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Safety and efficacy of Panaferd-AX (red carotenoid-rich bacterium *Paracoccus carotinifaciens*) as feed additive for salmon and trout[1]

Scientific Opinion of the Panel on Additives and Products or Substances used in Animal Feed

(Question No EFSA-Q-2006-173)

Adopted on 18 September 2007

PANEL MEMBERS

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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 Panaferd-AX. The product is a feed additive consisting of dried sterilised cells of a red carotenoid-rich bacterium (*Paracoccus carotinifaciens*) intended to provide farmed Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) with a source of astaxanthin which confers the characteristic red colour to the flesh. Panaferd-AX contains around 4 % total red carotenoids, predominantly astaxanthin (2.2 %), adonirubin (1.3 %) and canthaxanthin (0.4 %).

In salmon and rainbow trout astaxanthin, deposition in flesh from Panaferd-AX was less efficient than that from synthetic astaxanthin. However, equal astaxanthin doses from both sources resulted in a comparable flesh pigmentation in the dose range of 20 to 100 mg astaxanthin kg^{-1} feed, due to the contribution of the other red carotenoids, mainly adonirubin and canthaxanthin, which are also demonstrated to be deposited in fish flesh. The technological and organoleptic properties of flesh from Panaferd-AX-treated fish were not different from those treated with synthetic astaxanthin.

Panaferd-AX, at dietary incorporation rate of 12.5-fold greater than the proposed maximum incorporation rate (0.4 %) is safe for salmonids (trout and salmon). *Paracoccus carotinifaciens* is not a known pathogen, and no other concerns have been identified either in the limited literature available or in the data submitted in the dossier.

[1] For citation purposes: Scientific Opinion of the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on a request from the European Commission on safety and efficacy of Panaferd-AX (red carotenoid-rich bacterium *Paracoccus carotinifaciens*) as feed additive for salmon and trout. *The EFSA Journal* (2007) 546, 1-30

Panaferd-AX is a non-genotoxic additive of very low acute and sub-chronic toxicity. No specific risk for the consumer related to compounds arising from the fermentation process (other than red carotenoids) is likely to occur.

As consumer exposure to astaxanthin and canthaxanthin after administration of Panaferd-AX at the maximum dose proposed would be at the most equal or lower than that resulting from the use of other astaxanthin or canthaxanthin sources, there is no additional risk for the consumer.

The structural proximity of adonirubin, astaxanthin and canthaxanthin, suggests that the toxicological profiles of the three compounds should be similar. However, the studies on genotoxicity and sub-chronic toxicity performed on Panaferd-AX are only indicative of the safety of adonirubin. The possible deposition of crystals of adonirubin in the retina, similar to that occuring with canthaxanthin, cannot be excluded and has to be considered as worst case scenario. The calculated consumer exposure to adonirubin plus canthaxanthin derived from the use of Panaferd-AX in salmon and trout complies with the canthaxanthin ADI (37 %). Therefore, no additional risk due to adonirubin exposure resulting from the use of Panaferd-AX is likely to occur.

Panaferd-AX shows very low acute inhalation toxicity and no potential for skin irritation. However, Panaferd-AX is considered as an eye irritant. Considering the nature of the product it should also be considered as a respiratory sensitiser.

The FEEDAP Panel does not expect that the use of Panaferd-AX as a source of astaxanthin, canthaxanthin and adonirubin for salmon and trout will pose an added risk to the environment.

The FEEDAP Panel recommends a number of modifications to the Register entry as proposed by the applicant. In particular, it is recommended to consider for Panaferd-AX the sum of astaxanthin, adonirubin and canthaxanthin as subject to Regulation and apply for this sum a maximum content of 100 mg kg^{-1} complete feed.

Key words: sensory additive, colourant, Panaferd-AX, *Paracoccus carotinifaciens*, red carotenoids, astaxanthin, canthaxanthin, adonirubin, salmon, trout, safety, efficacy

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BACKGROUND

Regulation (EC) No 1831/2003[2] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lies down that any person seeking an authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from the company Nippon Oil Corporation[3] for authorisation of the astaxanthin-rich bacterium *Paracoccus carotinifaciens* (NITE SD 00017) (Panaferd-AX) to be used as a feed additive for salmon and trout (category: sensory additive; functional group: colourants) under the conditions mentioned under 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). EFSA received directly from the applicant the technical dossier in support of this application. According to Article 8 of that Regulation, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. The particulars and documents in support of the application were considered valid by EFSA as of 9 of February of 2007.

The additive Panaferd-AX consists of sterilised dried cells of astaxanthin-rich *Paracoccus carotinifaciens*. The synthetic astaxanthin (E 161j) is authorised without a time limit at Community level for salmon, trout and ornamental fish.[4] Astaxanthin-rich *Phaffia rhodozyma* (ATCC 74219) (E 191z) is authorised without a time limit for use in salmon and trout.[5] Astaxanthin-rich *Phaffia rhodozyma* (ATCC SD-5340) (E 191y) is authorised for use in salmon and trout until 3.08.2011.[6]

The Scientific Committee on Animal Nutrition (SCAN) issued opinions on specific questions on the efficacy and safety of synthetic (EC, 1989) and biosynthetic (*Phaffia rhodozyma* (ATCC 74219)) (EC, 2002a; EC, 2003) astaxanthin. The FEEDAP Panel has adopted three opinions on astaxanthin. One opinion 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, 2005); in a third opinion, the safety and efficacy of an astaxanthin-rich *Phaffia rhodozyma* (ATCC SD-5340) product were assessed (EFSA, 2006a).

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 efficacy and the safety for the target animal(s), user and consumer and the environment of the product consisting of sterilised dried cells of astaxanthin-rich *Paracoccus carotinifaciens* (NITE SD 00017) (Panaferd-AX) when used under the conditions described in Table 1.

ACKNOWLEDGEMENTS

The European Food Safety Authority wishes to thank the members of the Working Group on Colorants (Panaferd-AX) for the preparation of this opinion.

[2] OJ L268, 18.10.2003, p.29
[3] Nippon Oil Corporation. Nippon Oil (UK) Plc. 15, Eldon St., London EC2M 7LD, UK
[4] OJ L 298, 28.10.1998, p.4
[5] OJ L 243 15.7.2004, p.10
[6] OJ L 184 14.7.2007, p.12

Table 1. **Register entry as proposed by the applicant**

Additive	Panaferd-AX
Registration number/EC No/No (if appropriate)	-
Category of additive	2; Sensory additive
Functional group of additive	A ii; Colourant

Description			
Composition, description	**Chemical formula**	**Purity criteria (if appropriate)**	**Method of analysis (if appropriate)**
Sterilised dried cells of astaxanthin-rich *Paracoccus carotinifaciens* (NITE SD 00017)	-	Containing at least 20 g astaxanthin per kilogram of additive	Solvent extraction, HPLC

Trade name (if appropriate)	Panaferd-AX
Name of the holder of authorisation (if appropriate)	

Conditions of use				
Species or category of animal	**Maximum Age**	**Minimum content**	**Maximum content**	**Withdrawal period (if appropriate)**
		mg kg^{-1} of complete feedingstuffs		
Salmonidae; salmon, trout	-	-	85 mg kg^{-1} as astaxanthin	-

Other provisions and additional requirements for the labelling	
Specific conditions or restrictions for use (if appropriate)	The maximum content is expressed as astaxanthin. Use permitted from the age of 6 months onwards. The mixture of the additive with astaxanthin or canthaxanthin is allowed provided that the total concentration astaxanthin and canthaxanthin does not exceed 100 mg kg^{-1} in complete feedingstuff.
Specific conditions or restrictions for handling (if appropriate)	-
Post market monitoring (if appropriate)	-
Specific conditions for use in complementary feedingstuffs (if appropriate)	-

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

ASSESSMENT

1. Introduction

The product (Panaferd-AX) is a feed additive consisting of dried sterilised cells of a red carotenoid-rich bacterium (*Paracoccus carotinifaciens* NITE SD 00017). It contains around 4 % total red carotenoids, predominantly astaxanthin (2.2 %), adonirubin (1.3 %), and canthaxanthin (0.4 %). According to the applicant, the purpose of the additive is to provide farmed Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) with a source of astaxanthin which confers the characteristic red colour to the wild salmonids that in nature is primarily obtained from crustaceans which form a major part of their diet.

The applicant recommends an incorporation of Panaferd-AX in feedingstuffs for fish at the maximum concentration of 3.86 g kg^{-1}, corresponding to 85 mg astaxanthin kg^{-1}.

2. Characterisation of the product

The additive is a dark red granular powder made of sterilised dried cells of the bacterium *Paracoccus carotinifaciens* (NITE SD 00017) and its fermentation medium.

Paracoccus carotinifaciens, an aerobic gram-negative bacterium isolated from the soil, belongs to the Protobacteria sub-class. The parent strain was originally obtained from a natural source. The strain NOC-18 was developed using classical mutation and selection techniques from the wild strain E-396, IFO 16121. The modified strain NOC-18 is deposited at the Japanese National Institute of Technology and Evaluation (deposit no. NITE SD 00017).

2.1. Physical and chemical properties

The product contains (average values from five batches) 50.4 % crude proteins (including non-protein nitrogen from nucleic acids), 33.6 % nitrogen-free extract including carbohydrates (10 %) and polyhydroxybutyric acid (13 %) from the fermentation medium, 9.3 % ash, 3.8 % crude fat, and 2.8 % moisture.[7]

Due to the heat treatment at 80 °C, no viable cells of *Paracoccus carotinifaciens* remain in the product. The analysis of five batches of the additive indicates the absence of *Salmonella* (in 25 grams of the product).[8]

Mean arsenic, cadmium, lead and mercury concentrations were reported to be <0.1, <0.01, 0.08 and <0.01 mg kg^{-1}, corresponding to the respective limits of detection. Total dioxins and dioxin-like PCBs, i.e. PCDDs + PCDFs + Co-PCBs, amounted to 0.0053 pg TEQ g^{-1}.[9] PCBs were below 0.1 mg kg^{-1}.

Particle size analysis determined in three batches indicates that 94 % of the particles are comprised between 100 and 500 µm, less than 0.5 % being < 63 µm.[10] The bulk density is in the range of 0.6 to 0.8 g mL^{-1}.[11]

2.2. Method of production

The additive is produced by classical fermentation.[12] The strain is cultivated in a sterilised medium. After cultivation, the final fermentation product is pasteurised. It is adjusted to an

[7] Technical Dossier, Section II, Appendix II-D
[8] Technical Dossier, Section II, Appendix II-D
[9] Technical Dossier, Section II, Appendix II-D
[10] Technical Dossier, Section II, Appendix II-H
[11] Technical Dossier, Section II
[12] Technical Dossier, Section II, Appendix II-I

astaxanthin content of 20-21 g kg^{-1} using calcium carbonate. The product is then spray-dried (110-200 °C).

2.3. Carotenoids in the additive

The product contains (average of five batches) 43.6 g total carotenoids kg^{-1}.[13] The detailed carotenoid composition is given in Table 2.

Table 2. **Composition of total carotenoids in Panaferd-AX**

Carotenoids	Content (g kg^{-1})
Astaxanthin	21.8
Adonirubin	12.9
Canthaxanthin	4.1
Adonixanthin	1.7
Echinenone	1.4
ß-carotene	0.7
Asteroidenone	0.7
3-hydroxyechinenone	0.4

The three main red carotenoids are: astaxanthin, representing about 50 % (21.8 g with a range from 20.7 to 22.9 g), adonirubin, 30 % (12.9 g with a range from 10.2 to 15.1 g), and canthaxanthin, 9 % (4.1 g with a range from 3.0 to 4.9 g). Molecular structures are presented in Figure 1.

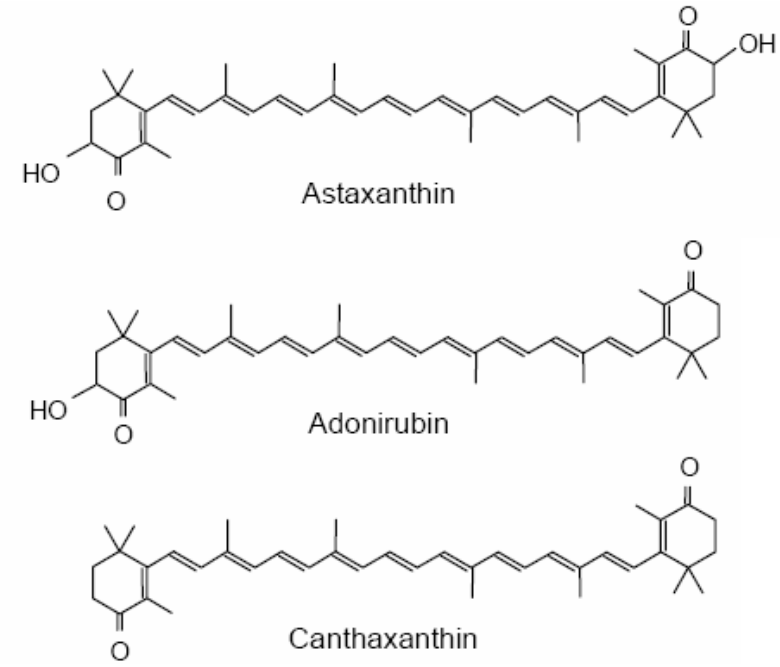

Figure 1. **Molecular structure of astaxanthin, adonirubin and canthaxanthin**

[13] Technical Dossier, Section II. Appendix II-C

2.3.1. Astaxanthin

Astaxanthin ($C_{40}H_{52}O_4$) occurs in three enantiomeric forms: 3R,3'R, 3S,3'S and 3R,3'S. Astaxanthin from *Paracoccus carotinifaciens* occurs as free, non-esterified form, and predominantly (>99 %) under the 3S,3'S configuration[14] which prevails in wild Atlantic or Coho salmon (Turujman *et al.*, 1997). Analytical data (average of three batches) indicate that astaxanthin in the product is essentially in the all-E (trans) configuration (95.5 %), the cis-forms (9Z and 13Z) representing only 1.7 and 2.8 %, respectively.[15]

The occurrence of astaxanthin in nature, its physical and chemical properties, are extensively described in a previous opinion of the FEEDAP Panel on the safety of use of colouring agents in animal nutrition (EFSA, 2005).

2.3.2. Adonirubin (Phoenicoxanthin)

Adonirubin, as well as asteroidenone and adonixanthin, are naturally occurring intermediates in bacterial, yeast and algal biosynthesis of astaxanthin. They are found in lower marine life forms, readily absorbed by higher marine species and present in a number of crustacea and fish, including those that are part of the human food chain. They are also found in bird plumage.

Studies with the carotenoid biosynthesis gene cluster for the production of astaxanthin from the marine bacterium *Agrobacterium aurantiacum* (Misawa *et al.,* 1995*)*; enzyme studies using *E. coli* strains (Fraser *et al*., 1997) expressing these genes showed rather similar ways of biosynthesis of astaxanthin from ß-carotene (Figure 2). Other experiments indicated that the enzymes were strictly oxygen-requiring. In anaerobic conditions, *A. aurantiacum* enzymes predominantly formed adonirubin. In contrast, astaxanthin was the main product under aerobic conditions.

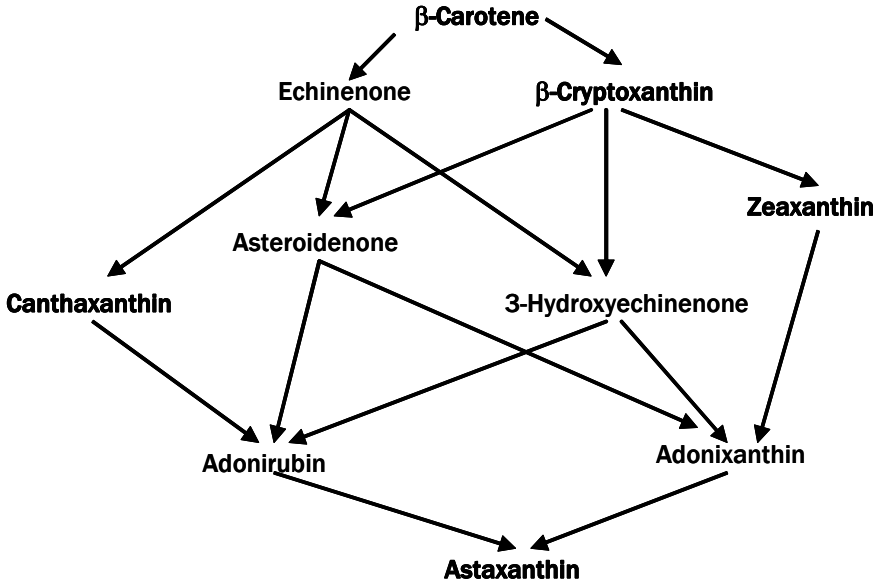

Figure 2. **Anticipated biosynthetic pathways of red carotenoids** (Misawa et al., 1995)

[14] Technical Dossier, Section II, Appendix II-J
[15] Technical Dossier, Section II, Appendix II-J

Adonirubin, together with asteroidenone and adonixanthin, is also synthesised in the algae *Cholorococcum sp.* and *Haematococcus pluvialis*. The astaxanthin-rich yeast *Phaffia rhodozyma* (ATCC 74219) also contains adonirubin (2.9-4.1 % of total carotenoids) but at a much lower level than the bacterium product (EC, 2002a; EC, 2003).

2.3.3. Canthaxanthin

Canthaxanthin is the β,β-carotene 4,4'-dione. Its occurrence in nature and its physical and chemical properties have been extensively reviewed by the SCAN (EC, 2002b). An ADI of 0.03 mg kg^{-1} bw was established by the Scientific Committee on Food (EC, 1997). A maximum content of 25 mg canthaxanthin kg^{-1} complete feedingstuff is set for trout and salmon.

Canthaxanthin is also a colour additive for food (E-161) used for colouring Strasbourg sausages.

2.4. Stability

The applicant proposes a shelf life of the product of 12 months. Stability studies of the product, carried out with one batch at room temperature for 24 months, showed maximum astaxanthin losses of 6 % of the original content.[16] Typically, shelf life is concluded on the basis of three batches.

The applicant also submitted tests on the effects of different conditions (light, moisture, temperature (25 to 60 °C), storage (aluminium film protection, polyethylene bags, open/closed)), for three to six months, on product stability. Astaxanthin analyses indicated good stability (losses up to 4 %) in closed bags.[17]

Stability of the product in premixtures was not tested but it should be noted that the product can be directly incorporated into compound feedingstuffs.

Carotenoids are known to be heat susceptible. Stability studies with the product have been performed during feed processing. Astaxanthin losses due to extrusion were as high as 32 to 36 %.[18] However, astaxanthin losses ranging from 8 to 11 % and canthaxanthin losses from 8 to 22 % were found in another study.[19]

The effect of feed storage on astaxanthin and canthaxanthin stability has been investigated in feedingstuffs containing different concentrations of the product kept for 12 weeks at 25 °C.[20] A weekly loss of 1 % for both pigments could be concluded from the data.

2.5. Homogeneity

Data to examine losses during processing (extrusion), but also to assess astaxanthin homogeneity in feed, were provided. Three samples, each of two intended levels, showed astaxanthin concentrations between 82 and 85 mg kg^{-1}, and 100 to 110 mg kg^{-1} after mixing, and after processing (from extrusion to packaging) of 53.5 to 54.7 and 70 to 71 mg kg^{-1}, respectively.[21] The product can therefore be considered homogeneously distributed.

[16] Technical Dossier, Section II, Appendix II-O
[17] Technical Dossier, Section II, Appendix II-P
[18] Technical Dossier, Section II, Appendix III-E
[19] Technical Dossier, Section II, Appendix II-S
[20] Technical Dossier, Section II, Appendix II-U
[21] Technical Dossier, Section II, Appendix III-E

2.6. Evaluation of the analytical methods by the Community Reference Laboratory (CRL)

EFSA verified the report and its amendment submitted by the Community Reference Laboratory (CRL) concerning the analytical method(s) for Panaferd-AX. The method (normal phase liquid chromatography coupled to UV detection) allows simultaneous determination of astaxanthin, adonirubin and canthaxanthin in feedingstuffs and fish tissues. The executive summary of the report and its amendment are attached in the Appendix.

3. Efficacy

The ability of astaxanthin to effectively colour fish flesh, in particular that of salmonids, has been already demonstrated in the former opinion of the FEEDAP Panel on the safety of use of colouring agents in animal nutrition (EFSA, 2005).

3.1. Bioavailability of astaxanthin from *Paracoccus carotinifaciens*

In a preliminary study with Coho salmon (*Oncorhynchus kisutch),* the bioavailability of astaxanthin from a pilot product (spray-dried cells of *Paracoccus carotinifaciens*)[22] was tested in comparison with synthetic astaxanthin.[23] The products were included in a common feed mixture for salmon at a level of 50 mg astaxanthin kg^{-1} diet, analytically verified. The experimental period lasted for 221 days, at a water temperature of 9.3 to 18.5 °C, in marine net cages with about 600 salmon per experimental group. Astaxanthin was determined by HPLC in the flesh of four fish from each group. Growth performance parameters and astaxanthin retention are given in Table 3.

Table 3. **Efficacy of spray-dried cells of Paracoccus carotinifaciens in Coho salmon** (body weight at start: 208 g; duration of the trial: 221 days)

	Synthetic astaxanthin	Astaxanthin from *Paracoccus carotinifaciens*
Astaxanthin (mg kg^{-1} feed)	44	45
Total weight gain (g)	1733	1771
Feed/gain (g g^{-1})	1.66	1.61
Astaxanthin in flesh (mg kg^{-1})	9.20	10.20

The study indicates that the additive contains bioavailable astaxanthin and did not adversely affect the performances of Coho salmon.

3.2. Efficacy for the target species

Besides the standard zootechnical endpoints, pigmentation of fish flesh was evaluated by Roche colour fan and reflectance spectrometry (Minolta colourimeter). Deposition of red carotenoids in fish flesh was also measured; however, the FEEDAP Panel focused the assessment on the sum of the three main red carotenoids (astaxanthin, adonirubin and canthaxantin).

[22] In early trials Panaferd-AX was also known as NM-Pigment
[23] Technical Dossier, Section III, Appendix III-A

3.2.1. Efficacy trials in salmon

A small-scale preliminary study was conducted in Coho salmon with 240 fish (80 per group) using two doses of NM pigment[24] and one dose of synthetic astaxanthin.[25] This trial was not considered further because of the low number of animals (two in each group) investigated for pigmentation.

A large-scale trial was performed with a total of 11,700 Atlantic salmon (*Salmo salar*) with an initial weight of 90 ± 18 g, kept in 18 cages (650 fish per cage, three cages per group).[26] The experiment contained two phases: the first phase lasted 12 months, followed by a two-month interruption due to technical reasons –when the fish were not fed–, then a second phase which lasted about three months. A standard diet was supplemented with target levels of 40, 70 and 100 mg astaxanthin from Panaferd-AX or from synthetic astaxanthin kg^{-1} feed. The average measured levels of astaxanthin in five batches fed consecutively were 47, 72 and 93 mg kg^{-1} in the Panaferd-AX-supplemented groups and 53, 78 and 104 mg kg^{-1} in the synthetic astaxanthin-supplemented groups. The pattern of red carotenoids was also quantified in the final batches. The sum of the three main carotenoids (i.e. astaxanthin, adonirubin and canthaxantin) of the Panaferd-AX diets was 87, 134 and 146 mg kg^{-1}, and that of the synthetic astaxanthin diets 55, 60 and 89 mg kg^{-1}. Zootechnical parameters were measured. Twelve fish per group (four from each replicate) were collected for determination of pigmentation parameters and astaxanthin deposition at three-month intervals.

There were no significant differences in fish weight, specific growth rate or feed to gain ratio between the two sources of astaxanthin.

Colour fan scores indicated an increase of flesh colour from the start to six months, without further changes up to 12 months. Colour fan values were not dose–dependent, and differences between the two astaxanthin sources were not observed, despite the fact that the content of main red carotenoids in the Panaferd-AX groups was considerably higher than that of the synthetic astaxanthin groups.

The reflectance spectrometry evaluation showed that both products, Panaferd-AX and synthetic astaxanthin, effectively increased the red colour of salmon flesh at all supplemented levels. The highest increase in Minolta redness (a*) was observed in the first six months (Table 4); it further increased after 12 months. One hundred mg synthetic astaxanthin per kg resulted in significantly higher values than 40 mg astaxanthin of both sources or 70 mg astaxanthin from Panaferd-AX.

Table 4. **Reflectance spectrometry scores (Minolta redness (a*)) of salmon flesh**

Months	Panaferd-AX (mg astaxanthin kg^{-1} feed)			Synthetic astaxanthin (mg astaxanthin kg^{-1} feed)		
	40	**70**	**100**	**40**	**70**	**100**
0	14.3	14.3	14.3	14.3	14.3	14.3
6	24.1	24.1	23.4	23.5	24.5	25.1
12	25.5^{a}	25.3^{a}	$25.9^{a,b}$	25.5^{a}	$26.1^{a,b}$	26.6^{b}

a, b: Values in a row with different superscripts differ significantly (P≤0.05)

The total red carotenoid deposition in flesh showed a tendency to a dose-dependent increase of total carotenoids deposition in the synthetic astaxanthin groups, not in the Panaferd-AX groups (Table 5). In contrast to the Roche colour fan values, the carotenoids deposition increased in a

[24] See Footnote 22
[25] Technical Dossier, Section III, Appendix III-B
[26] Technical Dossier, Section III, Appendix III-F

time-dependent manner until month 12. One hundred mg astaxanthin from the synthetic source resulted in a significantly higher deposition after three, six and nine months than the same amount of astaxanthin from Panaferd-AX.

Table 5. **Total red carotenoid content (mg kg^{-1}) of salmon flesh along the experimental astaxanthin diet supplementation**

Months	Panaferd-AX (mg astaxanthin kg^{-1} feed)			Synthetic astaxanthin (mg astaxanthin kg^{-1} feed)		
	40	**70**	**100**	**40**	**70**	**100**
0	0.3	0.3	0.3	0.3	0.3	0.3
3	3.3^{a,b}	3.1^{a}	3.1^{a}	3.2^{a}	3.7^{b}	4.3^{c}
6	4.0^{a,b,c}	3.7^{a,b}	3.4^{a}	3.5^{a,b}	4.1^{b,c}	4.4^{c}
9	4.6^{a,b}	4.3^{a}	4.6^{a,b}	4.8^{a,b,c}	5.2^{b,c}	5.4^{c}
12	6.4	6.1	6.7	6.2	6.8	7.2

a, b, c: Values in a row with different superscripts differ significantly ($P \leq 0.05$)

The deposition ratios for total red carotenoids (flesh:diet) for the groups fed synthetic astaxanthin declined, as expected, with increasing dietary concentration (0.13, 0.10, and 0.07 for 40, 70, and 100 mg astaxanthin kg^{-1}, respectively). The corresponding ratios for the Panaferd-AX group were considerably lower (0.05, 0.04, and 0.04, respectively).

The main individual red carotenoids were measured in the fish flesh at the end of the second phase of the experiment (Table 6); their proportions reflect those in the astaxanthin sources of the supplemented diets.

Table 6. **Red carotenoids content (mg kg^{-1}) of salmon flesh at the end of the experimental period (18 months ~ 16 feeding months)**

Carotenoid	Panaferd-AX (mg astaxanthin kg^{-1} feed)			Synthetic astaxanthin (mg astaxanthin kg^{-1} feed)		
	40	**70**	**100**	**40**	**70**	**100**
Total carotenoids	6.3^{a}	6.8^{a,b}	7.0^{b}	6.6^{a,b}	7.0^{b}	7.7^{c}
Canthaxanthin	0.6	0.5	0.5	0.1	0.1	0.1
Adonirubin	1.8	2.0	2.0	0.1	0.1	0.1
Astaxanthin	3.7	4.0	4.2	6.4	6.8	7.5

a, b, c: Values in a row with different superscripts differ significantly ($P \leq 0.05$)

The highest total carotenoid deposition was obtained by supplementation of 100 mg astaxanthin from the synthetic source (significantly different to all other groups); 70 mg astaxanthin from the synthetic source and 100 mg from Panaferd-AX resulted in the same total carotenoid deposition. Table 6 also shows that total carotenoids from synthetic astaxanthin originated predominantly from astaxanthin (97 %), whereas the corresponding figures for astaxanthin from Panaferd-AX represent about 60 %. Adonirubin amounted to 29 % of total carotenoids in the flesh of salmon fed Panaferd-AX.

3.2.2. Efficacy trials in trout

One dose-response study and one field trial were carried out with rainbow trout using Panaferd-AX- and synthetic astaxanthin-supplemented diets.

3.2.2.1. Dose-response study

A dose-response study was carried out with a total of 1320 non-pigmented rainbow trout (*Oncorhynchus mykiss*) with an initial average weight of 100 g, and allocated to 22 tanks (60

fish per tank, two replicates per group) for 12 weeks.[27] Fish were fed a standard diet supplemented with target levels of 20, 40, 60, 80, and 100 mg astaxanthin plus canthaxanthin from Panaferd-AX kg^{-1}, and 20, 40, 60, 80, and 100 mg astaxanthin kg^{-1} from synthetic astaxanthin, respectively. A negative control was included. All dose levels were analytically measured and found to be lower than the targets (20 to 33 %). Zootechnical parameters, pigmentation and red carotenoids deposition in flesh were determined.

Differences observed (sometimes significant) in specific growth rate and feed/gain ratio between treatment groups were not dose-related and irrespective of the astaxanthin source.

The initial colour fan score for all groups was 19 and was maintained at that level for the unsupplemented group. In the supplemented groups, it increased up to 21 to 24 after four weeks; at the end of the experiment, colour fan scores were between 22 and 25, without showing significant differences between the Panaferd-AX and synthetic astaxanthin fed fish, irrespective of the dose.

Reflectance spectrometry analysis ('Redness' (a*)) showed a dose-related trend to moderate increase in flesh colour, but not reaching significance. There were no differences for Minolta b* or L* scores.

Red carotenoids deposition in flesh showed a dose-response for the groups fed synthetic astaxanthin, the linear regression being y (mg carotenoids in kg flesh) = 0.087x (mg astaxanthin in kg feed) + 0.701 (R^2 = 0.524). The corresponding equation for the Panaferd-AX groups showed a lower slope (0.023) and a higher y intercept (4.066) with R^2 of 0.033. This supports the conclusion that no dose-dependent effect for Panaferd-AX was observed in this experiment, unlike for synthetic astaxanthin (Table 7).

Table 7. **Red carotenoid content (mg kg^{-1}) in trout flesh after 12 weeks of feeding a red carotenoid-supplemented diet**

Group	Astaxanthin	Canthaxanthin	Adonirubin
Negative Control	0.9	n.d	n.d
Panaferd-AX 20*	3.4	0.2	1.1
Panaferd-AX 40	2.6	0.3	1.3
Panaferd-AX 60	3.4	0.4	1.8
Panaferd-AX 80	3.3	0.4	1.9
Panaferd-AX 100	3.4	0.4	2.0
Synthetic astaxanthin 20*	1.6	n.d.	n.d.
Synthetic astaxanthin 40	4.5	n.d.	n.d.
Synthetic astaxanthin 60	3.7	n.d.	n.d.
Synthetic astaxanthin 80	6.1	n.d.	n.d.
Synthetic astaxanthin 100	7.6	n.d.	n.d.

n.d.: Not determined

(*) Panaferd-AX 20: 20 mg astaxanthin plus canthaxantin kg^{-1} feed. Synthetic astaxanthin 20: 20 mg astaxanthin kg^{-1} feed

3.2.2.2. Field trial

A field trial was carried rainbow trout *(Oncorhynchus mykiss)*.[28] Fish were allocated to 24 cages (eight cages per treatment group) and were fed a commercial trout diet supplemented either with synthetic astaxanthin at a target level of 80 mg kg^{-1} or with Panaferd-AX at target levels of 60 and 80 mg astaxanthin kg^{-1}. No negative control group was included. The measured astaxanthin content of the feed samples (nine samples per treatment) was slightly

[27] Technical Dossier, Section III, Appendix III-C

[28] Technical Dossier, Section II, Appendix III-E

lower than the target level. The pattern of carotenoids was also quantified. Pigmentation and carotenoid accumulation in flesh were recorded.

Pigmentation of trout flesh was determined at the end of the trial by Roche colour score and by reflectance spectrometry (Minolta colourimeter). Colour fan score showed no significant differences between Panaferd-AX- and synthetic astaxanthin-fed fish. There were also no significant differences for Minolta L*, a* or b* scores between the treatment groups.

After eight months, no significant differences were found in the astaxanthin content of the flesh of trout receiving both levels of astaxanthin from Panaferd-AX-supplemented groups, but astaxanthin was significantly higher ($P<0.01$) in the group fed synthetic astaxanthin. However, the main carotenoids content was not significantly different, regardless of the source and level of astaxanthin in the diet. The red carotenoids profiles of flesh reflected the carotenoids profile of diets.

The deposition ratios for astaxanthin in the group fed synthetic astaxanthin showed a higher value (0.22 for 80 mg astaxanthin kg^{-1}) than those of the Panaferd-AX groups (0.17 and 0.14 for 60 and 80 mg astaxanthin kg^{-1}, respectively).

3.3. Organoleptic findings

3.3.1. Stability of flesh pigmentation

Flesh samples from the large-scale trial with Atlantic salmon (see Section 3.2.1.) were stored for three months at -10 or -20 °C and for seven months at -20 °C, respectively (n = 5 per treatment).[29] The final coloration of flesh, based on carotenoid content, was slightly lower in one Panaferd-AX sample, in both series of flesh treatment; however, statistical analysis was not reported. Another study showed that stimulation with fluorescent light for seven days reduced flesh colour values in Panaferd-AX- and synthetic astaxanthin-treated fish by the same rate, irrespective of the source.[30]

Fillets from the field trial with rainbow trout (see Section 3.2.2.2.) were stored at -10 or -35 °C for two and a half to six months.[31] No significant differences in total red carotenoids content and in visual colour were observed between the groups fed Panaferd-AX and those fed synthetic astaxanthin.

3.3.2. Effects of technological treatments

The applicant provided a study on the effects of cooking, smoking and freezing on the sensory properties of fillets from Atlantic salmon fed diets supplemented with Panaferd-AX or synthetic astaxanthin.[32] The assessment by a trained panel comprised the determination of colour values and the sensory evaluation of randomly selected samples. No measurable differences were found between the two treatments.

3.4. Conclusion on efficacy for target species

Astaxanthin from spray-dried cells of *Paracoccus carotinifaciens* was shown to be bioavailable and efficient in colouring the flesh of Coho salmon, Atlantic salmon and rainbow trout.

[29] Technical Dossier, Section III, Appendices III-M, III-N and III-P
[30] Technical Dossier, Section III. Appendix III-O
[31] Technical Dossier, Section III, Appendices III-G, III-H, III-I, III-J, III-K and III-L
[32] Technical Dossier, Section III, Appendices III-S, III-T, III-W

In salmon and rainbow trout, astaxanthin deposition in flesh from Panaferd-AX was less efficient than from synthetic astaxanthin, particularly for higher doses. However, equal astaxanthin doses from both sources, in the dose range of 20 to 100 mg astaxanthin kg^{-1} feed, resulted in a comparable flesh pigmentation measured by Roche colour fan and colour reflectance spectrometry. The FEEDAP Panel concludes that the reason for the difference between the above findings can only be attributed to the content of other red carotenoids in Panaferd-AX, mainly adonirubin and canthaxanthin, which are also demonstrated to be deposited in fish flesh.

The technological and organoleptic properties of flesh from fish treated with Panaferd-AX were not different from those of fish treated with synthetic astaxanthin.

4. Safety

4.1. Safety for the target species

The safety of astaxanthin for salmonids has been assessed in the former opinion of the FEEDAP Panel on the safety of use of colouring agents in animal nutrition (EFSA, 2005). Canthaxanthin has been assessed by the SCAN (EC, 2002b).

The intermediate xanthophylls, adonirubin and adonixanthin have not been assessed yet for their safety for salmonids. However, adonixanthin, in particular, is found in a variety of wild fish.[33] Adonirubin was reported as being present in the integument of the Goldfish (*Carassius auatus*) by Shahidi *et al.* (1998). Adonirubin has been identified as one of the carotenoids present in the flesh of trout fed a commercial diet that contains, amongst other carotenoids, adonirubin, particularly form the algae *Haematococcus pluvialis* (Sommer *et al.*, 1992). The absorption of adonirubin was also described by Schiedt *et al.* (1985) in the rainbow trout. The lack of references to adonirubin, asteroidenone and adonixanthin reflects most likely their low commercial value, specificity of analytical methods and not necessarily their lack of presence in fish tissue.

4.1.1. Tolerance studies

Two tolerance studies on the rainbow trout (*Oncorhynchus mykiss*) and one on the red sea bream (*Pagrus major)* were performed.

4.1.1.1. Ninety-day study on rainbow trout

The study was designed to evaluate the toxicity of Panaferd-AX to the rainbow trout.[34] The maximum inclusion rate, 0.4 % Panaferd-AX in the final feed, was considered, assuming a maximum allowable usage rate of 80 mg astaxanthin kg^{-1} complete feed for salmonids.

The dose range tested included dietary incorporation levels of 0, 1, 3, and 5 %, the latter being approximately 12.5-fold greater than the proposed maximum dietary incorporation rate for Panaferd-AX. The study was designed with consideration of the OECD guideline for Bioconcentration (Flow-through Fish Test, OECD No. 305) and the principles of Sub-Chronic Oral Toxicity (OECD No. 408).

The study used Panaferd-AX batch number ASXGB-3510 (astaxanthin content, 1.9 % and total carotenoid content, 4.32 %). The test diets for this study were manufactured to give final

[33] Technical Dossier, Section IV, Appendix IV-S
[34] Technical Dossier, Section IV, Appendix IV-A

concentrations of Panaferd-AX of 0, 1, 3 and 5 %, in 2 mm pellets. Proximal analysis confirmed similar nutritional content between the diets. On arrival at the study location, the diet was analysed and dietary levels of Panaferd-AX were found to be 1.07 %, 2.89 % and 4.81 %, based on the astaxanthin content of Panaferd-AX. The diets were stored at -20 °C and analysed monthly throughout the study; the levels of astaxanthin stood within a range of 92 to 106 % of the original values, confirming frozen stability.

One hundred and four rainbow trout, approximately 6-7 g in weight, were randomly allocated to four groups of 26 (in two replicate tanks, each of 13). The fish were maintained in aerated, freshwater, flow-through tanks. Water temperature was maintained at approximately 14 °C. The control and test diets were fed in two equal portions, daily, at a rate of 2 % group mean body weight per day. Any uneaten feed, together with any excretory material, were removed daily from all vessels.

During the study, daily observations were done, and body weight and length were recorded weekly for a representative sample of ten fish, randomly selected from each vessel (20 per group). Environmental parameters (pH, dissolved oxygen, temperature and flow rates) were measured daily. At the end of the study, fish were killed. The toxicological assessment included a full necropsy with organ weights (heart, liver and gills) on ten fish from each group (five per vessel), and histopathological examination from the control and high dose groups. An additional ten fish in each group (five per vessel) were killed and subject to necropsy.

No mortalities or changes in appearance or behaviour that were indicative of toxicity were seen. The body weight and length of fish increased in a manner consistent with the controls showing that Panaferd-AX had no adverse effect on growth and development (average body weight at start: 6.4 g, at end: 66.8 g; length at start: 7.2 cm, at end: 15.8 cm).

At the end of the treatment period, no post-mortem abnormalities were observed. Analysis of organ weights found a statistically, significantly lower relative liver weight ($P<0.05$) in the treated groups compared to control groups (mean weights were 76, 73 and 86 % of the control value (0.0176 % of body weight) in fish given 1, 3 and 5 % Panaferd-AX, respectively). However, individual weights were variable and, in the absence of dose relationship or any corresponding pathological changes, this finding is considered to be wihtout toxicological significance.

Histological examination found no changes indicative of toxicity in fish given the highest dose of 5 % Panaferd-AX.

4.1.1.2. Fifty-six-day study in rainbow trout

This study assessed the safety of Panaferd-AX (Batch NM-001, Lot No. 980806) in the rainbow trout at concentrations of 0.33 % and 1 % of feed.[35] The upper level corresponded to the 2.5-fold of the maximum recommended dose of the applicant.

Astaxanthin content in the product was 1.57 % and total carotenoid content 2.79 %. The product was mixed with a basic diet to obtain 3 mm-size pellets, containing 0, 50 or 150 mg astaxanthin kg^{-1} feed. A proximal analysis of feed confirmed similar levels of water, protein and fat in the three diets. The fish were fed twice daily, to satiety, for 56 days.

Juvenile rainbow trout, approximately 7.5 g in weight, were allocated to 60 L water tanks in replicated groups of 40. Temperature was maintained between 16 and 18 °C. Ten fish were randomly taken from each replicate tank (20 per group) and weighed individually every two weeks; all fish were weighed every four weeks. At the end of the study, five fish from each

[35] Technical Dossier, Section IV, Appendix IV-C

tank (10 per group) were killed and the muscle was analysed for water, protein and lipid content.

Panaferd-AX was not associated with an increase in mortality, although deaths occurred in 6.3 % of control fish and 2.5 % of fish given 1 % Panaferd-AX. Other than reddening of the body surface in both of the treated groups, no behavioural, morphological or other abnormalities were noted. Daily feed intakes were similar amongst all groups. Steady body weight increases were noted in all groups, with negligible differences in body weight or muscle mass at termination (control group: 31 g, 0.33 % Panaferd-AX: 32 g, 1.0 % Panaferd-AX: 29 g).

At necropsy, no abnormalities of the gills, digestive organs, liver or kidney were observed. Analysis of the muscle revealed similar water, protein, lipid and ash content amongst all groups.

4.1.1.3. Study in the red sea bream

The study assessed the effect of the product on red sea bream at the dose of 72 mg astaxanthin kg^{-1} feed and five-fold (350 mg astaxanthin kg^{-1} feed). The study was conducted with an early Panaferd-AX batch containing only 1.76 % total carotenoids among which 0.76 % were astaxanthin.[36] The fish were fed for 30 days only.

Due to the short duration of the trial, the FEEDAP Panel did not further consider this study.

4.1.2. Microbiological safety of the additive

To the FEEDAP Panel knowledge, the only published study on the species *Paracoccus carotinifaciens* is the paper from Tsubokura *et al.* (1999) which describes the isolation and taxonomical characterisation of the organism. Except for two human infections by *Paracoccus yeei* reported in the dossier, there have been no reports associating the genus *Paracoccus* with clinical infections in either humans or animals.

No production of antibiotic or antimicrobial substances has been reported in the literature. Two agar diffusion tests were performed to identify eventual inhibitory effects of Panaferd-AX (as dimethylsulfoxide solution) and the fermentation broth, on a yeast (*Candida albicans* ATCC No. 10231*)* and selected bacteria (*Staphylococcus aureus* ATCC No. 6538*, Escherichia coli* ATCC No. 8739 or *Pseudomonas aeruginosa* ATCC No. 9027). No inhibitory effect was observed at the maximum solubility of Panaferd-AX (5 mg L^{-1}).

As there was no indication of antimicrobial activity, the potential of the product to select for antimicrobial resistances or interfering with the use of antibiotics is remote.

No studies have been reported on the presence of antibiotic resistance in the strain. Due to the nature of the product (a non-viable dried cell preparation), the transfer of any resistance would, in practice, be very unlikely.

No studies have been reported in the dossier concerning the possible effects of the additive on the intestinal bacterial community of salmonids. Since relatively little is known about the gut flora of salmonids (Ringø, 2004), such effects are difficult to evaluate.

[36] Technical Dossier, Section IV, Appendix IV-B

4.1.3. Conclusion on safety for the target species

In the 90-day study on rainbow trout, Panaferd-AX was tolerated at dietary incorporation rate of 12.5-fold greater than the maximum incorporation rate (0.4 %, assuming an astaxanthin content in Panaferd-AX of 2 % and a target concentration in feed of 80 mg astaxanthin kg^{-1}).

In the 56-day study, no adverse effect was observed in the rainbow trout at the highest dose level of Panaferd-AX used (1 %, i.e. 2.5 times the maximum incorporation rate). However, the FEEDAP Panel does not consider this study as a demonstration of safety due to the short trial duration. Therefore, the study can be considered only in support of the findings of the 90-day study.

The FEEDAP Panel concludes that Panaferd-AX, at the proposed maximum dietary incorporation rate of 0.4 %, is safe for salmonids (trout and salmon).

Paracoccus carotinifaciens is not a known pathogen, and no other concerns have been identified either in the limited literature available or in the data submitted in the dossier.

4.2. Metabolism

No specific study on the metabolism of Panaferd-AX has been conducted in the target species; the applicant refers to the literature.

4.2.1. Astaxanthin

In its opinion on the the safety of use of colouring agents in human nutrition (EFSA, 2005), the FEEDAP Panel described the metabolism of astaxanthin in detail. The main conclusions are summarised in this section.

The apparent absorption of astaxanthin varies from 20 to 95 %, with most values comprised between 50 and 70 %. It is determined by several factors, such as: i) fish species and strains, ii) the astaxanthin form – astaxanthin esters being less bioavailable than free astaxanthin and the geometrical isomer all-E being absorbed more efficiently than the Z isomers, whereas no difference was observed for the (3S,3'S), (3R,3'R) or (3R,3'S; *meso*) enantiomers, and iii) dietary factors such as lipid levels.

Astaxanthin 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, then to adonixanthin and finally zeaxanthin. No oxidation occurs in salmonids and therefore, the conversion of zeaxanthin to astaxanthin does not occur.

Astaxanthin has been shown to be a vitamin A precursor for fish, which implies the cleavage of the polyene chain.

After astaxanthin supplementation of feed, the pigment deposited in the flesh of rainbow trout (*Oncorhynchus mykiss*) and chinook salmon (*Oncorhynchus tshawytscha*) is predominantly astaxanthin (about 95 %), and in the Arctic charr (*Salvelinus alpinus*) also idoxanthin (20-35 %). A dose-related increase of astaxanthin in the flesh of trout and salmon was observed, according to the astaxanthin levels in the diet; a plateau was reached in salmon. The composition of the carotenoids deposited in flesh reflects that of the dietary pray organisms or added carotenoids in terms of astaxanthin stereo isomers. All-E isomers are deposited mainly in flesh, whereas Z isomers are preferentially stored in liver and kidney.

4.2.2. Adonirubin

Generally, data are very scarce concerning the biological/chemical properties of adonirubin.

Adonirubin is the intermediary metabolite between canthaxanthin and astaxanthin in the biosynthetic pathway of astaxanthin in micro-organisms, which comprises a two-step hydroxylation at C-3 and C-3' of canthaxanthin (see Figure 1). It is also an intermediary between 3-hydroxyechinenone or asteroidenone and astaxanthin (see Figure 2). A similar metabolic pathway was confirmed in fish (Guillou, 1992) after administration of [^{14}C]-astaxanthin and [^{3}H]-canthaxanthin to mature female rainbow trout (*Oncorhynchus mykiss*) where [^{14}C]-adonirubin was identified in muscle, liver, skin and ovaries. [^{3}H]-astaxanthin was also found in liver, indicating that canthaxanthin is a precursor of astaxanthin, presumably via adonirubin.

A study by Sommer *et al*. (1992) demonstrated that when adonirubin was present in the diet, it was absorbed and found into fish tissues. Schiedt *et al*. (1985) and Matsumo (2001) reported a reductive pathway for the metabolism of adonirubin in the skin of rainbow trout, leading to 3'-hydroxyechinenone then to β-cryptoxanthin. Schiedt *et al*. (1985) showed that a similar metabolic pathway occurred in Atlantic salmon (*Salmo salar*).

4.2.3. Canthaxanthin

In its opinion on the use of canthaxanthin as a feed additive (EC, 2002b), the SCAN described the pharmacokinetics and metabolism of canthaxanthin in fish. This section is summarised as follows, without the corresponding literature references:

i) canthaxanthin is absorbed at a large extent and excreted mainly through the bile as metabolites, about 15 % being retained in the tissues. About 70 % of the dose absorbed is excreted in the feces;

ii) canthaxanthin undergoes reductive metabolism, the reduction of one oxo group leading to 4'-hydroxy-β,β-carotene-4-one and the reduction of the second oxo group to β,β-carotene-4,4'-diol, the end product being β,β-carotene. Furthermore, the loss of one oxo group gives rise to echinenone;

iii) in the rainbow trout, canthaxanthin and metabolites are distributed in tissues as follows, by decreasing quantitative order: skin, liver, kidney and flesh. In the Atlantic salmon, canthaxanthin represents more than 90 % of total canthaxanthin-derived compounds in the flesh, 4'-hydroxyechinenone being a minor metabolite. During sexual maturation, canthaxanthin is mainly deposited in the flesh and skin of males and in the eggs of females.

4.3. Studies on laboratory animals

4.3.1. Acute toxicity

An acute oral toxicity study was conducted in male rats with a *Paracoccus carotinifaciens* cell preparation containing 0.42 % astaxanthin (0.9 % total carotenoids) and suspended in 0.1 % carboxymethylcellulose.[37] Only the summary of the study was made available. No adverse effects were reported up to the top dose of 5556 mg kg^{-1} bw (corresponding to 23.3 mg astaxanthin kg^{-1} bw).

Information on acute toxicity was also provided by the dose-range study conducted as a preliminary to a micronucleus test[38] (see Section 4.3.4). A limit dose of 5000 mg Panaferd-AX dissolved in corn oil (astaxanthin content, 2.56 %, total carotenoid content, 5.97 %) kg^{-1} bw was administered to Sprague-Dawley rats, by oral gavage, on two consecutive days. Only increased salivation and red faeces were observed.

[37] Technical Dossier, Section IV, Appendix IV-F
[38] Technical Dossier, Section IV, Appendix IV-J

4.3.2. Pharmacokinetics

In a toxicokinetic study, 5000 mg Panaferd-AX (astaxanthin content of 1.9 % and total carotenoid content of 4.32 %) kg^{-1} bw were administered by oral gavage on two consecutive days to groups of six male and six female rats. Blood samples were taken prior to administration on both days, and then at two, four and eight hours after administration of each dose. Astaxanthin was detected in plasma at concentrations above the LOQ (0.0171 µg mL^{-1}) and slightly higher than 0.02 µg mL^{-1}, at four and eight hours after both administrations. No astaxanthin was detectable at sampling prior to the second administration, i.e. 24 hours after the first dose administration. The presence of canthaxanthin was detected at those time points, with values below the limit of quantification (0.0126 µg mL^{-1}).

4.3.3. Repeated dose oral toxicity

A 28-day study was conducted in male rats with *Paracoccus carotinifaciens* cell preparation. Since only the summary of the study was made available, no conclusions can be drawn.

In a 90-day study, Panaferd-AX (astaxanthin content of1.9 % and total carotenoid content of 4.32 %) was administered to groups of ten male and ten female Sprague-Dawley rats (five to eight weeks of age) at dietary incorporation levels of 0, 1, 3 and 5 %.[39] Study parameters included functional observation battery for neurotoxicity, ophthalmology, haematology, blood chemistry, urine analysis and full histopathology. Besides red discolouration of the faeces, the fur and the gastrointestinal tract, no adverse effects were observed up to the top 5 % dietary level, which corresponded to mean achieved dosages of 3413 mg kg^{-1} bw day^{-1} in males and 3858 mg kg^{-1} bw day^{-1} in females. This corresponded approximately to 65.0 and 74.4 mg astaxanthin kg^{-1} bw day^{-1}, 42 and 48 mg adonirubin kg^{-1} bw day^{-1}, and 8 and 10 mg canthaxanthin kg^{-1} bw day^{-1}, respectively.

4.3.4. Genotoxicity, including mutagenicity

The following tests were performed with Panaferd-AX:

- Reverse mutation assay (Ames Test)[40] on *Salmonella typhimurium* (strains TA1535, TA1537, TA98 and TA100) and *Escherichia coli* (strain WP2uvrA$^-$), with and without metabolic activation (3.53 % total carotenoid content, 1.66 % astaxanthin, 0.98 % adonirubin and 0.39 % canthaxanthin) (dose range: 50 to 5000 µg $plate^{-1}$);
- Chromosome aberration test[41] in human lymphocytes *in vitro* with and without metabolic activation (3.53 % total carotenoid content, 1.66 % astaxanthin, 0.98 % adonirubin and 0.39 % canthaxanthin) (dose range: 93.8 to 3000 µg ml^{-1});
- L5178Y TK +/- mouse lymphoma cell assay for gene mutation[42] with and without metabolic activation (3.53 % total carotenoid content, 1.66 % astaxanthin, 0.98 % adonirubin and 0.39 % canthaxanthin) (dose range: 250 to 3000 µg ml^{-1});
- Rat bone marrow micronucleus test,[43] conducted at the oral limit dose of 5000 mg kg^{-1} bw of Panaferd-AX (5.97 % total carotenoid content, 2.56 % astaxanthin, 1.51 % adonirubin and 0.67 % canthaxanthin) in corn oil.

No genotoxic activity was observed in any of those tests.

[39] Technica Dossier, Section IV, Appendix IV-L
[40] Technical Dossier, Section IV, Appendix IV-G
[41] Technical Dossier, Section IV, Appendix IV-H
[42] Technical Dossier, Section IV, Appendix IV-I
[43] Technical Dossier, Section IV, Appendix IV-J

4.3.5. Chronic toxicity/Carcinogenicity

No studies were provided in the technical dossier.

4.3.6. Reproduction toxicity, including teratogenicity

No studies were provided in the technical dossier.

4.4. Safety for the consumer

Panaferd-AX is non genotoxic and exhibits very low acute and sub-chronic toxicity. Therefore, the FEEDAP Panel considers that no specific risk for the consumer related to compounds arising from the fermentation process (other than red carotenoids) is likely to occur.

Astaxanthin and canthaxanthin have been assessed by the FEEDAP Panel (EFSA, 2005) and SCAN (EC, 2002b), respectively. The FEEDAP Panel considers that the consumer exposure to astaxanthin and canthaxanthin through the consumption of flesh from salmonids administered Panaferd-AX at the maximum dose (84 mg astaxanthin kg^{-1} feed) would be at the most equal to that resulting from the use of the other astaxanthin-rich additives already authorised, or much lower in the case of canthaxanthin (see Section 3.4.1.). These exposures would be even lower if the suggestion to calculate each xanthophyll in proportion to the maximum content authorised was retained (see Section 3.4.1.).

The safety of adonirubin has not been established. Adonirubin has an intermediary chemical structure between canthaxanthin and astaxanthin (see Section 4.2.2.), which suggests that the toxicological profiles of the three compounds are similar. The available data indicate that this compound is not genotoxic at a maximum dose of 75.5 mg adonirubin kg^{-1} bw in the *in vivo* micronucleus test. No specific data were provided to rule out a possible genotoxicity of adonirubin at higher concentrations up to limit doses usually employed in standard tests (e.g., up to 5000 mg kg^{-1} bw in the micronucleus test). No signs of sub-chronic toxicity were observed at Panaferd-AX doses corresponding to 42 and 48 mg adonirubin kg^{-1} bw day^{-1}.

However, a specific toxicological endpoint concerning the possible crystallisation in retina is worth considering. There is no indication that astaxanthin forms crystals in the retina whereas, for this reason, an ADI has been set for canthaxanthin, based on its lowest NOEL deduced from effects on monkey retina. Taking a worst case approach, it can be hypothesised that adonirubin could behave equally as canthaxanthin in the retina. Consequently, the canthaxanthin ADI should be applied to the sum of canthaxanthin plus adonirubin on a precautionary basis. In that case, the human exposure resulting from the consumption of flesh from fish administered Panaferd-AX at the maximum dose should comply with the ADI. Considering that the deposition of adonirubin in fish flesh represents 2 and 6 mg kg^{-1} and that of canthaxanthin, 0.5 and 1.1 mg kg^{-1}, in salmon and trout respectively (see Sections 3.2.1. and 3.2.2.), the corresponding exposure of the human consumer –refined according to the FEEDAP Panel opinion on MRL's of canthaxanthin (EFSA, 2007)– would be 0.66 mg representing 37 % of the ADI.

4.5. Safety for the user

The dusting potential was not determined. However, the applicant claimed it to be low since a small proportion of the particles were less than 100 µm and the mechanical handling of the product is limited. This claim was confirmed in the worker safety assessment where it was necessary to grind the granules for the inhalation studies. All studies were made with the same product batch as that used for the 90-day toxicity study in trout.

4.5.1. Effects on the respiratory system

An acute inhalation toxicity study was performed in five male and five female rats exposed for four hours to a dust atmosphere of Panaferd-AX, obtained after grinding the product with a nose-only exposure system.[44] The animals were then observed for 14 days before termination. The mean atmospheric concentration was 4.92 (± 0.90) mg L^{-1}.

No mortality, body weight changes or gross lesions were recorded at necropsy. Animals exposed to Panaferd-AX showed increased respiratory rate, laboured respiration, hunched posture, piloerection, fur staining and wet fur. All signs disappeared by day 6 to 8 post-exposure.

4.5.2. Effects on eye and skin

In a study conducted in rabbit, Panaferd-AX showed no potential for skin irritation,[45] and some potential for eye irritation; therefore, the product should be labelled as an eye irritant.[46]

4.5.3. Sensitisation

No study was conducted.

Due to the characteristics of the product Panaferd-AX, the FEEDAP Panel recommends that the product should be considered as a respiratory sensitiser and the appropriate labelling applied.

4.5.4. Systemic toxicity

No repeated-dose toxicity tests upon inhalation or percutaneous exposure were available.

However, due to the lack of significant acute inhalation toxicity (see Section 4.3.1) or sub-chronic oral toxicity (see Section 4.3.3), Panaferd-AX is unlikely to exert any systemic toxicity upon exposure through routes relevant to user safety.

4.5.5. Conclusion on the user safety

Panaferd-AX shows very low acute inhalation toxicity and no potential for skin irritation.

However, Panaferd-AX is considered as an eye irritant. Considering the nature of the product, it should also be considered as a respiratory sensitiser.

4.6. Safety for the environment

Panaferd-AX consists mainly of dried cells of *Paracoccus carotinifaciens* containing at least 20 g astaxanthin, 4-5 g canthaxanthin and 12-13 g adonirubin. The bacteria in the product are dead and are not expected to be of consequence to the environment. The applicant supplied studies from phase I and II of the environmental risk assessment of Panaferd-AX.[47] However, in line with previous opinions of the SCAN (EC, 2003) and the FEEDAP Panel (EFSA, 2004), the organic matter is not of concern but only the red carotenoids.

In its opinion on the safety of use of colouring agents in animal nutrition issued afterwards (EFSA, 2005), the FEEDAP Panel concluded that:

[44] Technical Dossier, Section IV, Appendix IV-N
[45] Technical Dossier, Section IV, Appendix IV-O
[46] Technical Dossier, Section IV, Appendix IV-P
[47] Technical Dossier, Section IV, Appendix IV-T

> 'No data are available for a qualified assessment on the environmental impact of astaxanthin in salmonid feed. Astaxanthin occurs naturally in the habitat of wild living salmonids. Astaxanthin as feed additive for farmed fish substitutes natural sources. As astaxanthin is insoluble in water and susceptible to oxido-reduction it will mainly bind to faeces and sink to the seabed. Idoxanthin and zeaxanthin, the main metabolic excretion products of astaxanthin, occur frequently and in considerable quantities in the environment. In that respect the FFEDAP Panel does not expect that the use of astaxanthin as feed additive to salmon and trout will pose a significant risk to the environment.'

The FEEDAP Panel considers that this conclusion also applies to the other major red carotenoids contained in Panaferd-AX, namely adonirubin and canthaxanthin.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Astaxantin from spray-dried cells of *Paracoccus carotinifaciens* was shown to be bioavailable and efficient in colouring the flesh of Coho salmon, Atlantic salmon and rainbow trout.

In salmon and rainbow trout, astaxanthin deposition in flesh from Panaferd-AX was less efficient than that from synthetic astaxanthin. However, equal astaxanthin doses from both sources, in the dose range of 20 to 100 mg astaxanthin kg^{-1} feed, resulted in a comparable flesh pigmentation. The FEEDAP Panel concludes that the reason for the difference between the above findings can only be attributed to the content of other red carotenoids in Panaferd-AX, mainly adonirubin and canthaxanthin, which are also demonstrated to be deposited in fish flesh.

The technological and organoleptic properties of flesh from Panaferd-AX-treated fish were not different from those of fish treated with synthetic astaxanthin.

In the 90-day study on rainbow trout, Panaferd-AX was tolerated at a dietary incorporation rate of 12.5-fold greater than the maximum incorporation rate. A 56-day study in rainbow trout was not considered in detail due to the short trial duration; however, the results of this study support the conclusions of the 90-day study. The FEEDAP Panel therefore concludes that Panaferd-AX is safe for salmonids (trout and salmon), considering the authorised maximum contents of astaxanthin and canthaxanthin in feed.

Paracoccus carotinifaciens is not a known pathogen and no other concerns have been identified either in the limited literature available or in the data submitted in the dossier.

Panaferd-AX is a non-genotoxic additive of very low acute and sub-chronic toxicity. No specific risk for the consumer related to compounds arising from the fermentation process (other than red carotenoids) is likely to occur.

As consumer exposure to astaxanthin and canthaxanthin after administration of Panaferd-AX at the maximum dose proposed would be at the most equal or less than that resulting from the use of other astaxanthin or canthaxanthin sources, there is no additional risk for the consumer.

The structural proximity of adonirubin, astaxanthin and canthaxanthin suggests that the toxicological profiles of the three compounds should be similar. However, the studies on genotoxicity and sub-chronic toxicity performed on Panaferd-AX are only indicative of the safety of adonirubin. The possible deposition of crystals of adonirubin in the retina, similar to that occuring with canthaxanthin, cannot be excluded and has to be considered as worst case scenario. The calculated consumer exposure to adonirubin plus canthaxanthin derived from the use of Panaferd-AX in salmon and trout complies with the ADI for canthaxanthin (37 %).

Therefore, no additional risk due to adonirubin exposure resulting from the use of Panaferd-AX is likely to occur.

Panaferd-AX shows very low acute inhalation toxicity and no potential for skin irritation. However, Panaferd-AX is considered as an eye irritant. Considering the nature of the product it should also be considered as a respiratory sensitiser.

The FEEDAP Panel does not expect that the use of Panaferd-AX as a source of astaxanthin, canthaxanthin and adonirubin for salmon and trout will pose additional risk to the environment.

RECOMMENDATIONS

Astaxanthin is authorised in the EU as a feed additive[48] to be used with a maximum content of 100 mg kg^{-1} complete feed for salmon and trout. The mixture of astaxanthin with canthaxanthin is allowed provided that the total concentration does not exceed 100 mg kg^{-1} in the complete feedingstuff. In consequence, Panaferd-AX could be used (calculated with the values of Table 2) at a maximum level of 3.86 g kg^{-1} feed, providing 84 mg astaxanthin and 16 mg canthaxanthin kg^{-1} feed. This corresponds to the maximum dose of Panaferd-AX proposed by the applicant. But the total carotenoids would then amount to approximately 160 mg kg^{-1} feed.

In its opinion on the safety of use of colouring agents in animal nutrition (EFSA, 2006b), the FEEDAP Panel suggested that when a mixture of red pigmenting xanthophylls is used, the amount of each xanthophyll should be calculated in proportion to the maximum content authorised. Applying this proposal, Panaferd-AX could be used only at a maximum level of 2.63 g kg^{-1} feed, providing 57 mg astaxanthin (57 % of maximum authorised content) and 11 mg canthaxanthin kg^{-1} feed (43 % of maximum authorised content).

However, it is evident from the efficacy studies with Panaferd-AX that a xanthophyll not listed as a colourant in the EU regulations (adonirubin) plays a significant role in flesh pigmentation. Adonirubin occurs in most biological astaxanthin sources in varying and generally low amounts, whereas in Panaferd-AX it represents about 30 % of the total carotenoids. Information to the fish farmer and the official control (labelling) must refer to the content of astaxanthin, canthaxanthin and adonirubin in the product. Therefore, the FEEDAP Panel recommends that the sum of astaxanthin, adonirubin and canthaxanthin should be considered as the colouring pigments in the additive. For this sum, a maximum content of 100 mg kg^{-1} complete feed for salmon and trout could be applied. The resulting maximum content of Panaferd-AX would then be 2.58 g kg^{-1} feed, providing 56 mg astaxanthin (56 % of the maximum authorised content), 33 mg adonirubin and 10 mg canthaxanthin (42 % of the maximum authorised content).

MODIFICATIONS TO THE REGISTER ENTRY

1. Additive

The applicant proposes Panaferd-AX as the name of the additive. In the view of the FEEDAP Panel, the additive is a red carotenoid-rich bacterium *Paracoccus carotinifaciens* (NITE SD 00017).

2. Description

2.1. Composition, description. The strain *Paracoccus carotinifaciens* NITE SD 00017 should be described as a red carotenoid-rich bacterium. Mentioning astaxanthin alone would not

[48] See Footnotes 4, 5 and 6

characterise the product, considering the significant amount of adonirubin and canthaxanthin in the additive.

2.2. Purity Criteria. The applicant proposes to state a minimum level of astaxanthin of 20 g per kg of the additive. In consistency with previous opinions, the FEEDAP Panel stresses the necessity to give also a maximum content of astaxanthin in the product.

In the view of the FEEDAP Panel, a proper use of the product is only possible if the (maximum) content of astaxanthin, adonirubin and canthaxanthin is given.

The FEEDAP Panel proposes also to add the following purity criteria: As<0.1 mg kg^{-1}, Cd<0.01 mg kg^{-1}, Pb 0.08 mg kg^{-1} and Hg<0.01 mg kg^{-1}. Total dioxins and dioxin-like PCBs (PCDDs + PCDFs + PCBs)≤0.006 pg TEQ g^{-1}.

3. Conditions of use (maximum content)

Because adonirubin from Panaferd-AX essentially contributes in pigmenting fish flesh and represents about 30 % of total carotenoids, the FEEDAP Panel recommends to consider for Panaferd-AX the sum of astaxanthin, adonirubin and canthaxanthin as subject of Regulation. For this sum, a maximum content of 100 mg kg^{-1} complete feed for salmon and trout could be applied.

4. Other provisions and additional requirements for the labelling

The specific conditions or restrictions for use should be adjusted accordingly.

The FEEDAP Panel recommends labelling Panaferd-AX as a respiratory sensitiser and eye irritant.

IMPLICATIONS FOR MRLS

As a worst case scenario, the FEEDAP Panel attributed to adonirubin the same biological properties as canthaxanthin, resulting in a common ADI for canthaxanthin and adonirubin. Consequently, the procedure of setting MRLs is also influenced. In a previous opinion (EFSA 2007), the FEEDAP Panel proposed MRL values for canthaxanthin in salmon flesh of 10 mg kg^{-1} and in trout of 5 mg kg^{-1}, representing 76 % of the ADI. Taking into account the adonirubin contribution, this MRL would be exceeded in trout only, i.e. 7 mg kg^{-1} (measured value). It is therefore proposed to set an MRL for the sum of canthaxanthin plus adonirubin of 8 mg kg^{-1} for trout and of 10 mg kg^{-1} for salmon. The corresponding consumer exposure from fish consumption will increase from 76 % to 84 % of the ADI; the total exposure, including eggs consumption (EFSA, 2007), would not exceed the ADI. In consequence, each analysis of flesh for control of canthaxanthin MRL should include the determination of adonirubin.

Due to potential consequences for consumer safety, any change in the production process that would influence the content, the pattern and the bioavailability of the three main red carotenoids should be subject to a new application for authorisation.

DOCUMENTATION PROVIDED TO EFSA

1. Panaferd-AX (sterilised cells of astaxanthin-rich *Paracoccus carotinifaciens*). July 2006. Submitted by Food Chemical Risk Analysis.
2. Additional documentation for the application for Panaferd-AX (sterilised cells of astaxanthin-rich *Paracoccus carotinifaciens*). January 2007. Submitted by Food Chemical Risk Analysis.

3. Evaluation report of the Community Reference Laboratory on the analytical methods of Panaferd-AX. May 2007.
4. Amendment to CRL report on the dossier related to EFSA-Q-2006-173 (Panaferd-AX). July 2007.
5. Comments from the Member States received through the EFSAnet.

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APPENDICES

APPENDIX A

Executive Summary of the Evaluation Report of the Community Reference Laboratory Feed Additives Authorisation on the Method(s) of Analysis for Panaferd-AX

Panaferd-AX is a feed additive for which authorisation is sought under the category "sensory additives", functional group "colorants: substances which, when fed to animals, add colours to food of animal origin", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Panaferd-AX contains astaxanthin as active substance.

Panaferd-AX is composed of sterilised dried cells of astaxanthin-rich *Paracoccus carotinifaciens* containing at least 20 g/kg of astaxanthin and is intended to be added to salmonid fish feed at a rate providing up to 85 mg/kg as astaxanthin in the final feedingstuff. Panaferd-AX will be added directly to the final feedingstuffs and not prepared in pre-mixtures.

An HPLC (high performance liquid chromatography) method with spectrophotometric detection is proposed for the quantification of the active substance (astaxanthin) in the *feed additive*, *feedingstuffs* and *fish tissues*. The validation of the proposed method has been performed according to the requirements laid down by Commission Directive 2001/79/EC in fish pellet feed and fish tissues. For the determination of astaxanthin in *feedingstuffs* the following performance characteristics were obtained. The percentage of the recovery rate was estimated through blank feed samples fortified with the feed additive at different concentrations and ranged between 80 and 93 %. The obtained precision values, expressed as relative standard deviation were below 3.8 %. The limit of detection (LOD) and limit of quantification (LOQ) were 0.124 and 0.412 mg/kg, respectively. These performance characteristics are considered acceptable and the method is therefore considered suitable for official control purposes in *feedingstuffs*. Performance characteristics have also been provided for the method for the determination of the target analyte in *fish tissue*. However, since there are no Maximum Residue Limits (MRLs) for astaxanthin, the CRL cannot evaluate the suitability of the proposed method for official control of astaxathin in *fish tissue*.

Different control methods are proposed for the identification and quantification of impurities. Most of the proposed methods are classical methods that are often part of the relevant legislation, therefore the proposed methodologies can be considered suitable for the intended purposes.

Further testing or validation by the CRL is not considered necessary.

APPENDIX B

Amendment to CRL report (D08/FSQ/CVH/GS/D(2007) 11114) on the dossier EFSA-Q-2006-173 (Panaferd-AX)

In its report (D08/FSQ/CVH/GS/D(2007) 11114) the CRL-FA evaluated analytical methods based on normal phase High Performance Liquid Chromatography (HPLC) coupled to UV detection for the determination of astaxanthin in feedingstuffs and in fish tissue. Evaluating these methods against their suitability for the determination of other carotenoids showed that the methods also allow for the simultaneous measurement of canthaxanthin and adonirubin – in addition to astaxanthin. Performance characteristics were estimated at various concentrations of the target analytes in feed [1] and fish tissue [2] and the following results were obtained:

For the method for the determination of canthaxanthin in *feedingstuffs* the limit of quantification (LOQ) was 0.25 mg/kg, the percentage of the recovery rate (RR) was at least 87 % or higher and the obtained precision values expressed as relative standard deviation (RSD) were below 3.9 %. For the determination of adonirubin in *feedingstuffs* the RR was at least 87 % and the values for the RSD were below 3.3 %. The validation report did not indicate a LOQ for this compound, but it is most likely below 6.7 mg/kg which is the lowest concentration level at which the experiments for assessing the RSD and RR have been conducted.

For the method for the determination of canthaxanthin in *fish tissue* the limit of quantification (LOQ) was 0.053 mg/kg. The RR was about 59 % at a concentration of 0.09 mg/kg and above 75 % at 5 mg/kg and at higher concentrations. The values for the RSD were below 14 %. For the determination of adonirubin in *fish tissue* the RR was at least 87 % and the values for the RSD were below 12.7 %. The validation report did not indicate a LOQ for this compound, but it is most likely below 0.24 mg/kg which is the lowest concentration level at which the experiments for assessing the RSD and RR have been conducted.

[1] Technical dossier (EFSA-Q-2006-173), Section II, Appendix II-Z12
[2] Technical dossier (EFSA-Q-2006-173), Section II, Appendix II-Z11