d)]$	$a > 0, d > 1$
Model 4	3	$y = a [1 + (c-1)x/(b + x)]$	$a > 0, b > 0, c > 0$
Model 5	4	$y = a [1 + (c-1)x^d/(b^d + x^d)]$	$a > 0, b > 0, c > 0, d > 1$
Quantal data [(b)]			
Logistic	2	$y = 1/(1 + \exp(– a – bx))$	$b > 0$
Probit	2	$y = \text{CumNorm}(a + bx)$	$b > 0$
Log-logistic	3	$y = a + (1 – a)/(1 + \exp(–\log(x/b)/c))$	$0 \leq a \leq 1$, $b > 0$, $c > 1$
Log-probit	3	$y = a + (1 – a)\ \text{CumNorm}(\log(x/b)/c)$	$0 \leq a \leq 1$, $b > 0$, $c > 0$
Weibull	3	$y = a + (1 – a) \exp((x/b)^c)$	$0 \leq a \leq 1$, $b > 0$, $c > 1$
Gamma	3	$y = a + (1 – a)\ \text{CumGam}(bx^c)$	$0 \leq a \leq 1$, $b > 0$, $c > 1$
Linearized multistage (LMS) family [(c)]			
One-stage	2	$y = a + (1 – a) \exp(– bx)$	$a > 0, b > 0$
Two-stage	3	$y = a + (1 – a) \exp(– bx – cx^2)$	$a > 0, b > 0, c > 0$
Three-stage	4	$y = a + (1 – a) \exp(– bx – cx^2 – dx^3)$	$a > 0, b > 0, c > 0, d > 0$

(a): Model 1 can be regarded as a model that is nested within any dose-response model: it reflects the situation of no dose-response (= horizontal line).
(b): For the constraints given here, the models result in increasing dose-response curves.
(c): The one-stage model is identical to the quantal linear model as implemented in BMDS; note that in BMDS, this model is called "multistage" and the number of stages has to be defined by setting the degree of the polynomial in this model, e.g. 2 for a two-stage model.
a, b, c, d, unknown parameters that are estimated by fitting the model to the data.
CumNorm, cumulative (standard) normal distribution function.
CumGam, cumulative Gamma distribution function.

For quantal data, a benchmark dose response (BMR) of 10 % extra risk is used and the BMD_{10} and its 95 % lower confidence limit $BMDL_{10}$ were calculated.

The outputs of the analysis were checked and the assessment of the results focused only on the accepted models. The following parameters were taking into consideration: the log-likelihood and the Akaike's Information Criterion (AIC) (EFSA, 2009). In addition, the BMD/BMDL ratios were also considered as an indication of the uncertainty of the data (the higher the BMD/BMDL ratio, the higher the uncertainty (Muri et al., 2009). A ratio of < 2 indicates acceptable uncertainty. Considering the above, for each endpoint analysed, a $BMDL_{10}$ has been selected, if possible.

It should also be noted that the data under assessment are quantal data. EFSA's Scientific Committee Opinion (2009) recommended the use of different sets of models depending on the data type (quantal or continuous), with Hill and Exponential family models suited for continuous data. Considering the

flexibility of such models (Hill and Exponential), they have also been used here to model quantal data. This is possible owing to the procedure used to fit the models when using PROAST 40.7, which constructs the likelihood function based on the distribution specified (in this case, binomial for quantal data), and the probability that characterises the distribution is then modelled using different model expressions, which are those listed above. In principle, there is no restriction of which model expression to use. Each model expression is linked to potential interpretation, which could be lost when using different models as the one proposed for each data type, but if the purpose is purely to fit a dose–response model and to estimate BMD and BMDL, there is no need for interpretation of the parameters in the dose–response model. One requirement that should be fulfilled by the model expression is for the estimated probability to be bounded between 0 and 1, which it is for these sets of data.

APPENDIX B2

BMD analysis of liver adenomas in female rats in a two-year carcinogenicity study

The BMD approach, as described in Appendix B1, was used to analyse the incidence of liver adenomas in female rats (two control and three treated groups) as reported in Table B2.1.

Table B2.1: Incidence of liver adenomas in female rats treated with astaxanthin in a two-year carcinogenicity study

Dose of astaxanthin (mg/kg bw/day)	0 (control)	0 (placebo)	40	200	1 000
Number of animals examined	50	49	50	50	50
Number of animals with hepatocellular adenomas	2	1	5	9	14
Prevalence of hepatocellular adenomas (%)	4.00	2.04	10.0	18.0	28.0

Three different sets of dose–response models were also fitted here, considering each control group separated together with the other doses and merging the results from the two control groups. The results of the three models were very similar. The results obtained for the fitted dose–response model with the lowest AIC value, when the placebo control was used in combination with the other doses, are reported below (Figure B2.1 and Table B2.2).

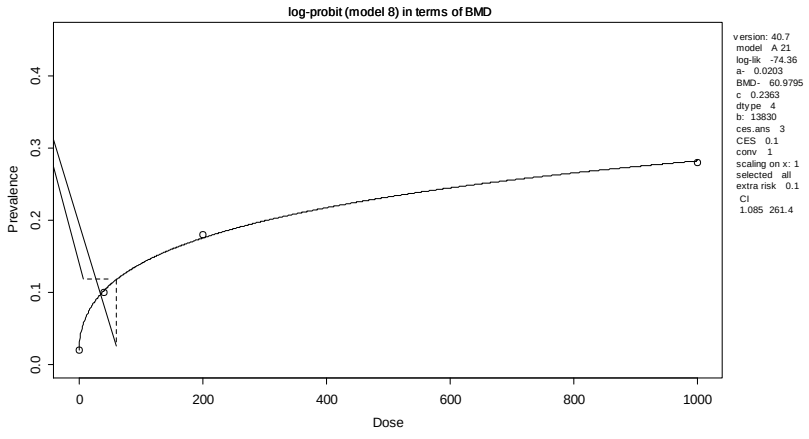

Figure B2.1: Log probit model fit together with the observed prevalences and estimated parameters

Table B2.2: Different models fitted, goodness of fit, BMD together with confidence bounds (a)

Model used	No of parameter	Log-likelihood	Accepted	AIC	BMD_{10}	$BMDL_{10}$	BMDU	BMD/ BMDL
Null	1	–82.63	–		NA	NA	NA	
Full	4	–74.35	–		NA	NA	NA	
Two-stage	3	–76.47	No	158.94	341	NA	NA	
Log.logist	3	–74.37	Yes	154.74	60.5	1.41	277	42.9
Weibull	3	–74.37	Yes	154.74	59.8	2.23	283	26.8
Log.prob	3	–74.36	Yes	154.72	61	1.08	261	56.5
Gamma	3	–74.38	Yes	154.76	59.2	3.49	287	17.0
Logistic	2	–82.57	No	169.14	65600	NA	NA	
Probit	2	–81.9	No	167.80	523	NA	NA	
LVM: E3	3	–74.39	Yes	154.78	58.1	0.461	303	126.0
LVM: E4	3	–75.06	Yes	156.12	115	22.1	346	5.2
LVM: H3	3	–74.37	Yes	154.74	60.2	0.772	284	78.0
LVM: H4	3	–74.47	Yes	154.94	63.9	15.7	262	4.1

(a): It should be noted that Proast 40.7 did not provide results for the one-stage model and BMDS 2.4 indicated that the standard deviation of the parameters was not calculated. Owing to these events, results were not taken into consideration.

Four quantal models and four continuous models were accepted considering their fitness by the log-likelihood. The lowest $BMDL_{10}$ identified by the quantal models were in the range of 1.1 to 3.5. However, the uncertainty of this estimate was very high, and the ratio of BMD/BMDL ranged from 17 to 56.5. Therefore, none of the $BMDL_{10}$ values could be retained. The continuous models H4 and E4 resulted in a $BMDL_{10}$ of 15.7 and 22.1, respectively. The estimates of the continuous models resulted in a less conservative $BMDL_{10}$ and a lower level of uncertainty than the quantal models. However, the ratio of BMD/BMDL (4.1 to 5.2) was still above the currently accepted maximum value of 2.

APPENDIX B3

BMD analysis of liver hypertrophy in female rats in a two-year carcinogenicity study

The BMD approach, as described in Appendix B1, was used to analyse the incidence of liver hypertrophy in female rats (two control and three treated groups) as reported in Table B3.1.

Table B3.1: Incidence of liver hypertrophy in female rats treated with astaxanthin in a two-year carcinogenicity study

Dose of astaxanthin (mg/kg bw/day)	0 (control)	0 (placebo)	40	200	1 000
Number of animals examined	50	49	50	50	50
Number of animals with liver hypertrophy	1	3	21	37	37
Prevalence of liver hypertrophy (%)	2.00	2.04	42.00	74.00	74.00

Three different sets of dose–response models were fitted, considering each control group separated together with the other doses and merging the results from the two control groups (also here the similar results are obtained for the different set of data). The results, when the placebo control was used in combination with the other doses, are reported below (Figures B3.1 and B3.2, and Table B3.2).

In order to illustrate the differences between the constrained and unconstrained models, Table B3.2 presents results for the unconstrained and constrained models, confirming that unconstrained models provide a better fit. The exponential model with four parameters, as well as the Hill model, which also has four parameters, produced the best fit to the data.

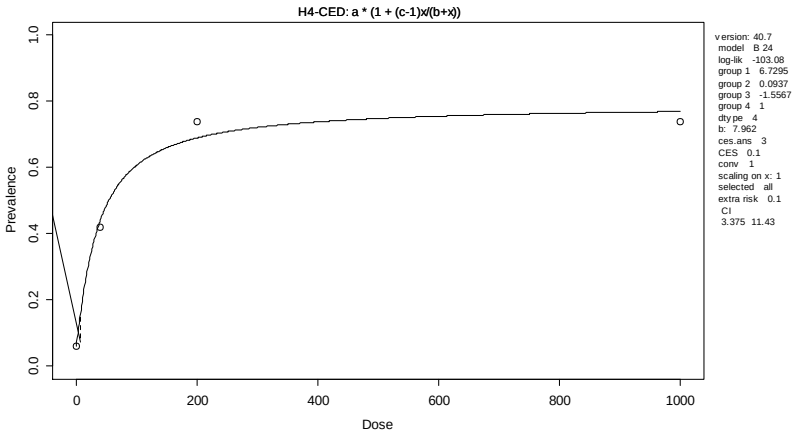

Figure B3.1: Model plot for H4

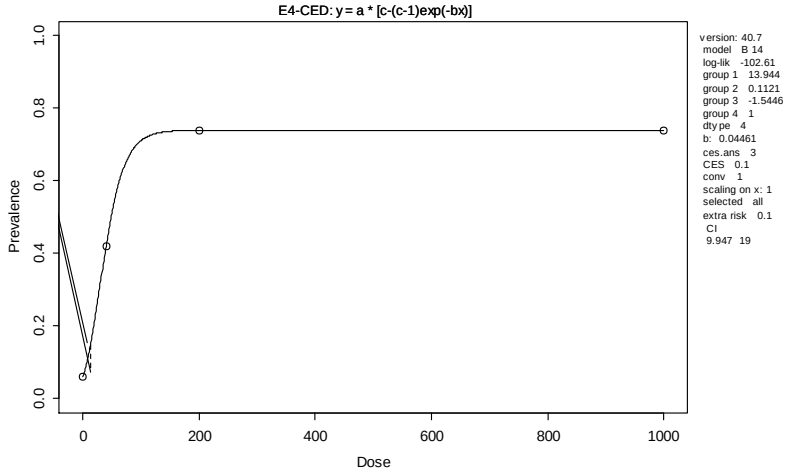

Figure B3.2: Model plot for E4

Table B3.2: Different models fitted, goodness of fit, BMD together with confidence bounds

Model used	No of parameter	Log-likelihood	Accepted	AIC	BMD_{10}	$BMDL_{10}$	BMDU	BMD/ BMDL
Null	1	–137.91	–		NA	NA	NA	
Full	4	–102.61	–		NA	NA	NA	
One-stage	2	–120.61	No	245.22	67.4	47.9	102.74	1.41
Two-stage	3	–120.61	No	247.22	67.4	47.9	102.74	1.41
Log.logist	3	–104.27	Yes	214.54	0.596	0.00184	4.68	323.91
Log.logist const	3	–110.21	No	226.42	8.81	13.23	21.6	0.67
Weibull	3	–104.6	No	215.2	0.0745	1.57E–05	1.456	4 745.2
Weibull const	3	–120.61	No	247.22	67.4	47.9	102.88	1.41
Log.prob	3	–104.34	Yes	214.68	0.716	0.00253	5.32	283
Log.prob const	3	–123.45	No	252.9	100.6	65.66	170.33	1.53
Gamma	3	–104.93	No	215.86	0.003	2.50E–09	0.403	12E05
Gamma const	3	–120.61	No	247.22	67.4	47.9	103.7	1.41
Logistic	2	–124.01	No	252.02	137	105.6	189.7	1.3
Probit	2	–124.1	No	252.2	140.55	111.06	NA	1.27
LVM: E4	3	–102.61	Yes	211.22	13.9	9.95	19	1.4
LVM: H3	3	–104.43	Yes	214.86	0.239	2.39E–04	2.69	1000
LVM: H4	3	–103.08	Yes	212.16	6.73	3.38	11.4	1.99

Two quantal equations were initially accepted based on the log-likelihood, but were not considered acceptable owing to a BMD/BMDL ratio of about 300. The same criteria lead to the exclusion of the continuous model H3 (BMD/BMDL of 1 000). Two estimates remained, both from the continuous models (E4 and H4). Model E4 with a $BMDL_{10}$ of 10.0 showed the lowest uncertainty (BMD/BMDL ratio of 1.4), whereas model H4 gave the most conservative $BMDL_{10}$ of 3.4 with a somewhat higher uncertainty (BMD/BMDL ratio of 2.0).