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 a 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 Isomers of Astaxanthin

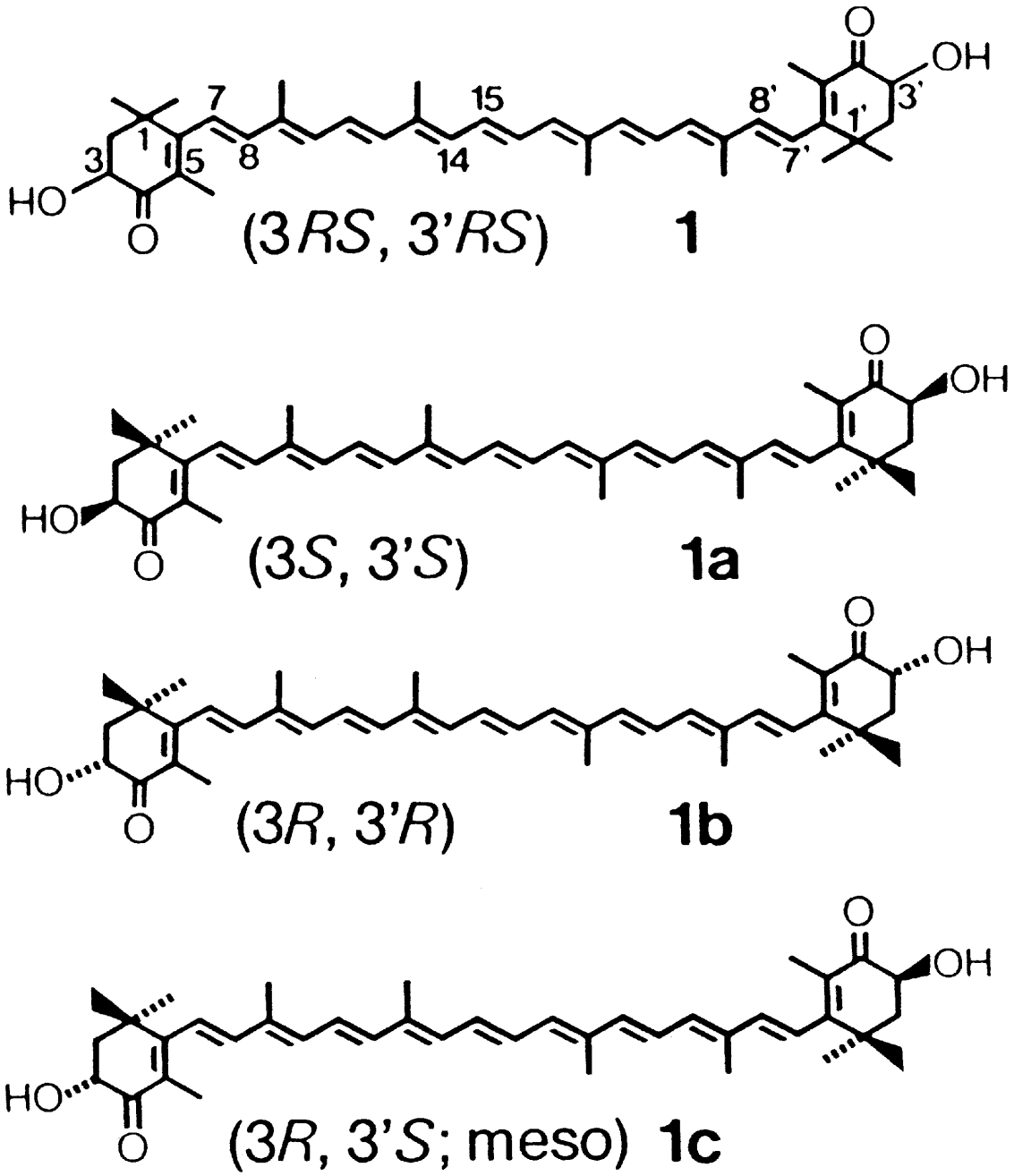

Figure 2 Astaxanthin pathway of *Haematococcus*

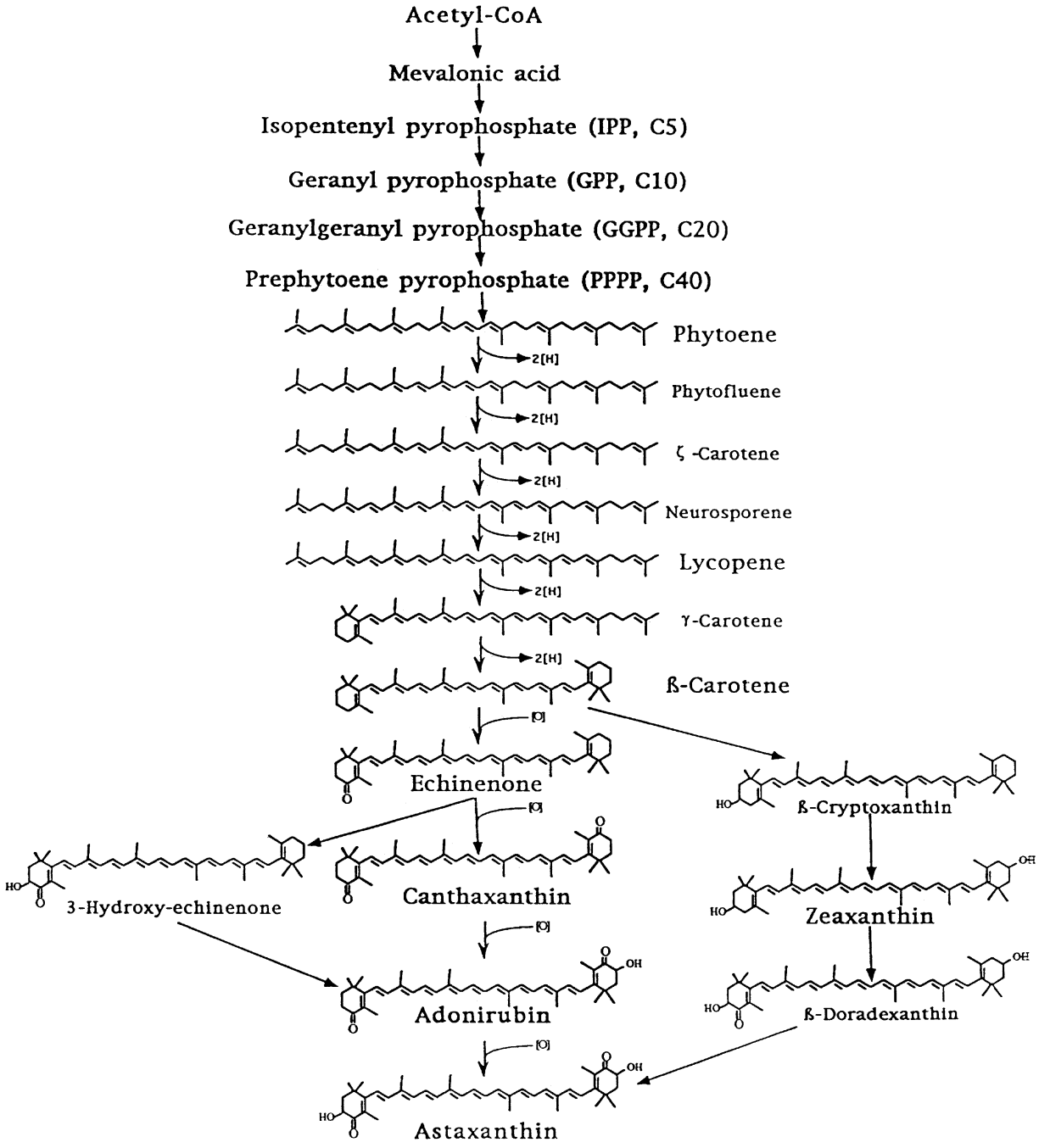

Figure 3: ***Haematococcus*** **algae Carotenoids**