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Directorate C - Scientific Opinions
C2 - Management of scientific committees; scientific co-operation and networks

Report of the Scientific Committee for Animal Nutrition on the use of astaxanthin-rich *Phaffia rhodozyma* in feedingstuffs for salmon and trout

Adopted on 6 February 2002

1. BACKGROUND

Astaxanthin-rich *Phaffia rhodozyma* is currently authorised by Commission Regulation (EC) N°2316/98 at national level on a provisional basis not exceeding five years as a colouring matter in feedingstuffs under the conditions set out in table 1.

Table 1: Annex entry proposed by the Company

EEC No.	Additive	Chemical formula, description	Species or category of animal	Maximum Age	Minimum Content	Maximum content	Other provisions
					mg/kg complete feedingstuff		
12	Astaxanthin-rich *Phaffia rhodozyma* (ATCC 74219)	Concentrated biomass of the yeast *Phaffia rhodozyma* (ATCC 74219), killed, containing at least 4.0g astaxanthin per kilogram of additive and having a maximum ethoxyquin content of 2000 mg/kg.	Salmon	-	-	100	The maximum content is expressed as astaxanthin. Use permitted from the age of 6 months onwards. The mixture of the additive with canthaxanthin is allowed provided that the total concentration of astaxanthin and canthaxanthin does not exceed 100 mg/kg in the complete feedingstuff. Ethoxyquin content to be declared.
			Trout	-	-	100	

A new dossier has been submitted in order to get a permanent Community authorisation without time limit.

2. TERMS OF REFERENCE

The Scientific Committee for Animal Nutrition (SCAN) is requested to answer the following questions:

2.1. Product *Phaffia rhodozyma* is claimed to affect the characteristics of salmon and trout products by colouring them. Is the efficacy of this astaxanthin-rich product demonstrated, when used in the feedingstuffs for salmon and trout under the conditions laid down in the background?

2.2. On the basis of the toxicological data, is the use of astaxanthin-rich *Phaffia rhodozyma* safe for:

- the target animals *i.e.* salmon and trout?
- the user (workers' exposure)?
- the consumer, taking into account total dietary exposure?

In assessing the safety of the product for the consumer, the Committee should in particular address the following aspects:

- The metabolic fate of astaxanthin-rich *Phaffia rhodozyma* in salmons and trouts
- The presence of residues in animal tissues, and their qualitative and quantitative composition

2.3. What are the nature and the persistence of the excreted products derived from astaxanthin-rich *Phaffia rhodozyma*? Can these products be prejudicial to the environment?

3. OPINION OF THE COMMITTEE

The dossier contains documents for ***Phaffia rhodozyma***, the products „**Red Star®** ***Phaffia*** **Natural Color**“, “**Red Star®** ***Phaffia*** **Yeast**” and „**Ecotone®** ***Phaffia*** **natural color**” and is extended over a time span of 10 years. The entire dossier consists of an older (April 1996) but comprehensive part (1645 pages), a supplementary dossier (113 pages, June 1997), a more recent (May 1999) supplementary dossier II (95 pages), a small "communication" and a supplementary dossier IV (4 pages) from June 2001.

Before formulating an opinion of the SCAN, it seems helpful to have an overview on the documents (App. = Appendix) with particular attention to the type and origin of the *Phaffia* products tested. The summary of the information provided is presented in tables A, B, C and D in the annex.

3.1. Characteristics of the product

Phaffia yeast is the asexual form of the yeast *Xanthophyllomyces dendrorhous* (Golubev, 1995) which name now takes precedence.

The products „**Red Star**® ***Phaffia*** **Natural Color**“, “**Red Star**® ***Phaffia*** **Yeast**”, later called „**Ecotone**® ***Phaffia*** **natural color**” consist mainly (> 80 %) of killed (at a temperature of 80 ± 5° C, for at least 2 minutes) spray-dried cells of the yeast *Phaffia rhodozyma* (ATCC 74219) and to a minor extent (< 20 %) of brewers yeast (*Saccharomyces cerevisiae)* and contains 2,000 mg Ethoxyquin/kg product. In the supplementary dossier IV it is stated, that the cells are pasteurized at 88° C in a continuous kill loop and heated again before spray drying for 20 – 30 minutes at 82 – 85° C.

The microbiological characteristics are described by a total bacterial count of < 15,000/g, yeast and mould < 60/g, *E.coli* < 3/g, Salmonella negative and live *Phaffia rhodozyma* count < 1/g.

“**Ecotone**® ***Phaffia*** **natural color**” is manufactured basically equally **as „Red Star**® ***Phaffia*** **Natural Color**“, but a further processing step, a kind of wet milling, is made to improve the bioavailability of astaxanthin.

The parent strain was originally obtained from a natural source. The production strain was modified by mutagenesis to improve pigment production. No rDNA techniques were applied. It is stated in the dossier that ATCC 74219 (without more detailed information) “will be used for the commercial product”. In the supplementary dossier it is explicitly stated that ATCC 74219 is used for the production.

Brewers yeast *Saccharomyces cerevisiae* is added to standardize the astaxanthin level of the product, ethoxyquin for protecting astaxanthin against oxidation. However, the dossier **Red Star**® ***Phaffia*** **Natural Color** is not quite clear. It is mentioned that feed yeast is (or can be) probably replaced by “other materials approved for feed use” as well as ethoxyquin by “other approved antioxidants”.

In a later communication (Section I) it is stated that “one or more substances may be added to the product to (a) standardise, or (b) to stabilise the pigment in the product”. Also the Ethoxyquin content of **Ecotone**® ***Phaffia*** **natural color** is given with "max. 2,000 ppm" and not with a definite level.

3.1.1. Chemical composition (Analytical data: 1993)

Red Star® ***Phaffia*** **Natural Color** (Original report of analysis: *Phaffia rhodozyma* yeast, new formulation) contains about (averages of 4 samples) 29-31 % crude protein, 21-29 % lipids. 2.9-3.6 % ash and 39-41 % carbohydrates.

The amino acid pattern shows the common characteristics of yeast proteins. The fatty acid pattern and the mineral content of **Red Star**® ***Phaffia*** **Natural Color** were determined.

It is mentioned that "chemical analysis has shown the heavy metals content of this product to be within the following limits: Total heavy metals (by H_2S) < 10 ppm; Arsenic < 1ppm; Lead < 1ppm; Mercury < 0.01ppm and Cadmium < 1ppm. However details of these analyses are not found.

3.1.2. Astaxanthin

The product contains the carotenoid pigment Astaxanthin (empirical formula: $C_{40}H_{52}O_4$) as naturally synthesised by *Phaffia rhodozyma* during fermentation. The astaxanthin molecule has three enantiomeric forms: 3R,3'R; 3S,3'S and 3R,3'S. *Phaffia rhodozyma* contains predominantly the 3R,3'R enantiomeric form. On the other hand, astaxanthin amounts to > 70 % of the total carotenoids present in *Phaffia rhodozyma*. The nature and amount of the other carotenoids in the product is not reported.

Andrewes, Phaff and Starr (1976) reported that astaxanthin was by far the most abundant carotenoid in *Phaffia rhodozyma* with 83-87 % of the total pigment mixture. Although astaxanthin was isolated principally as the *E*-isomer, every culture of *Phaffia rhodozyma* contained a *Z*- isomer. ß-carotene (2-2.5 % of total carotenoids), echinenone (2-4 % of total carotenoids), 3-hydroxyechinenone (3-4.5 % of total carotenoids) and phoenicoxanthin (5-7 % of total carotenoids) were also isolated and identified. γ-carotene, neurosporene and lycopene were present only in traces. The authors presented evidence for a new carotenoid, 3-hydroxy-3'4'-didehydro-β,ψ-caroten-4-one.

However no comparable data for the production strain ATCC 74219 obtained by classical mutagenesis are submitted.

Red Star® *Phaffia* Natural Color is stated to contain > 4,000 mg astaxanthin/kg, **Ecotone® *Phaffia* natural color** obviously 4,000-6,000 mg astaxanthin/kg *Phaffia* yeast. Finally a table of the dossier showing recommendations for the inclusion of **Red Star® *Phaffia* Natural Color** in fish feed operates with 4 different astaxanthin levels in **Red Star® *Phaffia* Natural Color** (3,000, 4,000, 5,000 and 6,000 ppm).

3.1.3. Stability

Data on the stability of **Ecotone® *Phaffia* natural color** in feed were presented. The studies were started in April 1998. Samples (number not given) were vacuum packed in thermo sealed bags and held at constant temperatures in darkness until assayed by extraction followed by HPLC. The astaxanthin content at the beginning was determined as 4,390 mg/kg. At 6° C storage temperature, the astaxanthin content after 42 days was 97 % of the level at the beginning, after 83 days 75 %, after 112 days 99 %, after 149 days 90 % and after 180 days 96 %. Regression analysis allowed the interpretation, that at 6° C there is nearly zero loss of astaxanthin indicating good stability under refrigeration.

At 30 ° C storage temperature, **Ecotone® *Phaffia* natural color** lost 7 % of its astaxanthin content in 42 days, 33 % after 83 days and about 50 % after 149 and 180 days.

Data on the stability in feed were also presented. Feed processing led to astaxanthin losses depending on the astaxanthin inclusion in the feed. For an inclusion of 40 mg astaxanthin/kg feed the losses by pelleting were 6 % while for an inclusion of 70 mg astaxanthin/kg feed they reached about 11 % (by pelleting or by extrusion). Two weeks after pelleting the astaxanthin in feed was about 75 % of the original level, after 4 weeks about 61 %. Comparable data for the extruded fish feed are not given.

3.1.4. Homogeneity in feed,

The studies were performed with the "**New additive F-266**" (Astaxanthin content reported to the study conductor: 6,380 mg/kg). The additive was studied for its mixing homogeneity in a commercial salmon feed in a plough share mixer and for its segregation behaviour in a pneumatic conveying system. The results show that mixing homogeneity is satisfactory (coefficient of variation 0.066). Segregation was not observed.

3.2. Efficacy trials (fish flesh pigmentation)

Ten trials are listed in the dossier, but two are only protocols (or project descriptions). Another trout experiment is designed as a tolerance study on young fingerlings, it cannot be considered as an efficacy trial under the conditions laid down in the background. Among the remaining eight trials, there are four experiments with rainbow trout and four with salmon (but different species).

Table E in annex summarises the eight fish feeding trials the aim of which was to show the efficacy of astaxanthin-rich yeast as a dietary source of astaxanthin for the pigmentation of salmonid fishes.

From these eight experiments, four are related to rainbow trout (*O. mykiss*), two to atlantic salmon (*S. salar*), one to chinook salmon (*O. tshawytscha)*, one to coho salmon (*O. kisutch*) (these two species are not common in EU). One experiment has some "genus" errors since fish are sometimes called salmon and sometimes trout.

On a scientific point of view only three experiments are to be taken into account. All these three experiments are related to rainbow trout. All other experimental data on salmon can not be accepted due to serious lacks in protocol, in study conduct, disease occurrence and the failure of statistical evaluation, as shown in table E.

3.2.1. Experiments with rainbow trout - Astaxanthin content in fish flesh

Table 2 provides further information on the design of the three experiments with rainbow trout (*O. mykiss*) given in the dossier carried out to show the efficacy of astaxanthin-rich yeast as a dietary source of astaxanthin for the pigmentation of salmonid fish.

Table 2: Summary of the three experiments with rainbow trout *(O. mykiss)*

Criteria	Experiment A (appendix 11)	Experiment B (appendix 12)		Experiments C (appendix II)
Date of trial	1989	1=1989; 2=1991		1998
Fish species	Rainbow trout	Rainbow trout		Rainbow trout
Initial fish weight (g)	180-200	240		255
Astaxanthin sources	*Phaffia*	*Phaffia*/synthetic		*Phaffia*/synthetic
Phaffia process	not mentioned	mechanical/chemical		milled/alkali/enzyme treated
Feed	extruded	pelleted		extruded
Theoretical astaxanthin concentration in the feed (mg astaxanthin/kg feed)	diet "A"= 67 [(1)] diet "B"= 16 diet "C"= 0	diet "A1"= 100 [(2)] diet "B1"= 100 [(3)] diet "C1"= 0	diet "A2"= 30 [(2)] diet "B2"= 30 [(3)] diet "C2"= 0	diet "A"= 60 (intact) diet "B"= 60 (treated) diet "C"= 60 [(3)]
Duration	12 weeks	12 weeks		12 weeks
Water temperature (°C)	8-12	10.6		8-9
Feeding rate (%BW/d)	1.2	1.5		feed in excess
Colour measurement	Minolta	Minolta/Salmo fan		Minolta/Salmo fan
Astaxanthin measurement	Spectrophotometry	Spectrophotometry		HPLC

[(1)] 10 % phaffia [(2)] phaffia origin [(3)] synthetic origin

All trials show serious lacks for further consideration of the experiments. Experiment A was performed with unformulated "*Phaffia rhodozyma*" (probably raw product), experiment B with "*Phaffia* yeast" (product not precisely described) and experiment C with 4 *Phaffia* pilot products. The pilot product "milled yeast" probably corresponds to **Ecotone® *Phaffia* natural color** and another one (untreated yeast) to the original **Red Star® *Phaffia* Natural Color**. But product identity is not given in the trial report (also lacking the name of the dilution-feedstuff and the antioxidant).

Production and formulating time of the *Phaffia* products, storage time were also not known. No analytical data on the astaxanthin content of the products were presented neither data on the potential loss by feed extrusion or pelleting. Finally, for the experiments A and B details of the astaxanthin analysis are not given.

In experiment C, a rather unusual feeding regime was applied.

For the experiments with trout (table 2), the astaxanthin source *Phaffia* seems to give adequate pigmentation of trout flesh after a 12 week feeding period comparable to synthetic astaxanthin as it was shown by physical and chemical methods (experiment A (67 mg astaxanthin/kg feed): 5.54±1.66 mg total pigment (95 % astaxanthin)/kg trout flesh (unpigmented control group: 1.04±0.18); experiment B (100 mg astaxanthin/kg feed): 3.2±0.8 mg carotenoids/kg trout flesh (unpigmented control group: 0.3±0.2; 100 mg synthetic astaxanthin/kg feed: 3.6±1.9 mg/kg); experiment C (61 mg astaxanthin from "milled yeast"/kg feed): 8.2±0.1 mg astaxanthin/kg trout flesh, but also (56 mg astaxanthin from "untreated yeast"): 3.7±0.2 mg astaxanthin/kg trout flesh and (58 mg synthetic astaxanthin/kg feed): 8.6±0.9 mg/kg).

3.2.2 Other data - Astaxanthin content in fish flesh

Table 3 gives some information on the astaxanthin content of fish flesh after feeding rainbow trout and salmon. Different levels of astaxanthin from Phaffia were incorporated in the feed for trout and salmon.

Table 3: Other trout and salmon data - Astaxanthin in fish flesh

Appendix	Species	Duration	Pigment source	Dosis (mg/kg feed)	Astaxanthin in fish flesh (mg/kg)
12	Rainbow trout	12 weeks	*Phaffia*	30	2.0 (1)
13	Chinook salmon	150 days	*Phaffia* ± Ethoxyquin	50	11 (2)
23	Rainbow trout	90 days	Red Star *Phaffia* yeast	53	0.2
I (sup. Dossier (II))	Rainbow trout	12 weeks	4 differently treated *Phaffia* products	55-59	2.7-7.3
III (sup. Dossier (II))	Atlantic salmon	5 months	Ecotone	60	3.6 (1)

(1) determined as total carotenoids (the data of appendix 11 give the ratio of total carotenoids to astaxanthin with 100 : 95).

(2) Initial Astaxanthin ca. 8 mg/kg.

The data of tables 2 and 3 show despite a remarkable variation that astaxanthin from Phaffia preparations colours fish flesh. For **Ecotone®** ***Phaffia*** **natural color** only one experiment is submitted. The four different preparations of Appendices I and II (of the supplementary dossier (II)), table 3 and table 2, respectively, can not clearly be attributed to a commercial product.

3.3. Safety aspects

3.3.1. Safety for the target animal

One experiment on rainbow trout was performed with 2.5, 5.0 and 10.0 % **Red Star® *Phaffia* Yeast** (2 x 20 fish/group) for 91 days. Survival, growth and tissue histology were assessed during and following the 90-day-test. No evidence was found of adverse effects of the test diets to rainbow trout.

3.3.2. Toxicological data

3.3.2.1. Mutagenicity

The results of the Salmonella Reverse Mutation Assay (Ames Test) performed on TA1535, TA100, TA1537, TA1538 and TA98 strains indicate that **Red Star® *Phaffia* Yeast** did not cause an increase in the number of histidine revertants per plate of any of the tester strains either in the presence or absence of microsomal enzymes prepared from Aroclor-induced rat liver (S9).

A mutagenicity test on **Red Star® *Phaffia* Yeast** was performed measuring chromosomal aberrations in chinese hamster ovary (CHO) cells. The product was considered negative for inducing chromosomal aberrations with

and without metabolic activation. A L5178Y TK+/- mouse lymphoma forward mutation assay confirmed **Red Star® *Phaffia* Yeast** as nonmutagenic both with and without S9 metabolic activation.

On the same product also an *in vivo* mouse micronucleus assay was performed**. Red Star® *Phaffia* Yeast** did not induce a significant increase in micronuclei in bone marrow polychromatic erythrocytes.

3.3.2.2.Toxicity

Tests on mice and rats showed acute oral toxicity being > 5000 mg/kg BW.

A 13-week toxicity study was performed with 0, 2.5, 5.0 and 10.0 % **Red Star® *Phaffia* Yeast** on Crl:CD (SD)BR VAF/Plus® rats (20/sex/group). All animals survived until the terminal sacrifice. There were no apparent *Phaffia*-related observations during the course of the experiment. Body weight of the *Phaffia*-groups was higher than that of the controls, but no differences in feed consumption were observed. No test material related ophthalmic observations were noted (examined: corneal ulcerations /dystrophy, acute and chronic dacryadenitis, developmental and cortical lens cataract, retinal atrophy generalized and acute anterior uveitis).

Blood and urine samples were collected for haematology, clinical chemistry and urine analysis tests from 10 animals/sex/group during weeks 5 and 14. At the end, the animals were necropsied. In the control and the 10 % *Phaffia* group, selected organs were weighed (adrenals, brain, kidneys, liver, ovaries, testes, spleen and thymus) and a complete set of relevant organs microscopically examined. Lungs, liver, kidneys and macroscopic lesions were also examined from each animal in the 2.5 and 5 % *Phaffia* groups.

10 % *Phaffia* was associated with mildly higher red blood cell count, haemoglobin concentration, haematocrit and platelet count and mildly lower cholesterol level in males after 13 weeks. Dietary administration of **Red Star® *Phaffia* Yeast** did not cause any biologically important changes in the anatomical pathology data. Macroscopic and microscopic findings noted in the organs and tissues examined indicated no major differences between control and treated animals in the incidence of any finding. All findings were considered incidental and unrelated to the test material.

In conclusion, feeding **Red Star® *Phaffia* Yeast** up to 10 % in diet for 13 weeks does not result in adverse effects in rats.

3.3.2.3.Dermal irritation

A skin irritation study was conducted on intact rabbit skin (in compliance with Council Directive 67/548/EEC, Method B4). Well defined or very slight erythema were apparent at two dermal test sites, very slight erythema persisted for at least 48 hours. No oedematous reactions or other dermal changes were observed.

The product does not require labelling as a skin irritant.

3.3.3. Metabolism in fish

Astaxanthin is a complex mixture of stereo and geometrical isomers. The composition of astaxanthin deposited in fish muscle, skin and ovaries reflects the feeding sources of the animal. Wild Pacific salmon flesh contains predominantly either the 3R,3'R or the 3S,3'S stereoisomers depending on the dietary prey organism, while only a small percentage of the 3R,3'S isomer is present. The percentage of all-E- and Z- forms (structural) of each isomer varies slightly with fish species, feeding regimes and environment, the Z form representing less than 15 % of the E one.

Astaxanthin from *Phaffia rhodozyma* is mainly (97 %) composed of the free, non esterified, 3R,3'R stereoisomer, with an E/Z ratio of 60:40. It must be noted that astaxanthin from crustaceans is under the same isomeric form, but esterified by long chain fatty acids as mono and diesters.

In comparison, synthetic astaxanthin (Carophyll Pink) used in industrial fish farms is a racemic mixture of (3R,3'R), (3R,3'S *meso*) and (3S,3'S) in a 1:2 :1 ratio, with an E/Z ratio of 75 : 25.

The metabolic fate of astaxanthin has been studied in salmonids. The deposition of substantial amounts of the pigment in different tissues and organs indicates that it is largely absorbed, absorption values are in a range between 17 and 97 % depending on the astaxanthin source and fish species (table 4).

Astaxanthin apparent absorption, defined as the difference between ingested astaxanthin and the amount recovered in the faeces, is given in **Table 4** for various salmonid species.

Table 4: Intestinal absorption (%) of astaxanthin in different salmonid species

Astaxanthin source	**Rainbow trout**	**Atlantic salmon**	**Sea trout**	**Reference**
Shrimp meal	79	--	--	Choubert, 1977
Capelin red oil	17-94	--	--	Choubert, 1977
Mixture (50:50) of synthetic astaxanthin + canthaxanthin	91-97	--	74-96	Foss *et al.,* 1987
Synthetic	51-70	--	--	No and Storebakken, 1991
Synthetic	--	45-74	--	Storebakken *et al*, 1987
Synthetic	--	46-59	--	Bjerkeng and Berge, 2000

It has been shown that astaxanthin and/or metabolites are excreted through the bile to a very significant extent, but the nature of these compounds has not been established. The significantly higher fecal carotenoid concentration (ca. 50 %) of trout fed an all-E/Z astaxanthin diet when compared to those receiving an all-E diet was due to a higher concentration of astaxanthin Z

isomers. Together with a similar fecal all-E-astaxanthin concentration for both treatments, it suggests that intestinal absorption of 9Z- and 13Z-astaxanthin might be lower than for the all-E isomer (Osterlie et al., 1999). Similarly, other authors have found that the digestibility coefficient of all-E-astaxanthin in salmon was higher than that of the 9Z isomer (Bjerkeng and Berge, 2000). When astaxanthin esters are concerned it appears that the hydrolysis of the ester linkage is a limiting step of the bioavailability of astaxanthin for trout and salmon (Torrissen and Braekkan, 1979; Schiedt and Leuenberger, 1981; Storebakken et al., 1987). However, the hydrolysis step is more efficient for (3R,3'R)- than for (3S,3'S)-astaxanthin esters.

Astaxanthin is metabolised by salmonids to zeaxanthin through a double step reduction at the 4 and 4'-oxo groups leading to the successive intermediary compounds idoxanthin then adonixanthin. The configuration of astaxanthin does not influence the reduction of the 4'-oxo group, but the enzymatic reduction is stereospecific leading to the 4'R-hydroxy group irrespective of the configuration at C (3'). Therefore, the reduction of the 3 astaxanthin stereoisomers leads to 4 idoxanthin isomers instead of 8 theoretically, i.e. the Z forms (3S,3'S,4'R) and (3R,3'S,4'R) and the E forms (3S,3'R,4'R) and (3R,3'R,4'R), which proportions are identical to those of the original astaxanthin. Racemic astaxanthin (1 :2 :1) gives rise to 3S,3'S-zeaxanthin and to a lesser extent 3R,3'S-zeaxanthin, while the 3R,3'R isomer appears at very low level (Schiedt *et al.*, 1988). In the trout, the (3S,3'S)-astaxanthin leads to the (3R,3'R)-zeaxanthin. These data indicate that the epimerization from 3S- to 3R- and *vice versa* occurs *in vivo* (Katsuyama *et al.*, 1987). No signifiant *in vivo* E/Z isomerization has been observed (Schiedt *et al.*, 1981 and 1989; Osterlie *et al.*, 1999).

Following the administration of astaxanthin (*Phaffia rhodozyma* as the source or synthetic Carophyll Pink) at 50 ppm level in diets for rainbow trout (Section II, 2.1., ref. Appendix 12, 1991) and chinook salmon (Section III, 2.2., ref. Appendix 13, 1993) for 84 and 150 consecutive days respectively, 95 % and 96 % of the pigments deposited in the flesh have been found as unchanged astaxanthin, with very minor quantities of idoxanthin and zeaxanthin. In the Arctic charr (*Salvelinus alpinus*) fed racemic (1:2:1) synthetic astaxanthin under similar experimental conditions, free non esterified astaxanthin comprised 64-79 % of flesh carotenoids, idoxanthin accounting for 20-35 %. The corresponding figures for the skin were 85 % and 10 %, but astaxanthin and idoxanthin consisted mainly of esters (diesters: 82-87 %, monoesters: 7-13 %). Minor amounts of tunaxanthin, lutein and zeaxanthin were present. In the ovaries of sexually maturing or immature female charr, idoxanthin was the major carotenoid (56 %) followed by crustaxanthin (20 %) and astaxanthin (< 5 %) (Bjerkeng et al., 2000).

The incidence of the distribution of the stereo and geometrical isomers of both sources of astaxanthin, *i.e. Phaffia rhodozyma* (R,R' ; E/Z ratio 60:40) and the synthetic compound Carophyll Pink (R/S racemic 1:2:1 ; E/Z ratio 75:25) on the composition of the deposits in the flesh of the chinook salmon has been studied (Section III, 2.2., ref. Appendix 13, 1993). The results are summarized in Table 5.

Table 5: Percentage of astaxanthin isomers in the flesh of chinook salmon fed different astaxanthin sources supplying 50mg/kg diet

	Phaffia rhodozyma	Synthetic astaxanthin	Not supplemented (natural control population)
All-E ; R,R'	42	23	23
All-E ; R,S' and R',S	30	42	40
All-E ; S,S'	19	29	29
Z ; R,R'	4	1	2
Z ; S,S'	2	2	3

Both the Z-(3R,3'R) and Z-(3S,3'S) geometrical astaxanthin isomers brought by *Phaffia rhodozyma* and synthetic astaxanthin do not accumulate in the flesh. No major change in the distribution of the all-E-(R,S and S,S)-astaxanthin isomers is observed when comparing the composition of the flesh of fish receiving a non supplemented diet to that of those fed the two different pigment sources. Only increase of the all-E-(3R,3'R) isomer content is observed with the yeast that corresponds to the specific composition of the derived astaxanthin.

It must be noted that very limited information is available concerning the metabolic fate of astaxanthin in the rat. It has been shown recently that astaxanthin biotransformation by rat hepatocyte primary cultures consists in the asymetrical cleavage of the polyene chain of the molecule at the C9 position (Wolz et al., 1999). These preliminary results indicate a metabolic pathway which is very different from that described in fish.

3.3.4. Safety for workers

Studies on the dusting behaviour were performed with F-225. In studies (4 runs) with the Stauber-Heubach method (EC-Directive 70/524/EEC) no dust could be detected. By sieving, particle size could not be investigated due to the agglomerate characteristic of F-225. Results obtained by laser diffraction showed, that the percentage of mass for particles < 10 µm is 10 % by liquid dispersion and 6 % by dry dispersion. The laser test in oil may reflect a measure of particle size distribution of the bulk powder before any dispersion in air, dry dispersion the human exposure during handling. The laser test procedure was repeated with **Ecotone** (Lot #99K1). Less than 7 % of the particles (% under the cumulative volume distribution curve) are smaller than 11 µm.

The company assures that the product label will also contain provisions for labour protection and safety advices as follows:

– wearing of respiratory protection device with particle filter class P1 required

– wearing of eye protection devices required

– avoid direct skin contact

3.3.5. Safety for the consumer

The exposure of the human consumers to astaxanthin through fish consumption is qualitatively the same whatever the astaxanthin source in fish diet, *i.e.* naturally occurring, synthetic or *Phaffia rhodozyma* origin. As the objective of astaxanthin supplementation of fish diet is to obtain a sufficient but limited flesh deposit satisfying market needs for coloured flesh, the synthetic and *Phaffia rhodozyma* sources of that pigment can be considered as substitutive, and therefore the quantitative exposure of the consumer should remain the same. However no data for the maximum residue after feeding *Phaffia* at the highest authorised level of inclusion of astaxanthin in feed are submitted. Although no ADI has been established yet for astaxanthin, it can be concluded that if any risk would exist, the introduction of astaxanthin through *Phaffia rhodozyma* would not contribute to increase it.

As the compound *Phaffia rhodozyma* is not intentionally a biomass as feedingstuff, but a colouring agent with the biomass as a "carrier", and as the carrier will not exceed 2.5 % in the complete feedingstuff due to the limitations of astaxanthin in complete feedingstuffs set by Directive 70/524/EEC, the 90 day study is considered sufficient.

3.3.6. Impact on environment

Data with environmental concern are not provided in the dossier. The company states that there would be no need to study the excreted residues, because (i) astaxanthin is present in nature, (ii) the product is a true (dead) yeast and as yeast an accepted feed ingredient.

Undigested yeast compounds will be excreted. SCAN agrees that these yeast compounds will probably contribute to the organic load of water only to a negligible extent.

However, SCAN is of the opinion that there is no justification for the absent-ce of any data, because not absorbed astaxanthin could amount up to 50 % of the ingested (table 4). Its fate as well as its influence on other water living organisms need not to be without any environmental impact, as long as no data are presented.

4. CONCLUSIONS

The company submitted a dossier for **Red Star® *Phaffia* Natural Color** and **Ecotone™** (**Ecotone® *Phaffia* natural color**), products which in all cases are described as a mixture of the fermentation product *Phaffia* yeast with an antioxidant and a (suitable) dilution agent**. Red Star *Phaffia* Yeast** (formulation not given), "**Red Star® *Phaffia* Yeast Natural Color**" and "**Ecotone® *Phaffia* natural color**" are not identical products. Even the formulation of "**Red Star® *Phaffia* Natural Color**" will not be maintained constant according to the statement of the manufacturer. Therefore product conformity is not supported by the dossier.

Obviously, ***Phaffia* yeast** and **Ecotone™** or **Red Star® *Phaffia* Natural Color** are different. Even **Red Star® *Phaffia* Natural Color** and **Ecotone** seem to be different because of a different manufacturing process. However, the (proprietary) manufacturing process for Phaffia in **Ecotone** to weaken the cell structure is not described. If the manufacturing process applied for **Ecotone** improves the availability of astaxanthin from Phaffia, then it can not be excluded that the bioavailability of other (all?) cell constituents is consequently also changed (may be improved).

Because astaxanthin in *Phaffia* yeast is not stable during storage, the addition of an antioxidant is foreseen by the company. Information, in particular through labelling, on the quality and quantity of the antioxidant used is missing. This lack should be corrected.

The carotenoid fraction of the product is not characterised. The primary coloring substance in *Phaffia* yeast is astaxanthin. Several Studies (Johnson *et al*., 1980; and unpublished but submitted data in the dossier) show that *Phaffia* yeast pigments the flesh of fish when it was fed to salmonid fish.

The supplementation level of *Phaffia* yeast (**Ecotone**), the company wants to apply for, is also not given. In practice, for the purpose of management of flesh colour, an astaxanthin containing product can only be used if its astaxanthin content is clearly established.

According to Directive 70/524 EEC, the astaxanthin concentration should not exceed 100 mg/kg fish feed. The Phaffia product/products (**Red Star**, **Ecotone**) are stated to contain > 4,000 ppm astaxanthin, but the variation is given with 4,300 – 5,500 ppm. This variation may result in difficulties for the feed manufacturer to guarantee the maximum content of 100 ppm in a complete feed is not exceeded as well as to label the realistic astaxanthin concentration, which the feed manufacturer has to label. In addition, from a consumer point of view, dietary astaxanthin variations as a result of the content of astaxanthin in *Phaffia* will also impact its level in fish flesh.

The data presented for efficacy of the *Phaffia* products in rainbow trout can not be accepted for evaluation. Generally all experiments concerning Phaffia should contain statements on (a) the nature of the yeast (and composition of the yeast preparation) used, (b) the technological process of yeast treatment (and of the preparation, if treated), (c) the time period between production of the yeast and the start of the experiment, (d) the amount of the yeast preparation included in the feed,

(e) the analysed amount of astaxanthin in the yeast (yeast preparation), (f) the analysed amount of astaxanthin in fish feed (preferably at the start and at the end of the feeding trial) and (g) the concentration of astaxanthin in fish flesh feed (preferably at the start and at the end of the feeding trial). These informations are not given.

The safety aspects for the yeast *per se* are satisfactorily demonstrated. Concerning the active ingredient astaxanthin, questions on the maximum quantitative residues in fish products and their consequences for the human consumer, as well as on its environmental impact remain still open.

The SCAN wishes to draw the attention of the Commission

- to the fact that this report relates only to an astaxanthin rich mycelium and not to astaxanthin itself. The active colouring substance, astaxanthin, should undergo a dedicated risk assessment
- to the high degree of variation of biological products and consequently on the fact that the outcome of a scientific evaluation has to be linked with the dossier and the data considered. It cannot therefore be applicable to other products, although of the same kind, through a generic authorisation system.

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Annex: Summary of the reports

Table A: from the original dossier

App.	Date of report	Title (abbreviated)	Product	Asta (%)	Comments
1	11/04/95	Method for determination of particle size	Red Star® *Phaffia* Natural Color	no data	
3	04/95	Composition of Red Star *Phaffia* Natural Color	Red Star® *Phaffia* Natural Color	no data	4 samples from oct. 93
4	21/12/92	Spectrophotometric analysis of astaxanthin	Phaffia		limit of detection: <0.17 ppm limit of quantification 0.5 ppm
5	05/95	Storage stability test of Red Star *Phaffia* Natural	Red Star® *Phaffia* Natural Color	0.4-0.5	6 boxes of 5 batches
6	12/94	Astaxanthin stability in the production of extruded feed	Phaffia	0,47	
7	03/95	Storage stability in steam pelleted feeds for salmonid	Red Star® *Phaffia* Natural Color	0.3	
8	08/91	Stability in moist feeds for salmonid fish	Red Star® *Phaffia* Natural Color	0.2	
9	16/10/91	Material safety data sheet	Red Star™ *Phaffia rhodozyma* yeast		
10	12/92	Test for viable *Phaffia* rhodozyma cells			
11	1989	Evaluation of *Phaffia rhodozyma* for salmonids (trout)	*Phaffia rhodozyma*		Astaxanthin in flesh determined
12	1989–91	Evaluation of *Phaffia* rhodozyma for salmonids (trout)	*Phaffia* yeast		Carotinoids in feed and flesh determined
13	8/02/93	Assimilation of Astaxanthin from Phaffia rhodozyma by the Chinook Salmon	Formulated *Phaffia* "Red Star *Phaffia* Yeast"		Carotinoids in eggs and flesh determined, and astaxanthin
14	1991–92	Evaluation of *Phaffia* rhodozyma for salmon	*Phaffia rhodozyma*		ca. 30 % mortality
		Standard color card for salmonids			
	20/10/95	Feeding trial with atlantic Salmon	Red Star® *Phaffia*		40 - > 50 % mortality, both cages combined!
17	04/95	Organoleptic evaluation of fish fed diets containing	Red Star® *Phaffia* Natural Color		Chinook salmon
18	09/92	Effect of cold smoking	See 17		
19	01/93	Method for use of Minolta colorimeter			
20	23/02/94	Acute oral Toxcicity in mice	Red Star® *Phaffia rhodozyma* inactive dried yeast	> 0.4	LD_{50} >0.5 g/kg
21	23/02/94	Acute oral Toxicity in rats	See above	> 0.4	LD_{50} >0.5 g/kg
22	28/02/95	13-week toxicity in rats	Red Star® *Phaffia*		2.5 – 10 % in feed
23	1/08/95	A 90-day feeding study with the rainbow trout	Red Star® *Phaffia* Yeast		Tolerance study 2.5 – 10 % *Phaffia* By asta-analyses 1.3 – 7.2 % *Phaffia*
24	15/04/94	Mutagenicity test Chromosomal aberrations in Chinese Hamster Ovary	Red Star® *Phaffia* Yeast		
25	15/04/94	Mutagenicity test In vivo mouse micronucleus assay	Red Star® *Phaffia* Yeast		
26	20/05/94	Mutagenicity test Mouse lymphoma forward mutation assay	Red Star® *Phaffia* Yeast		
27	25/04/94	Mutagenicity test Salmonella/mammalian-microsome reverse mutation assay (Ames Test)	Red Star® *Phaffia* Yeast		

Table B: From the supplementary dossier of June 1997

App.	Date of report	Title (abbreviated)	Product	Asta (%)	Comments
1	June 6, 1997	ATCC statement			
2		Stability = APP. 5			Explanation
3	April 23, 1997	Skin irritation study	Phaffia Yeast		
4	May 20, 1997	Dusting behaviour	New Additive F-225*		
5	May 20, 1997	Particle size distribution	New Additive F-225*		
6	May 20, 1997	Particle size (by laser diffraction method)	New Additive F-225*		
7	June 4, 1997	Respiratory sensitation	Opinion on laser diffraction		
8	July 4, 1997 July 4, 1997	Mixing homogenity Mixing homogenity	New Additive F-266 New Additive F-266 See above		Mixed feed analysed for astaxanthin
9	Aug. 25, 1995	Analytical method for astaxanthin			
10		Flesh pigmentation (App. 12)			Corrected figures

Table C: From the Supplementary Dossier II of May 1999

App.	Date of report	Title (abbreviated)	Product	Asta (%)	Comments
1	Dec. 14, 1998	Assessment of improved *Phaffia* rhodozyma products	4 pilot products		Trout pigmentation study
2	March 2, 1999	Differently processed cells of the red yeast *Phaffia*	4 pilot products		Trout pigmentation study; feed analysed for astaxanthin
3	May 17, 1999	Ecotone efficacy in Atlantic Salmon compared to synthetic Astaxanthin	Ecotone Interim report	analysed	Astaxanthin in flesh; no growth
4	May 24, 1999	Protocol for commercial scale Salmon and sea trout feeding trial			
5	May 26, 1999	Project description: Biological evaluation of Ecotone Atlantic Salmon			
6	May 10, 1999	Ecotone stability study 6° and 30° C (in feed, storage) 180 days	Ecotone		
7	May 17, 1999	Particle size distribution (by laser diffraction)	Ecotone Lot #99K1		

* New Additive F-225 is described as *Phaffia* Yeast (p. 5, Supplementary Dossier)

Table D: From the supplementary dossier IV of June 2001

Statements on the manufacturing process
Statements on the effect of milling on particle size

Table E: Summary of the eight experiments given in the dossier carried out to show the efficacy of astaxanthin-rich yeast as a dietary source of astaxanthin for the pigmentation of salmonid fish.

Appendix.	**Dossier section**	**Title of the trial**	**Comments**
# 11	Book 2, 181-250	An evaluation of *Phaffia* rhodozyma as a dietary source of astaxanthin for salmonids, using the rainbow trout as a model.	***Can be considered***
# 12	Book 2, 252-295	An evaluation of *Phaffia* rhodozyma as a dietary source of astaxanthin for salmonids, using the rainbow trout as a model: A comparison to synthetic astaxanthin	***Can be considered***
# II	Supplementary dossier II, 36-49	Differently processed cells of the red yeast, *Phaffia* rhodozyma, in comparison with chemically synthesised astaxanthin as astaxanthin sources for rainbow trout (Oncorhynchus mykiss).	***Can be considered***
# I	Supplementary Dossier II, 13-35	Assessment of improved *PHAFFIA RHODOZYMA* in products to pigment rainbow trout *(ONCORHYNCHUS MIKYSS)*	*Not considered* *No statistics*
# III	Supplementary dossier II, 52-55	Ecotone efficacy in Atlantic salmon compared to synthetic astaxanthin	*Not considered* *Interim report*
# 13	Book 2, 297-417	Assimilation of astaxanthin from *Phaffia* rhodozyma by the Chinook salmon (Oncorhynchus tschawytscha)	*Not considered* *No replicates* *No statistics*
# 14	Book 2, 419-454	Evaluation of *Phaffia* rhodozyma as a nutritional source and pigment for salmon	*Not considered* *No replicates* *No statistics* *High mortality*
# 16	Book 2, 458-484	Commercial scale feeding trial with Atlantic salmon (Salmo salar) to test the efficacy of red star *Phaffia* natural color as a dietary source of astaxanthin under european aquacultural conditions	*Not considered* *No replicates* *Disease problem* *No statistics*