

A Technical Review of NatuRose™ *Haematococcus* Algae Meal

NatuRose™ *Haematococcus* algae meal is a natural source of astaxanthin derived from a unique strain of the microalgae, *Haematococcus pluvialis*. NatuRose™ is a spray-dried, dark red powder, and is currently used worldwide as a coloration and nutrition source for numerous species of animals. It has been successfully used for pigmenting shrimp (*P. monodon*, *P. japonicus*), rainbow trout, Coho, Atlantic salmon, sea bream, tropicals (marine and fresh water), Koi, poultry, and copepod/larval enrichment. This paper reviews the natural occurrence of *Haematococcus* algae, the composition of NatuRose™ and its safety.

History, Distribution and Classification of *Haematococcus pluvialis*

Observations of *Haematococcus* began in 1797 by Girod-Chantrons 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 least 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 of *Haematococcus* has ever been reported.

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 NatuRose™ *Haematococcus* algae Meal

The general composition of *Haematococcus* algae meal (NatuRose™) 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: Common Components of NatuRose™

	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
sol. dietary fiber (%)	-	-	1.2%
insol. dietary fiber	-	-	16.7%

Table 3: Physical Characteristics NatuRose™ *Haematococcus* Algae Meal:

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	1.5%

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

Table 4: Amino Acid Analysis of NatuRose™

	<u>Minimum value</u>	<u>Maximum value</u>	<u>Mean</u>
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

Carotenogenesis and Astaxanthin of *Haematococcus pluvialis*

In 1938, astaxanthin was first characterized from an extract of the lobster, *Homarus astacus*, and termed ‘astaxanthin’. The pigment in *Haematococcus* was called “haematochrom” until 1944 when Tisher correctly 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 granules results 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). NatuRose™ could be described as a type of “concentrated” krill or crawfish extract. Table 5 lists the individual fatty acids that are found in NatuRose™.

Table 5: Fatty Acid Analysis of NatuRose™

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

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 allow 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).

NatuRose has been approved by the Canadian Food Inspection Agency as *Haematococcus* algae meal in salmonid feeds (Registration Number 990535) and by the US Food and Drug Administration for use in salmonid feeds for pigmentation (21 CFR 73.185). Additionally, the FDA has completed their review of *Haematococcus* algae (BioAstin) on August 7, 1999 such that it is now permitted for marketing in the US as a dietary supplement. *Haematococcus* algae has also been approved in Japan for foods and animal feeds and Canada for salmonid feeds. Approval is pending for salmonid feeds in the US and Europe. 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 NatuRose™ *Haematococcus* Algae Meal

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 NatuRose™ *Haematococcus* algae meal 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). Due to the protective cell wall, astaxanthin is not readily bioavailable when whole cells are added to feeds. The NatuRose production process includes a technique which “cracks” greater than 95% of the cells to enable maximum bioavailability. The antioxidant, ethoxyquin, is added at 0.3% (w/w) concentration to enhance carotenoid stability, according to tolerance limitations in animal feed prescribed in § 573.380 by the Federal Food and Drug Administration. Because the process is biological, astaxanthin titer of individual batches may vary, thus total astaxanthin content is standardized to either 1.5% concentration (15,000 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 meal. Table 7 lists the maximum tolerances of the product as designated by the Federal Food and Drug Administration.

Table 7

Heavy Metals (as lead),	<10.0 mg/kg
Mercury	<1.0 mg/kg
Cadmium	<0.5 mg/kg
Arsenic	<2.0 mg/kg

Lead, <5.0 mg/kg

Safety Studies of *Haematococcus* Algae Meal

Animal studies have proven the safety of consuming *Haematococcus* algae, it has never been associated with any toxicity in the reported literature or in field studies. *Haematococcus* algae has been reviewed by the US FDA and cleared for marketing in August 1999 as a new dietary ingredient by means of the DSHEA (21 CFR Part 190.6). The algae has also been approved in Japan for use in both foods and animal feeds. No adverse effects have been reported from human consumption of the algae.

A different formulation of *Haematococcus* algae has already gained wide acceptance in the aquaculture markets as a pigmentation and vitamin source for salmon, trout, shrimp and ornamental fish. *Haematococcus* algae has been approved as a feed additive for salmonids by the Canadian Food Inspection Agency (CFIA 990535) and US Food and Drug Administration approval for salmonid feeds was achieved in August 2000 (21 CFR 73.185). Similar registrations are in progress in the European Community.

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

A number of standard toxicity and safety studies have been conducted with *Haematococcus* algae. Acute oral toxicity studies conducted on Charles River CD rats with a dosage level of 5 grams of *Haematococcus* algae/kg for 13 days. Groups were evaluated for mortality, pharmacotoxic signs, body weights, and necropsy examinations during the 13-day study. The results demonstrated that the LD₅₀ value of each lot was greater than the administered dose of 5 grams/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.

An oral toxicity study in rats was conducted in rats by administering 6 grams/kg of *Haematococcus* by oral route for 14 days. No treatment-related deaths occurred during the course of the study. Routine clinical and laboratory observations did not show any adverse changes in the test animals of either sex. The post-mortem examination showed no changes in organ weight or gross pathology. It was concluded that *Haematococcus* algae administered by oral route at the maximum dosage of 6 grams/kg/day was well tolerated and caused no adverse effects (Istituto di Ricerche Biomediche; Torino, Italy, RBM Exp. 950501). An acute toxicity

study was conducted in which rats were administered 12 grams/kg of *Haematococcus* algae. At the end of the 14-day observation period, there were no mortalities, adverse clinical signs or behavioral alterations noted in the animals. Body weight gain was unaffected by the treatment and a post-mortem pathology showed no appreciable macroscopic findings at the end of the 14 days. It was concluded that the LD₅₀ value was higher than 12 grams/kg with no pathological changes (Istituto di Ricerche Biomediche; Torino, Italy, RBM Exp. 950053).

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 LD₅₀ was judged to be 18,000 mg/kg or above. Mutagenicity tests under standard conditions were negative for *Haematococcus* algae. A published study with rats fed 400 ppm astaxanthin for 41 days showed no harmful effects on body/organ weight, enzyme activities, pregnancy, or litter size (Nishikawa *et al.*, 1997).

Astaxanthin is approved for use in salmonid feeds under 21 CFR section 73.35 up to a level of 80 mg/kg. This petition was reviewed by the FDA as CAP 7C0211 and included numerous safety studies with astaxanthin. Volume 2-6 of this petition contains the summaries and complete reports from the studies. The acute toxicity of 10 consecutive daily oral doses astaxanthin in rats was found to be greater than 2000 mg/kg. There was no mortality or symptoms of toxicity reported. In the Ames mutagenicity test, astaxanthin concentrations ranging from 0.03-5.0 mg/plate did not induce mutations in *Salmonella typhimurium* tester strains with or without activation by rat liver homogenate. Astaxanthin administered to mice at 500, 1000, and 2000 mg/kg did not induce chromosome breaks or mitotic disjunction. In teratology and embryotoxicity studies with rabbits, doses ranging from 100-400 mg/kg/day were administered to pregnant animals. There were neither overt signs of maternal sensitivity to the treatment nor significant changes in body weight development or malformations among the fetuses compared to the controls. Other safety studies included reproductive performance in rats with P and F1 generations, 13-week tolerance study in rats, and 13-week tolerance study in dogs all without toxic effects. The full volume of these safety studies is available in CAP 7C0211 at the FDA.).

Fish tissues from a NatuRose™ 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.

Summary

Haematococcus pluvialis is an alga found worldwide that produces high concentrations of astaxanthin for use in aquaculture and poultry feeds. NatuRose™ *Haematococcus* algae meal also

provides a balanced proportion of proteins, fats, carbohydrates, and minerals such that diets do not have to be adjusted when using the product. NatuRose™ *Haematococcus* algae meal provides 1.5% astaxanthin in an esterified form similar to that from krill and crawfish. A manufacturing process cracks cells and ensures maximum bioavailability and stability of the astaxanthin. Animal studies have proven that *Haematococcus* algae meal is safe and has never been associated with toxicity in the reported literature or field studies.

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NatuRose™ Technical Bulletin #050

Revision Date: January 10, 2001

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Figure 1 (NatuRose Technical Bulletin): Enantiomers of Astaxanthin

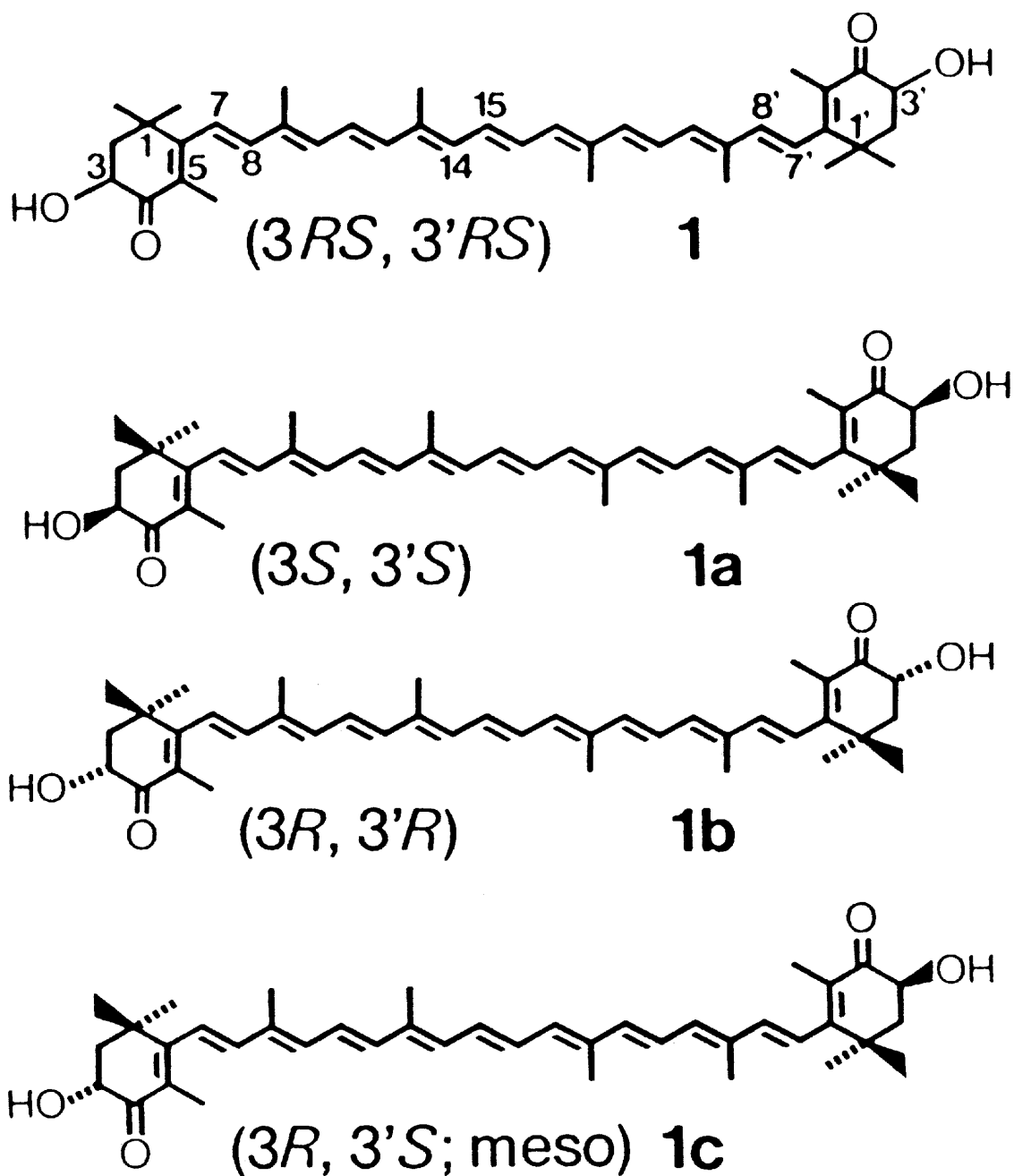


Figure 2 NatuRose Technical Bulletin): Astaxanthin pathway of *Haematococcus*

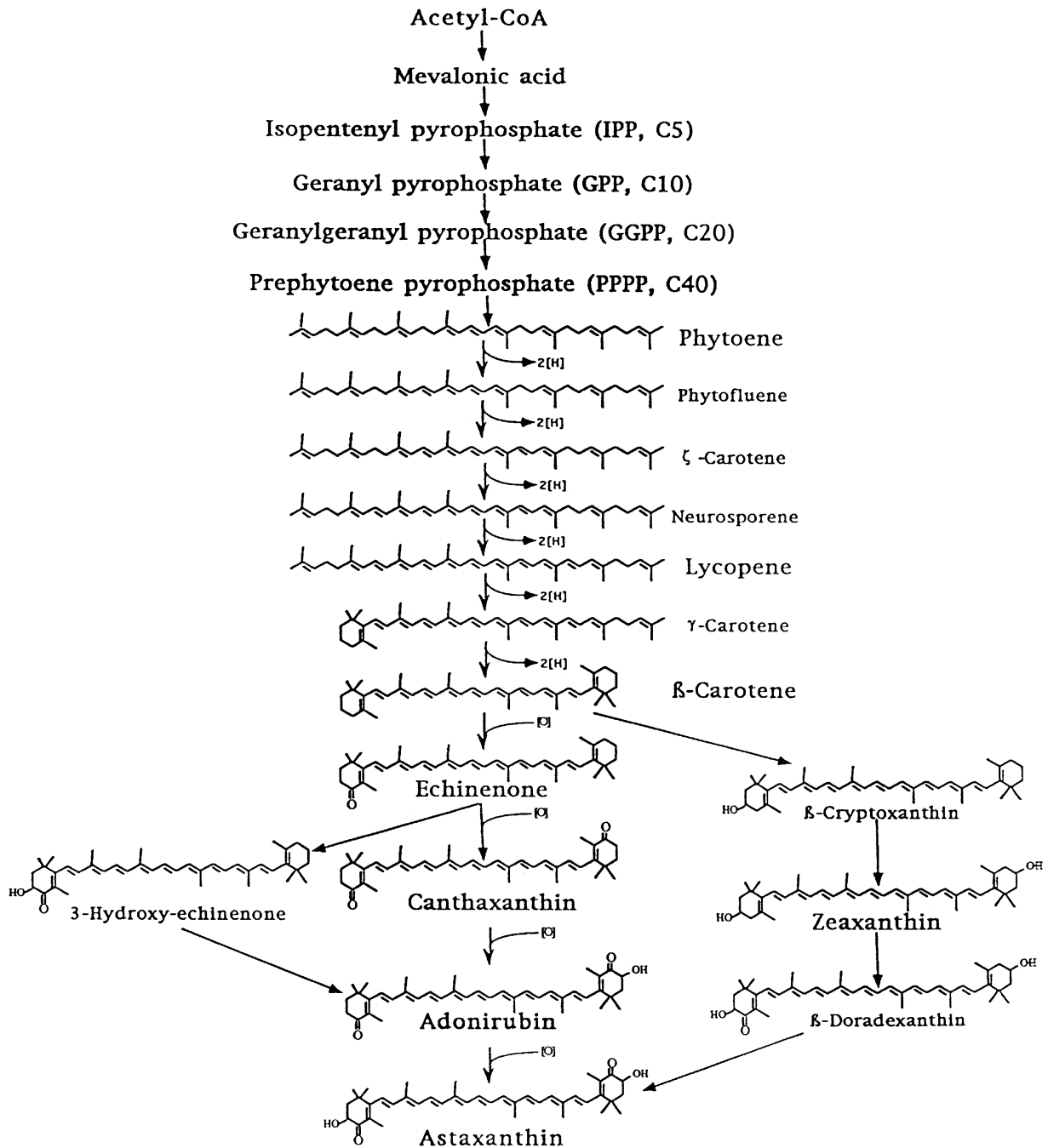
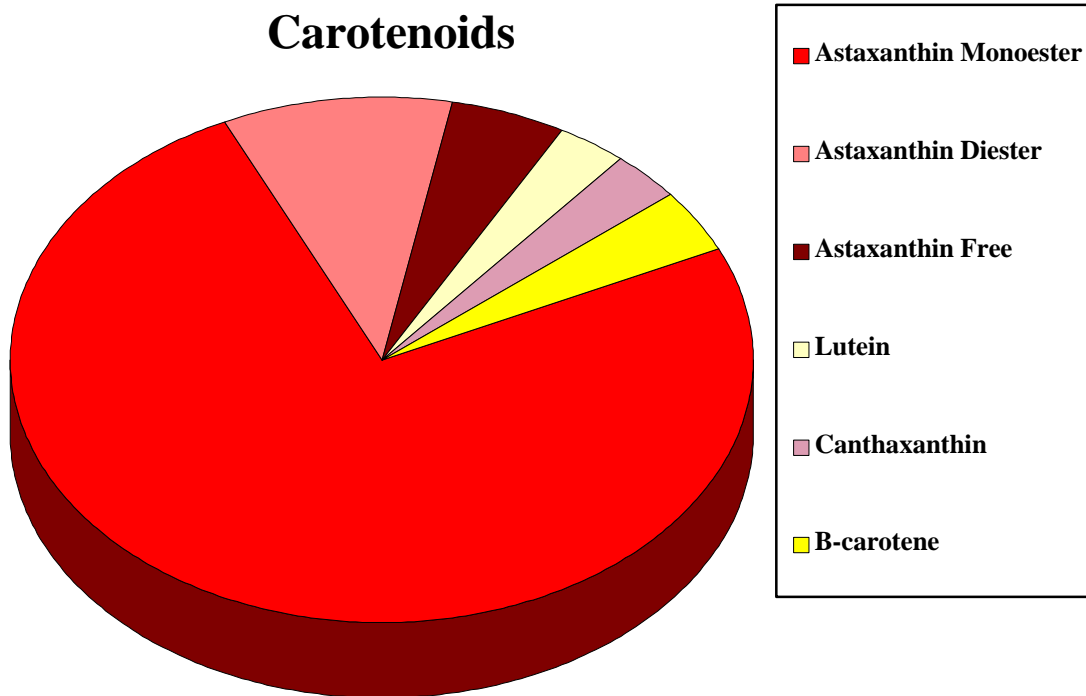


Figure 3: *NatuRose*- Natural Astaxanthin



- 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.