Appendix 3

CAP 7C0211

ROCHE VITAMINS AND FINE CHEMICALS
HOFFMAN-LA ROCHE INC.

ASTAXANTHIN AS A PIGMENTER IN SALMON FEED

VOLUME 2 OF 7

(D)(1) ASTAXANTHIN: HUMAN FOOD SAFETY SUMMARY

(See Volumes II through VI for complete reports)

In another trial, the acute toxicity of astaxanthin in rats was determined following administration of 10 consecutive daily oral doses ranging from [illegible] to 2000 mg/kg b.w. There was no mortality and no symptoms of toxicity were reported.

Lethal doses in mg/kg p.o.	24 h after compound administration	24 h after 5 days of daily compound administration	24 h after 10 days of daily compound administration	10 days after 10 days of daily compound administration
LD 10	over [illegible]	over 2000	over 2000	over 2000
LD 50	over [illegible]	over 2000	over 2000	over 2000
LD 90	over [illegible]	over 2000	over 2000	over 2000

II. Mutagenicity

a) Ames Test

Astaxanthin at concentrations ranging from 0.03 to 5.0 mg/plate did not induce mutations in Salmonella typhimurium tester strains TA 1535, TA 1537, TA 1538, TA 98, and TA 100 with or without activation by a rat liver homogenate fraction.

b) Micronucleus Test

Astaxanthin in the form of the 10% gelatin beadlet was administered orally to mice at 30 hours and again at 6 hours prior to sacrifice. Dosage was 500, 1000, and 2000 mg/kg b.w. of the 10% gelatin beadlets. No compound-related increase in micronuclei was observed. Astaxanthin at the doses tested induces neither chromosome breaks nor mitotic disjunction in vivo.

III. Teratology and Embryotoxicity Studies

a) Rabbits

Astaxanthin was tested for embryotoxic and teratogenic effects in rabbits in accordance with the guidelines established by the American FDA and English CSM. Doses of 100, 200 and 400 mg/kg/day were administered orally to pregnant animals from day 7 to 19 inclusive of gestation. A control group received the vehicle for the same treatment period. All females were sacrificed at day 30 of gestation. Fetuses were removed by ovariohysterectomy, tested for viability (24 hrs.) and examined for macroscopic, skeletal and visceral and soft tissue anomalies.

The test compound was well tolerated by all females receiving treatment. During the course of the study there were neither overt signs of maternal sensitivity to the treatment nor significant changes in body weight development between the dams from the treated groups and those of the controls. In the low (100 mg/kg) and intermediate (200 mg/kg) dose groups the measured reproductive and litter parameters plus, course and outcome of pregnancy were unaffected, with all values comparing favorably to the concurrent controls. There was also no evidence of drug-related malformations among the examined fetuses. At the highest dose of 400 mg/kg, the only abnormal finding among the various measured reproductive parameters was a nominal increase in the incidence of resorptions (37.7%). However, this finding was not dose-related and failed to achieve statistical significance, due mainly

IV. Reproductive Performance Study in Rats

Astaxanthin was tested in accordance with the guidelines of the American FDA and the English CSM for effects on fertility and general reproductive performance of the rat and on the in utero and postnatal development of the F1-offspring to time of weaning. The study includes the assessment of later development and of the reproductive capability of selected F1-offspring retained beyond weaning.

Doses of 25, 100 or 400 mg/kg/day were administered by oral gavage to 32 male rats/group, beginning 70 days prior to mating and continuing until sacrifice, and to 32 females/group beginning 14 days prior to mating and continuing through gestation until sacrifice or weaning. Control animals (32/sex) received the vehicle (rape-oil, 2 ml/kg) in a comparable regimen.

About half of the mated females in each group were sacrificed on about gestation day 14, while the remaining females were allowed to litter. F1-pups of selected litters were evaluated for developmental indices during lactation. On lactation day 23, selected weanlings were retained for learning and memory testing or the assessment of their reproductive capability.

The results of the study can be summarized as follows:

P-GENERATION

No substance-related mortality in males or females was observed in any of the dosage groups. The body weight gain of both P-males and P-females in all experimental groups matched that of concurrent controls.

The percentage of males which mated their partners, as well as the ratio of mated to pregnant females and the median precoital time were comparable between all groups.

Up to 400 mg/kg, the highest dose tested, the reproductive parameters of females sacrificed between gestation days 14 and 16 were within normal limits.

F1-GENERATION

In all experimental groups, the litter parameters such as the body weight gain of pups, the time of onset of developmental landmarks and the learning and memory ability matched that of the controls.

The neonatal mortality of the F1-generation in the highest dosage group (400 mg/kg) was at the upper limit of the biological range (25.6%). However, there was no statistical significance for this finding and no dose-relationship was evident. Therefore a substance-related impairment of pup viability was considered to be very unlikely.

In all dosage groups, the macroscopic and soft tissue examination of pups found dead during lactation showed isolated findings which were not considered to be substance-related. These included hematoma in the lung, empty stomach, and dilated renal pelvis and ureter. The gross examination of weanlings for malformations, as well as for liver and kidney weights, yielded normal findings. One neonate in the low-dose group (25 mg/kg) exhibited unilateral anophthalmia. This isolated anomaly was not considered to be substance-related.

The reproductive capability of F1-animals was not adversely affected in any of the experimental groups. The number of F2-pups which died or were cannibalized between lactation days 1 and 4 was unusually high in all groups, controls included, and, therefore, was considered to have resulted from other than substance-related influences.

It can be concluded from the results of this study that the no-effect-level of astaxanthin given by oral gavage to male and female rats during gametogenesis, mating, gestation and lactation was 400 mg/kg/day, the highest dose tested in this study.

V. 13-Week Tolerance Study in Rats

Astaxanthin in the form of gelatin beadlets was added to the feed of rats at concentrations of 6.25%, 12.5%, and 25% of beadlets. This corresponds approximately to an intake of 310, 620, and 1240 mg of astaxanthin/kg b.w./day, at the start of the study. Through the use of placebo beadlets, all groups, including the controls, received the same amount of beadlets in their feed. The concentrations of astaxanthin in the feed were kept constant during the whole study. Food wastage and avoidance of astaxanthin containing beadlets were minimal.

No astaxanthin related mortality occurred, body weights of dosed and control animals were similar.

Faeces of astaxanthin-fed animals were colored reddish. Yellow pigmentation of adipose tissue was observed at autopsy.

Focal to extensive alopecia with some tendency for reversal was observed in all groups.

There were no ophthalmoscopic findings related to the feeding of astaxanthin. Decreased organ weights in experimental groups were recorded for kidney (males, except at 6.25%), ovary (except at 6.25%), uterus, adrenals (females) and spleen (males and females). Histology of these organs was similar to the controls.

The hematology and blood chemistry parameters were within or close to the normal range, with the exception of decreased total serum proteins levels (week 13: males in 12.5% and 25% groups), and liver enzyme activities of several animals, which were sporadically increased, most probably as a result of parasite or Nosema infestation. Plasma cholesterol was elevated in all treated groups, but values were still within normal limits. Slight elevation of protein in urine (up to 200 mg/100 ml) was randomly found for animals of all groups with a number of cases in the 25% male group on week 13.

It can be concluded that the administration of astaxanthin at the above mentioned concentrations had been well tolerated and that no apparent toxicity, attributable to astaxanthin was observed.

VI. 13-Week Tolerance Study in Dogs

Astaxanthin in the form of gelatin beadlets containing 6.1% w/w of astaxanthin was administered for a period of 3 months to 3 groups of six dogs each (3 males + 3 females), at concentrations of 2.5%, 5% and 10%

w/w of beadlets by means of feed admix. Average astaxanthin intake for the 13-week period was 41, 76, and 162 mg/kg b.w./day for the 2.5%, 5%, and 10% groups, respectively.

Six additional dogs were kept as controls and received a feed admix containing placebo beadlets.

The test compound was well tolerated for the entire period of 3 months and did not cause any adverse effects or show any evidence of a systemic toxicity with regard to the general state of health, the body weight development, the behavior of the dogs, the hematological and clinical chemical parameters, the ophthalmoscopy, and the autopsy and histological appearance of the organs.

(E) HUMAN CONSUMPTION OF ASTAXANTHIN

(E) Human Consumption of Astaxanthin

The present Color Additive Petition provides for the use of nature-identical synthetic astaxanthin as a pigmenter in the feed of salmonid fish. Such a use will not change the consumption pattern of astaxanthin.

Astaxanthin is the natural pigment in salmonid fish, shrimp, and lobster, all of which have been part of the human diet for a long time. Replacement of a small fraction of the astaxanthin of crustacean origin by nature-identical synthetic astaxanthin will not change present human consumption of or exposure to astaxanthin.

(F) PROPOSED REGULATION

(F) Proposed Regulation

§73. _____ Astaxanthin

(a) Identity. (1) The color additive astaxanthin is 3,3'-dihydroxy-β,β-carotene-4,4'-dione.

(b) Specifications. Astaxanthin shall conform to the following specifications:

Physical state, solid.
0.05 percent solution in chloroform, complete and clear.
Absorption in chloroform, maximum 488-493 nm.
Residue on ignition, not more than 0.1 percent.
Total carotenoids other than astaxanthin, not more than 4 percent.
Cis-astaxanthin, not more than 2 percent.
Lead, not more than 5 parts per million.
Arsenic, not more than 2 parts per million.
Heavy metals, not more than 10 parts per million.
Assay, minimum 96 percent.

(c) Use and restrictions. (1) The color additive may be safely used in the feed of salmonid fish (salmon and trout) to enhance the pink to orange-red color of the flesh in accordance with the following conditions: (i) The quantity of astaxanthin incorporated in the feed shall not exceed 200 parts per million (180 grams per ton) of finished feed.

(d) Labeling requirements. The labeling of the color additive and any premixes prepared therefrom shall bear in addition to the information required by §70.25 of this chapter:

(1) Adequate directions to provide a final product complying with the limitations prescribed in paragraph (c) of this section.

(e) Exemption from certification. Certification of this color additive is not necessary for the protection of the public health, and therefore batches thereof are exempt from the certification requirements of section 706 (c) of the act.

(6) BATCH CERTIFICATION NOT REQUIRED

(G) Batch Certification Not Required

Astaxanthin is a nature-identical synthetic oxycarotenoid with a well-defined chemical structure, a clearly described synthesis with in-process controls, and with specifications which guarantee high standards of purity and identity.

Astaxanthin occurs naturally in salmon and trout, shrimp, and lobsters, all of which have long been part of the human diet. Synthetic astaxanthin, which will replace a small fraction of the naturally present astaxanthin, has been shown to be very safe for human consumption in extensive toxicity studies reported in the present Color Additive Petition.

Based on these considerations, there is no need for batch certification of astaxanthin.

I

000302

H

(H) REVISED REGULATION

(H) Revised Regulation

Does not apply.

(I) PRESCRIBED FEE

Astaxanthin
Color Additive

21

(I) Prescribed Fee

See Cover Letter.

000308

(J) ENVIRONMENTAL IMPACT ASSESSMENT REPORT

(J) Environmental Impact Assessment Report

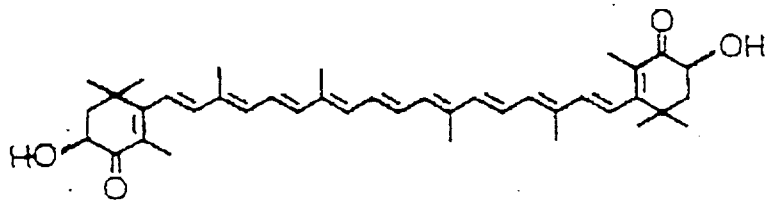
1. Date: August 1987

2. Name of Application/Petitioner: Hoffmann-La Roche Inc.

3. Address: 340 Kingsland Street
Nutley, New Jersey 07110

4. Description of the Proposed Action: The action is a Color Additive Petition for use of synthetic, nature-identical astaxanthin in the form of a stabilized gelatin beadlet in the feed of commercially raised salmonid fish (salmon, trout) at concentrations of up to 200 ppm in the finished feed. Salmon are raised commercially in net pens placed in the ocean, and trout in concrete raceways in running fresh water.

5. Identification of Chemical Substances that are the subject of the Proposed Action: Astaxanthin is 3,3'-dihydroxy-β,β-carotene-4,4'-dione. The molecular formula is $C_{40}H_{52}O_4$ and the molecular weight 596.86.



Astaxanthin

The composition of the stabilized beadlet is shown in Appendix I.

6. Introduction of Substances into the Environment:

The manufacture of astaxanthin is carried out at the facilities of F. Hoffmann-La Roche & Cie., Ltd. in Basle, Switzerland. The manufacture of the stabilized gelatin beadlets is carried out at the facilities of F. Hoffmann-La Roche & Cie, Ltd. in Village-Neuf, France. All manufacturing operations are performed in compliance with all the laws and regulations promulgated by Switzerland and by France for the protection of the environment. See Appendices II & III for statements of compliance.

7. Fate of Emitted Substances in the Environment:

See 6 above.

8. Environmental Effects of Released Substances:

Addition of synthetic astaxanthin to the feed of salmonid fish will have no effect on the environment for the following reasons:

a) Astaxanthin is a natural substance and is the principal oxycarotenoid present in the zooplankton of oceans and fresh water bodies. The total quantity of astaxanthin present in nature can only be estimated and may be of the order of millions of tons.

b) Astaxanthin is the natural pigment in the tissues of salmon and trout. Fish depend upon the presence of astaxanthin in components of their diet to achieve their characteristic pigmentation. Replacement of a small portion of the natural astaxanthin in their rations by nature-identical synthetic astaxanthin will have no effect whatsoever on the environment.

c) The principal excipients present in the stabilized astaxanthin beadlet need not be given separate environmental consideration since they are gelatin, sucrose, corn starch and dextrin. All of these materials (proteins and carbohydrates) are common and indeed essential components of feed. The antioxidant ascorbyl palmitate is hydrolyzed to vitamin C and palmitic acid (fatty acid), which are normal feed ingredients. Ethoxyquin is widely used as a preservative in feeds and is regulated under 21CFR §573.380.

9. Use of Resources and Energy:

It is the very nature of the chemical manufacturing process to transform certain natural resources in an irreversible manner. Fish farming is rapidly becoming an important source of wholesome protein food for man. The availability to aquaculturists of astaxanthin will help to make their products more appealing to the consumers. This is of some practical importance, since nutritional experts are recommending increased use of fish in human diets.

10. Mitigating Measures: Does not apply.

11. Alternatives to the Proposed Action: Does not apply.

12. List of Preparers: Josef Fellig, Ph.D.

8/7/87
Date

Signature of Responsible Official

Manager, Drug Regulatory Affairs
Title

APPENDIX II

F. HOFFMANN-LA ROCHE & CO.
AKTIENGESELLSCHAFT SOCIÉTÉ ANONYME LIMITED COMPANY

TO WHOM IT MAY CONCERN

This is to confirm that ASTAXANTHIN is manufactured by F. Hoffmann-La Roche & CO., Basel in conformance with cantonal and federal laws.

The firm adheres to WHO- and federal GMP-regulations and is inspected by swiss health authorities on a regular basis.

F. HOFFMANN-LA ROCHE & CO.
Limited Company

The Quality Assurance Manager

Dr. P. Fischer

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Bâle, Suisse Gruppe/Groupe/Group II/III
Basle, Switzerland

APPENDIX III

F. HOFFMANN-LA ROCHE & CO
AKTIENGESELLSCHAFT SOCIÉTÉ ANONYME LIMITED COMPANY

TO WHOM IT MAY CONCERN

This is to confirm that Caropohyll Pink (Astaxanthin 5%) is manufactured by Société Chimique Roche S.A., Village Neuf, France in conformance with French laws and with all requirements of the French government for the protection of the environment with regard to air, water, and disposal of solid wastes.

The firm adheres to WHO- and European GMP-regulations and is inspected by French health authorities on a regular basis.

Yours faithfully,

F. HOFFMANN-LA ROCHE & CO.
Limited Company

The Quality Assurance Manager

Dr. P. Fischer

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LA HAYE

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TO:	Dr. Todd Lorenz
FIRM:	Cyanotech
FROM:	Patsy Maltby
DATE:	June 2, 1998
SUBJECT:	**Synthetic Astaxanthin Toxicity Data**

CC: Michael C. Maloney, President & CEO

Fax Number: 808-329-3597

Number of pages including this cover page: (1)

MESSAGE

Dear Todd:

Per the request of Mike Maloney I have sent, under separate cover, the summary of the synthetic astaxanthin toxicity data that you and Mike spoke about in your meeting last week.

Should you have any questions, please feel free to contact Mike at your convenience.

Thank you.

Astaxanthin Safety Tests

Sincerely,

Patsy Maltby
Executive Assistant

La Haye Laboratories, Inc.

2205 152nd Avenue NE
Redmond, WA 98052-5519

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A Technical Review of *Haematococcus* Algae

History, Distribution and Classification of *Haematococcus pluvialis*

Observations of *Haematococcus* began in 1797 by Girod-Chantrans and were continued by other Europeans. The first description of *Haematococcus pluvialis* was conducted by Flotow in 1844, and in 1851 Braun added to the details and corrected a few errors of earlier observations. Herrick published some brief comments in 1899 on the life history of *Haematococcus*, noting the alternation of lifecycle between resting cells and motile cells.

The first extensive description of the life history of *Haematococcus* in English was by T.E. Hazen in 1899 in a published report of the Torrey Botanical Club. He noted that the algae is usually found as a blood-red crust adhering to the sides of urns or shallow pools near the ocean which were periodically filled with water. He went on to describe the life history of the alga through a red resting stage and green swimming stage followed again by a red resting stage. At this time the chemical nature of the red coloring matter within the alga was unknown, but was given the name "haematochrom", and is now known as astaxanthin. Hazen reported that the alga "is reported as very common and widely distributed in Europe, where it is found from Scandinavia to Venice...the alga is distributed from Vermont to Texas and from Massachusetts to Nebraska and probably farther West."

A few years later, Peebles (1901a, 1909b) published a life history of the alga with detailed drawings of changes occurring in the "haematochrom" throughout the life cycle. In 1934, Elliot added details of the cellular morphology to the life history of the alga. During the life cycle four types of cells were distinguished: microzooids, large flagellated macrozooids, non-motile palmella forms; and haematocysts, which are large red cells with a heavy resistant cell wall. The macrozooids predominated in liquid cultures with sufficient nutrients, but when environmental conditions become unfavorable the palmella stage results, followed by the resistant haematocysts and the accumulation of astaxanthin. Subsequently, after being exposed to a nutrient-favorable environment, haematocysts give rise to motile microzooids that grow into palmella or macrozooid stages.

Pocock (1937 and 1961) described the distribution and life history of *Haematococcus* strains isolated in Africa. Almgren (1966) described the ecology and distribution of *Haematococcus* in Sweden, where the alga is found in ephemeral rain pools made of rock, generally of small dimensions and based upon firm material, impermeable to water. Droop (1961) also noted that that *Haematococcus* typically inhabited rock pools, often, though not necessarily, within a few feet of the sea.

The widespread occurrence of *Haematococcus* in temporary rather than permanent bodies of water is due, at lease in part, to the fact that such pools are usually free of other competing algae, and not to any inherent characteristic of the pools. *Haematococcus* is considerably better suited for survival under conditions of expeditious and extreme fluctuations in light, temperature and salt concentration than most algae, due to its rapid ability to encyst (Proctor, 1957a).

Haematococcus pluvialis, also referred to as *Haematococcus lacustris* or *Sphaerella lacustris*, is a ubiquitous green alga of the order Volvocales, family Haematococcaceae (Table 1). It is now known that the alga occurs in nature worldwide, where environmental conditions for its growth are favorable. No toxicity associated with *Haematococcus* has ever been reported in the literature.

Table 1: Classification

Haematococcus is an ubiquitous green algae classified as:

Phylum:	Chlorophyta
Class:	Chlorophyceae
Order:	Volvocales
Family:	Haematococcaceae
Genus:	Haematococcus
Species:	pluvialis

General Properties and Composition of *Haematococcus* algae

The general composition of *Haematococcus* algae consists of common carotenoids, fatty acids, proteins, carbohydrates, and minerals, and is listed in Table 2. Some physical characteristics are listed in Table 3.

Table 2: Typical Common Components of *Haematococcus* algae

	Minimum	Maximum	Mean
protein	17.30	27.16	23.62
carbohydrates	36.9	40.0	38.0
fat	7.14	21.22	13.80
iron (%)	0.14	1.0	0.73
moisture	3.0	9.00	6.0
magnesium (%)	0.85	1.4	1.14
calcium (%)	0.93	3.3	1.58
biotin (mg/lb)	0.108	0.665	0.337
L-carnitine (ug/g)	7.0	12	7.5
folic acid (mg/100g)	0.936	1.48	1.30
niacin (mg/lb)	20.2	35.2	29.8
pantothenic acid (mg/lb)	2.80	10.57	6.14
vitamin B1 (mg/lb)	<0.050	4.81	2.17
vitamin B2 (mg/lb)	5.17	9.36	7.67
vitamin B6 (mg/lb)	0.659	4.5	1.63

vitamin B12 (mg/lb)	0.381	0.912	0.549
vitamin C (mg/lb)	6.42	82.7	38.86
vitamin E (IU/lb)	58.4	333	186.1
ash	11.07	24.47	17.71

Table 3: Physical Characteristics *Haematococcus* Algae:

Color	Red to Dark red
Particle size	5-25 microns
Moisture	4-9%
Bulk density	
loose value	0.303-0.345 g/ml
tapped value	0.370-0.435 g/ml
astaxanthin	1.0%

The amino acid profile of *Haematococcus* algae is listed in Table 4.

Table 4: Typical Amino Acid Analysis of *Haematococcus* algae

	Minimum value	Maximum value	Mean
tryptophan	0.05	0.56	0.31
aspartic acid	1.37	2.31	1.89
threonine	0.78	1.24	1.04
serine	0.73	1.06	0.94
glutamic acid	1.70	2.39	2.19
proline	0.69	1.00	0.89
glycine	0.84	1.32	1.17
alanine	1.30	1.92	1.73
cysteine	0.16	0.21	0.19
valine	0.83	1.94	1.36
methionine	0.32	0.43	0.40
isoleucine	0.55	0.97	0.79
leucine	1.21	1.84	1.67
tyrosine	0.40	0.63	0.52
phenylalanine	0.61	1.05	0.90
histidine	0.48	0.76	0.61
lysine	0.75	1.32	1.13
arginine	0.81	1.34	1.07

Table 5 lists the individual fatty acids that are found in *Haematococcus* algae.

Table 5: Typical Fatty Acid Analysis of Haematococcus algae

Fatty Acid	Mean	Minimum	Maximum
C12:0 lauric	< 0.01	<0.005	0.01
C14:0 myristic	0.07	0.04	0.10
C16:0 palmitic	3.82	2.078	6.15
C16:1 palmitoleic	0.08	0.02	0.17
C17:0 margaric	0.03	0.01	0.03
C17:1 margaroleic	0.17	0.09	0.23
C18:0 stearic	0.27	0.14	0.46
C18:1 oleic	3.41	1.66	5.31
C18:2 linoleic	2.74	1.44	4.40
C18:3 linolenic	1.47	0.86	2.11
C18:3 gamma linolenic omega 6	0.21	0.09	0.29
C18:4 octadecatetraenoic	0.19	0.09	0.25
C20:0 arachidic	0.08	0.04	0.12
C20:1 gadoleic	0.04	0.01	0.08
C20:2 eicosadienoic	0.16	0.06	0.21
C20:3 eicosatrienoic gamma	0.06	0.02	0.09
C20:4 arachidonic	0.18	0.082	0.31
C20:5 eicosapentaenoic omega 3	0.08	0.031	0.18
C22:0 behenic	0.05	0.02	0.08
C24:0 lignoceric	0.03	0.013	0.05

Carotenogenesis and Astaxanthin of *Haematococcus pluvialis*

The pigment in *Haematococcus* was termed "haematochrom" until 1944 when Tisher identified the principal carotenoid as astaxanthin. Goodwin and Jamikorn (1954) identified the other pigments produced in *Haematococcus* during carotenogenesis. In 1954, Droop described the conditions governing astaxanthin formation and loss in *Haematococcus*. He showed that the action of light and carbon dioxide were dependent on one another, but that of organic carbon (such as acetate) is independent of light. Thus, astaxanthin formation could occur in the dark when energy is derived from organic carbon. Droop (1955a; 1955b) determined that the conditions for encystment and carotenogenesis in the alga were the same, and that the two phenomena usually occur together. Encystment and astaxanthin production can be induced by low nitrate or phosphate, high temperature or light, or the addition of sodium chloride in the culture medium (Boussiba and Vonshak, 1991, Kobayashi et al. , 1992, Fan et al. , 1994, Kakizono et al., 1992).

Sestak and Baslerova (1963) used paper chromatography to follow the changes in pigment composition of *Haematococcus* during encystment and carotenogenesis. They found

that astaxanthin precursors and chlorophyll decreased as astaxanthin accumulated. In 1976 Donkin used radioactively labeled acetate to determine that biosynthesis of astaxanthin occurs in *Haematococcus* through the intermediates beta-carotene, echinenone and canthaxanthin. The process of accumulation of astaxanthin in *Haematococcus* has been analyzed by optical and electron microscopes (Lang, 1968; Santos and Mesquita, 1984). In motile cells, astaxanthin first appears in small spherical inclusions (with no true limiting biomembrane) in the perinuclear cytoplasm, the pigment granules are not within any specific organelle or vesicle. In maturing cysts the pigment deposits increase in number and take on a variety of shapes. Coalescence of the globular granule result from increasing quantities of astaxanthin formed as the cell ages. In mature cysts the cytoplasm is almost uniformly red with no pigment in the nucleus or chloroplast.

Astaxanthin disperses towards the periphery of *Haematococcus* cells under light induction, and moves back towards the center after illumination is discontinued (Yong and Lee, 1991). No major quantitative or qualitative changes occur during this migration. Red cysts are more resistant to photoinhibition than green cysts, strongly indicating a photoprotective role for astaxanthin. The specific rate of astaxanthin accumulation is a function of the photon flux density *Haematococcus* cultures are exposed (Lee and Soh, 1991). Continuous illumination is most favorable for astaxanthin formation, and carotenoid content is correlated proportionally to light quantity. Other studies support the major role of astaxanthin accumulation in *Haematococcus* as being a form of protection against high light and oxygen radicals (Kobayashi et al., 1992a).

In nature, algae synthesize the carotenoid pigment astaxanthin and concentrate it in the food chain through zooplankton and crustaceans, which are prey for salmon, trout and other aquatic animals. The composition of astaxanthin esters in *Haematococcus* is similar to that of crustaceans, the natural dietary source of salmonids (Lambertsen, C. and O.R. Braekkan, 1971, Foss et al., 1987, Maoka, T. et al., 1985).

The astaxanthin molecule has two asymmetric carbons located at the 3 and 3" positions of the benzenoid rings on either end of the molecule. Different enantiomers of the molecule result from the exact way that the hydroxyl groups (-OH) are attached to the carbon atoms at these centers of asymmetry (Figure 1). If the hydroxyl group is attached so that it projects above the plane of the molecule it is said to be in the R configuration and when the hydroxyl group is attached to project below the plane of the molecule it is said to be in the S configuration. Thus the three possible enantiomers are designated R,R', S,S' and R,S' (meso).Free astaxanthin and its mono- and diesters from *Haematococcus* have optically pure (3S,3'S)-chirality (Grung et al., 1992 and Renstrom et al., 1981).

Astaxanthin, is biosynthesized through the isoprenoid pathway which is also responsible for the vast array of lipid soluble molecules such as sterols, steroids, prostaglandins, hormones, vitamins D, K and E. The pathway initiates at acetyl-Co-A and proceeds through phytoene, lycopene, β-carotene, and canthaxanthin before the last oxidative steps to astaxanthin. The astaxanthin biosynthetic pathway of *Haematococcus* is described in Figure 2. Fatty acids are esterified onto the 3' hydroxyl group(s) of astaxanthin after biosynthesis of the carotenoid, and allows it to have more solubility and stability in the cellular environment.

The carotenoid fraction of green vegetative cells consists of mostly lutein (75-80%) and β-carotene (10-20%). Whereas in red cysts, the predominate carotenoid is astaxanthin (Renstrom et al., 1981).

Astaxanthin is presently exempt from certification under the US 21 CFR part 73.35 as a color additive in fish feed, and *Haematococcus* algae meal is currently in the approval process by the Food and Drug Administration as a color additive for aquaculture feeds. *Haematococcus* algae meal has been approved in Japan as a natural food color and as a pigment for fish feeds. The formal descriptions of astaxanthin are presented in Table 6.

Table 6: Formal Descriptions of Astaxanthin

Chemical name:	3, 3'-dihydroxy-β,β,-carotene-4, 4' dione.
Molecular formula:	$C_{40}H_{52}O_4$
Molecular weight:	596.82
CAS number:	472-61-7
EINECS number	207-451-4

Quality Control Standards of *Haematococcus* Algae

GMP (Good Manufacturing Practice) is employed for the manufacture of *Haematococcus* algae. Pure cultures of the algae are cultivated employing Cyanotech's proprietary closed culture technology known as PhytoMax PCS (Pure Culture System) which automatically regulates pH and temperature, before transfer to open ponds for the final stage of the process. Under the proper stress conditions, *Haematococcus* encysts and produces high concentrations of carotenoids, which facilitates its own protection against light and oxygen. The carotenoid fraction of *Haematococcus* algae contains about 70% monoesters of astaxanthin, 10% diesters of astaxanthin, 5% free astaxanthin, and the remaining 15% consists of a mixture of β-carotene, canthaxanthin, lutein and other carotenoids (Figure 3). The production process includes a technique which "cracks" greater than 95% of the cells to enable maximum bioavailability. Because the process is biological, astaxanthin titer of individual batches may vary, thus total astaxanthin content is standardized to either 1.0% concentration (10,000 ppm) by blending of various lots in large stainless steel tumbler cones.

All media ingredients for the cultivation of the algae are food grade or higher quality. Reliable manufacturers that include specifications for heavy metals and other possible contaminants supply all nutrients. No solvents, pesticides, herbicides or toxic substances are used during any cultivation or manufacturing step of the product. There are no carcinogens or compounds that may degraded or metabolized to carcinogens used in the manufacturing process or known within *Haematococcus* algae.

Safety Studies of *Haematococcus* Algae Meal

Acute oral toxicity studies have been conducted on Charles River CD rats. The dosage level was 5,000 mg/kg and was administered as a 0.5% aqueous methylcellulose solution. Each lot was administered to separate groups of 10 rats that consisted of five males and five females. Groups for each treatment effect were evaluated for mortality, pharmacotoxic signs, body weights, and necropsy examinations during the 13-day study.

The results demonstrated that the LD_{50} value of each lot was greater than the administered dose of 5,000 mg/kg. No visible abnormalities were observed, nor differences in body weights during the study. The postmortem examination did not reveal any abnormalities in rats sacrificed at the end of the study.

Additional acute oral toxicity studies were conducted with both male and female mice. *Haematococcus* algae meal was suspended in distilled water for injection to give a 30% solution (w/v). The solution was forced by oral administration once using a gastric probe. The dosages ranged from 10,417-18,000 mg/kg, no mortalities were observed. The postmortem examination did not reveal any abnormalities in the rats that were sacrificed at the end of the study. The oral LD_{50} was judged to be 18,000 mg/kg or above.

A mutagenicity test using *Salmonella typhimurium* strain TA100, TA1535, TA98, TA1537, TA1538 and *E. coli* WP2 uvr A. A sample of *Haematococcus* algae meal was formulated into a 50 mg/ml solution of dimethyl sulfoxide. The formulation was spread onto the test petri plates in the presence of the microbial cultures with positive controls. The positive controls 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, 1-ethyl-2-nitro-3-nitrosoguanidine, 9-aminoacridine, 2-aminoanthracene, and 2-nitrofluorene showed a remarkable increase in the number of reverent colonies compared with the solvent control.

In contrast to these results, the *Haematococcus* algae meal sample showed no significant increase in the number of reverent colonies in every case compared to the solvent control. This demonstrated that the mutagenicity of the sample under the employed conditions were negative.

Fish tissues from a *Haematococcus* algae feeding study of rainbow trout were analyzed for toxic effects and neoplasia. All tissues examined were normal in appearance with no indication of disease, toxicity or neoplasia. All fish examined were in excellent nutritional status with abundant body fat. Gross findings indicate that no adverse effects on health were observed from *Haematococcus* algae meal as the dietary source of astaxanthin.

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Figure 1 (NatuRose Technical Bulletin): Isomers of Astaxanthin

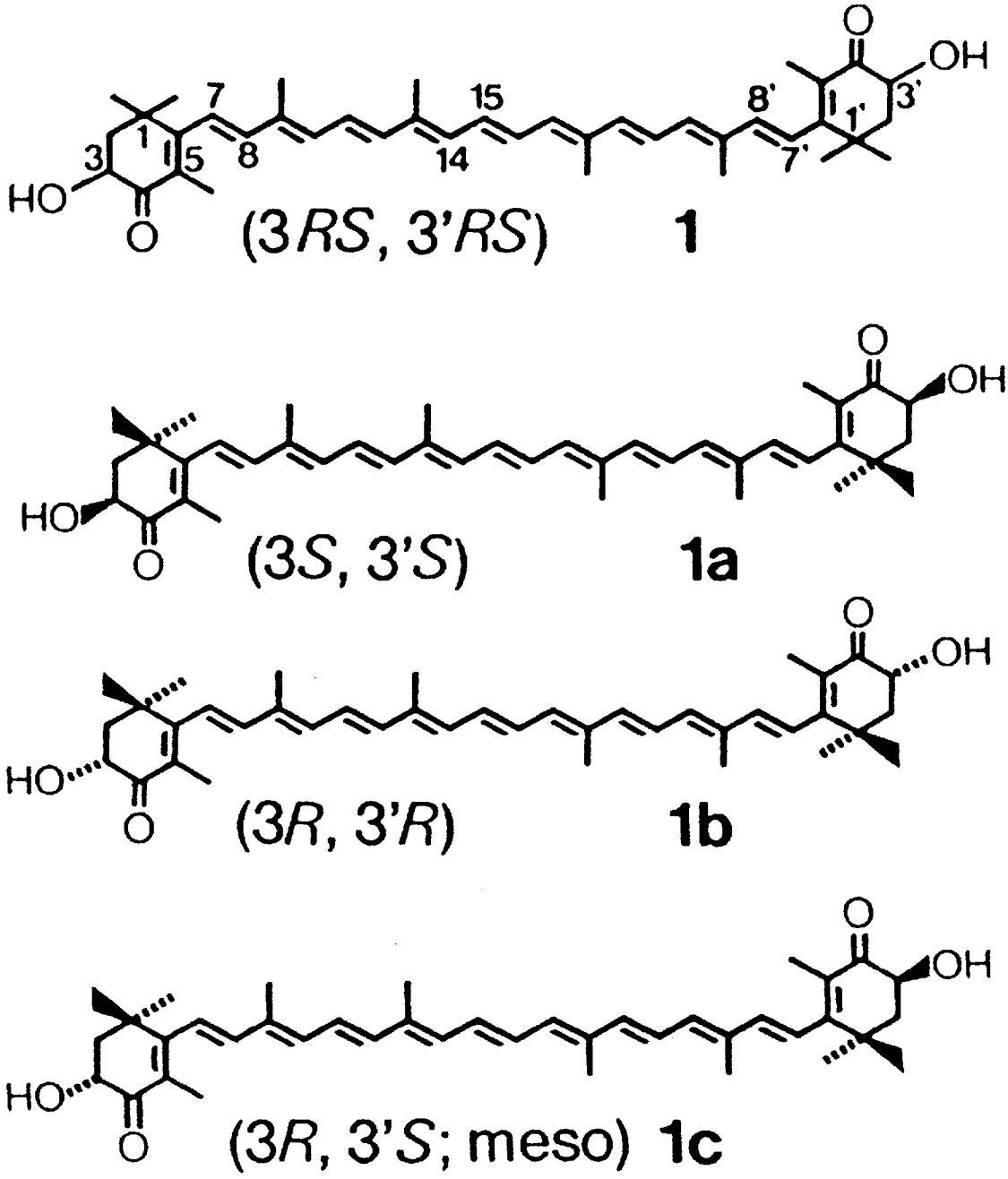


Figure 2 NatuRose Technical Bulletin): Astaxanthin pathway of *Haematococcus*

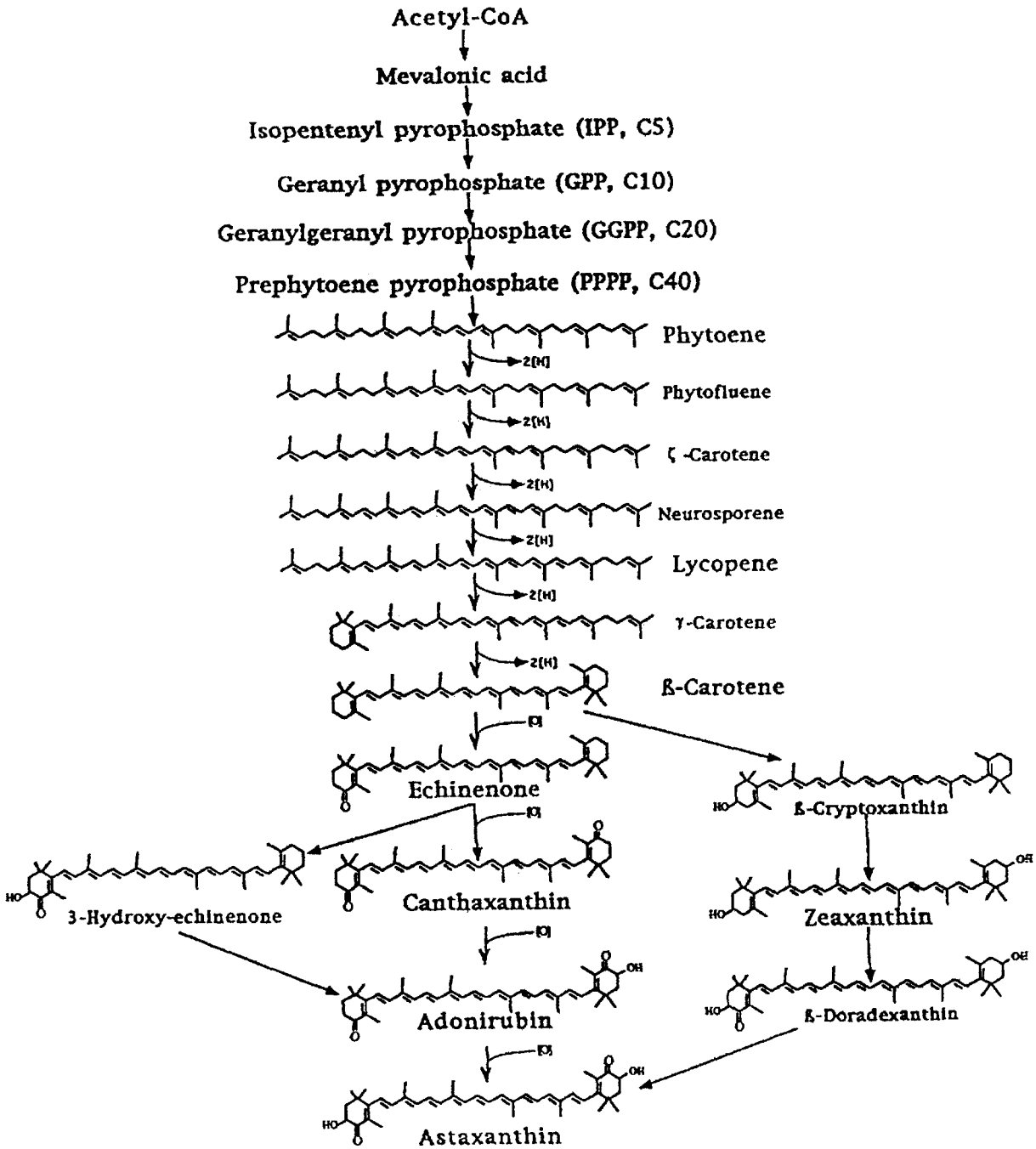
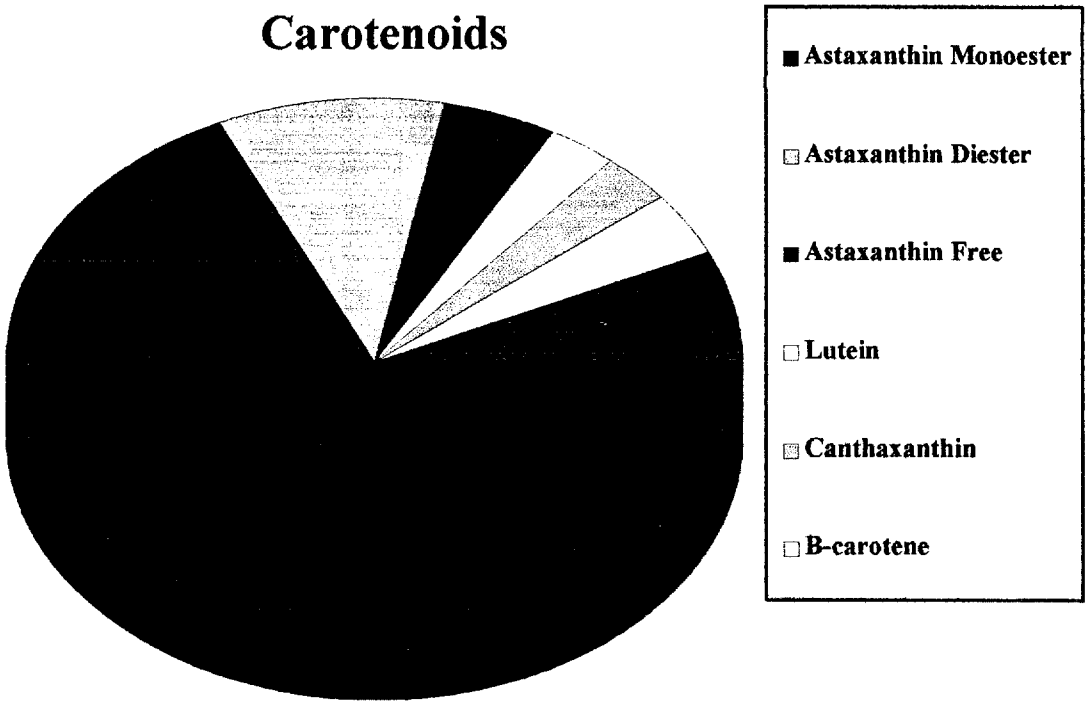


Figure 3: ***NatuRose*-Natural Astaxanthin Carotenoids**



- The carotenoid composition of *NatuRose* is similar to that of krill, shrimp, and crawfish.
- Esterified astaxanthin is inherently more stable to heat and oxygen.
- Canthaxanthin and β-carotene are converted to astaxanthin by shrimp and Koi species.