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A Stuydy of Astaxanthin

— Its Application for the Pigmentation of Salmonid Fish —

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ABSTRACT

1. The pink-red coloration of the flesh of salmonids is due to carotenoids, especially astaxanthin, and is an important factor to evaluate marketing value of cultured salmonids. For many years fish technologists and scientists have endeavored to enhance the coloration of salmonids through the use of crustacean waste, yeast, algae, flower patels and synthetic carotenoids.

2. Synthetic canthaxanthin and astaxanthin are the two most effecient carotenoids for pigmenting salmonids, and are now widly used in salmonids culture.

3. The metabolism of astaxanthin in salmonids has been studied. The reductive metabolism of astaxanthin to β-carotene have been proposed. The proposed pathways are ;

Astaxanthin ⟶ idoxanthin ⟶ adonixanthin ⟶ zeaxanthin,

and

Canthaxanthin ⟶ 4-hydroxyechinenone ⟶ echinenone ⟶ β-carotene.

4. One of well-known functions of carotenoids in fish is pro-vitamin A activity. Salmonid fish are able to convert β-carotene, canthaxanthin, lutein, zeaxanthin and astaxanthin, into vitamin A.

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INTRODUCTION

The red coloration of the flesh, which is ascribed to accumulation of dietary carotenoids, especially astaxanthin [5]*, is one of the important factors in the marketing of cultured salmonids. Aquacultural production of Atlantic salmon and rainbow trout is now estimated at over 160000 tons a year. These fish are pigmented in order to meet the expectation of consumeres for a product a having natural, red-pink color. It is thus of great economic importance to achive a "natural" pigmentation of farmed salmonids . In common with other animals, salmonids cannot synthesize carotenoids *de novo*.. Therefore, it is necessary to supplement dietary carotenoids, especially astaxanthin, to produce the natural pink coloration. Much effort has been put into testing and comparison of available natural and synthetic carotenoid sources.

Various authors have studied the pigmentation of salmonids with astaxanthin from marine sources[1-10]. Carotenoids from plant origin have also been examined[1,11-16]. However, the usage of crustacean waste materials and plant pigment sources have not been fully successful for the pigmentation of cultured salmonids mainly because of poor availability of astaxanthin. Since canthaxanthin [11] and astaxanthin were chemically synthesized, these two carotenoids have recieved considerable interest for the pigmentation of salmonids[17-24].

We have worked on the pigmentation of rainbow trout using crustacean waste materials, plant extract and pure pigments as pigmenters. In the present paper, the results from our current experiments on the pigmentation of rainbow trout and other investigator's studies on the pigmentation of salmonids are reviewed. In addition, metabolism and some functions of carotenoids in salmonids are also discussed.

Carotenoids in salmonid feed

Numerous diets for trout and salmon have appeared in the literature, and fish feeds are available from companies all over the world. The Oregon test diet[25] has been used as a vehicle for pigment incorporation studies, and its development is contained in a series of progress reports[26]. A various pigment sources, such as crustacean meal, plant materials and pure pigment preparation including synthetic astaxanthin and canthaxanthin, were applied for the pigmentation of salmonid fish.

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*Structures are shown at the end of the text

Crustacean meal

In processing shrimp, the head and the hard carapace are removed during semi-mechanized peeling operations. Waste material accounts for approximately 70% of the whole shrimp, and may approach to 85% in the processing of other crustaceans such as Atlantic deep sea crab. Thus the use of shrimp by-products becomes an economic as well as an environmental pollution problem. It has been reported that 90-95% of the pigments in the red crab processing waste[27], shrimp meal waste[2,28] and crawfish[29] are in some form of astaxanthin. One possible use of crustacean waste materials is as a source of carotenoid, protein, lipids and other nutrients for the fish raised in aquaculture. A number of investigators have studied the pigmentation of salmonids using crustacean waste.

Spinelli *et al*[4] fed the Oregon moist pellet containing pelagic red crab (*Pleuroncodes planipes*) meal at concentration of 10% and 20% to 7-month-old rainbow trout for 60 days. The fish fed on both diets as well as 25% shrimp diet had a hue similar to the reddish color of ocean-caught salmon. Saito and Regier[3] studied the effects of shrimp waste and snow crab waste on the pigmentation of salmonids. They fed a diet containing 20 to 30% shrimp waste and 20% snow crab waste (vacuum-dried below 50℃ for 48 h) to year old brook trout for 12 weeks. The 20% shrimp waste fed fish were ranked first in skin and flesh appearance, flesh order and taste. The crab waste was found to have only a slight effect on the pigmentation of the trout.

Kuo *et al*[7] fed rainbow trout the diet containing 20% freeze-dried Atlantic red crab (*Geryon quinquendens*) waste. They observed that the fish fed the crab meal diet grew as fast as the control, but there was no significant coloring effect over the control during 15 and 23 week trials. On the other hand, the fish fed a carotenoid extract showed an excellent red color after 6 weeks feeding. They concluded that *G. quiquedens* meal, even under the best of processing conditions, was not a good pigment source for salmonids because of poor availability of pigment from the Atlantic red crab.

Peterson *et al*[1] fed raw crayfish and a crayfish extract to rainbow trout and brown trout. They reported that the extract provided better coloration than the raw material althogh the amount of pigment was adjusted to be equal. Meyers and Thibodeaux[30] reported that crawfish meal was effective for the caloration of rainbow trout as well as extracted astaxanthin in soybean oil.

Steel[31] fed rainbow trout the diet containing 15% shrimp waste meal and 7 % of pigment extract over a 24-week period. The shrimp waste meal increased the pigmentation in the skin and musclar tissue of trout nearly 13 fold over the control fish. Sensory evaluation showed that the

fish which recieved the shrimp waste meal and the shrimp pigment extract were rated significantly higher in firmness, color, flavor and overall desirability.

Spinelli[32] prepared a dry feedstuff by co-drying a mixture of Atlantic krill (*Euphasia pacifica*), shrimp waste and soy oil containing 0.025% ethoxyquin (based on wet weight of the *E. pacifica*) and added it at the 20% level to two dry diets that contained 10 and 15% fat. Rainbow trout fed the diets were well pigmented after 75 days. The fish fed the diet containing 15% fat were pigmented faster than those consuming the diet containing 10% fat. The feeding experiment of raw Atlantic krill to rainbow trout also showed that Atlantic krill was effective for the pigmentation of fish. He concluded that *Euphausia pacifica* can be added to salmonid diets in either wet or dry form to use as a carotenoid source to pigment the flesh of pen-reared salmonids.

Antarctic krill (*Euphausia superda*) have become a potential new source of astaxanthin[33,34]. A prepared carotenoid oil from the krill has been successfully employed for the improvement of integumentary color of red sea bream and yellowtail[35-38]. Arai *et al*[10] fed juvenile coho salmon (*Oncorhynchus kisutch*) with a diets supplemented with oil extract from Antarctik krill at 1, 4.5 and 9 % corresponding to 1.6, 7.2 and 14.4 mg astaxanthin per 100 g diet. They observed a marked pigmentation in coho salmon (average weight 180 g) after feeding on the diet containing 4.5% krill oil.

Mori *et al*[39] studied the pigmentation of cultured coho salmon on a practical scale by feeding diets supplemented mainly with Antarctic krill and lettoral mysid and observed a good coloration in coho salmon. Two diets (Diet 1 and Diet 2) contained carotenoids at concentration of 0.1-0.4mg and 1.1-4.1 mg／100g, respectively. They found that on the 127th day the fish from Diet 1 group and as early as on about 50th day those from Diet 2 group accumulated carotenoids in the flesh to 0.6mg／100g. The flesh color was close to that of wild coho salmon to the same level as wild fish can be attained by feeding a high carotenoid level diet. Economically, however, it is desirable to reduce the required supplmentation of astaxanthin to the minimum. The evaluation of the flesh coloration assured that the astaxanthin level of 0.6mg／100g in the flesh produces marketable fish. They concluded that the suitable level of astaxanthin supplemented to a diet for pigmentation of cultured coho salmon in a commercial scale is no less than 0.4mg／100g.

The shrimp meal and crustacean waste materials could be useful for the pigmentation of salmonids. However, practical problems of feed preparation may be encountered when whole crustacean or crustacean waste are formulated into diets. In general, the carotenoid content in whole crustacean and processing waste are low and the availability of carotenoids is poor. Moreover, the drying of crustaceans or crustacean wastes involves a considerble loss of carotenoid pigments[2,28,40]. The inclusion of large amounts of crustacean meal to increase carotenoid content in the diet produces the preparation of nutritionally inbalanced moist diets and induce mineral in-

balances in the fish[41,42]. To alleviate this problem several researchers have tried to improve availability of pigment from crustacean waste.

Ruteldge[43] described a decalcification procedure for crustacean meals based on a differencial screening effect between the protein and skeletal materials. The protein level of the meals nearly doubled in this procedue. In the calcification procedure, the protein is solubilized with NaOH, separated from the chitinous residue, and precipitated at in isoelectric point with HCl. It is, however, anticipated that the astaxanthin content of this preparation might be low. In a similar procedue used by Marine Commodites International for the chitinous residues, base was not used and the amount of acetic acid used to bring to isoelectric point was carefully controled. The resulting protein cake was deep red. This protein was incorporated into a fish diet, and coloration of trout was achived[28]. Torrissen *et al*[9] treated shrimp by-product with acid (4.8% (v/w) of 50% sulphuric acid, 1.2% (v/w) of propionic acid) along with antioxidant. They observed that the digestibility of the astaxanthin in shrimp processing waste was improved by ensiling to about 71% as compared to 45% in the corresponding fresh or dried materials. The rate of accumulations of the pigment in the fish muscle was markedly higher in fish fed the silage diet than those given fresh or dried materials.

Other investigators have prepared concentrated carotenoid extracts from crustacean and crustacean waste. The pigment extracts from shrimp meal[30], crayfish[1] and red crab[7] produced a good coloration.

Spinelle and Mahnken[5] developed a process in which soybean oil was used for the recovery of astaxanthin from pelagic red crab waste. Nine parts of comminuted red crab waste were mixed with 10 parts of water and 1 part of soybean oil containing 0.025% ethoxyquin (based on the weight of red crab) for extraction of astaxanthin. A-3-stage batch counter-current process (same ratio of red crab, water and oil in each stage) was used to increase the amount of carotenoid in the oil and succeeded to obtain a highly pigmented oil (153 mg／100g of oil). They reported that carotenoid degradation during extraction is minimized by the addition of 0.025% ethoxyquin and extracting at a temperature not exceeding 90℃. Oil extracts were incorporated into the Oregon moist pellet diets at 3, 6, 9 mg% and fed to rainbow trout for 120 days. Diets containing either 6 or 9 mg% were judged to give to excellent pigmentation. Chen and Meyers[29] studied the effect of antioxidant and a protease enzyme for extraction of astaxanthin from crawfish waste with soybean oil using the same process developed by Spinelli and Mahnken[5]. They found that the addition of protease resulted in a 58% increase in astaxanthin release. The advantage of the procedue developed by Spinelli and Mahnken[5] and Chen and Meyers[29] are that the destractive drying step is eliminated and no recovery of solvent is needed. The oil can be fed either as a pigmented oil or as part of the protein solids.

Plant pigment sources

Crustacean waste may be used as a source of astaxanthin in salmonid diets. However, crustacean shells cotaine only about 10-100 μg／g astaxanthin[2,42], and are high in minerals, which causes severe practical problem in feed formulation.

A number of investigators have studied the utilization of plant pigment as a pigment source for salmonids. Extracts of paprika pepper has been added as a supplement to many animal diets including fish[1,44]. The main pigment in paprika is capxanthin [12], which has never been isolated from the fish. Peterson *et al*[1] fed paprika extrct to brook trout. A coloration was detected at 2 weeks and was fairly prominent at 4 weeks. The color was described as natural except for a small amout of an undesirable yellow. Lutein [24] obtained from marigold patel was fed to brook trout[1]. It was reported that lutein was rapidly deposited in the skin, fins and flesh without modification but resulted in an undesirable golden yellow color.

Neamtu *et al*[45] fed rainbow trout the diets containing 4-5 mg% pigment extracts from the floral parts of the chesnut flower, *Aesculus hippocastanum* and *A. parviflora*. The *Aesculus* pollen contained pentacyclic carotenes and xanthophylls. The incorporation of pigment from *A. hippocastanum* to trout was low and it was found that the extract contained some toxic compound. The fish fed *A. pulviflora* showed a slight pink coloration at 4 weeks but slower growth rate than the control fish was observed. Lee *et al*[46] used extracts of marigold and squash flowers as a pigment source for a rainbow trout. They reported that the fish fed with squash extracts showed better coloration, but the fish fed with marigold extract had undesirable yellowish coloration. Kamata *et al*[12] studied the utilization of *Hippophae rhamnoides* oil (5 mg% pigment in diet) as a pigment source for a rainbow trout. After 4 week feeding period, some red coloration was observed. The oil contained β-carotene [13], cryptoxanthin [18] and zeaxanthin [30] as the major carotenoids.

Kamata *et al*[13] fed rainbow trout with the diets containing *Adonis aestivalis* flower and its pigment extract. It has been reported that certain species of *Adonis* flower such as *A. annua* L[47-49] and *A. aestivalis*[50,51] contained astaxanthin as a major pigment. According to Neamtu *et al*[50] *A. aestivalis* contained astaxanthin esters [9] at the level of 80% of the total carotenoid and its absolute configuration was (3S, 3'S)-form[51] [6]. The fish fed the diet containing 10 mg% *Adonis* extract showed an excellent pigmentation. No mortalities were recorded and a similar growth rate between the fish fed the *Adonis* extract and the control fish was observed during three month feeding period. However, a direct feeding of flower (5 % in the diet) to fish caused high mortarity possibly because of some toxic compound, alkaloids or other harmful compounds.

They concluded that the pigment extract of *A. aestivalis* could be one of the best dietary pigment source for salmonids due to the high concentration of astaxanthin, but the direct use of *A. aestivalis* would reduce production of fish.

The yeast, *Rhodotorula saneii*[11,52] and *Phaffia rhodozyma*[15,53] were also used for the study of pigmentation.

Savolainen and Gyllenberg[11] fed rainbow trout with dried *R. sanneii* cells, which contained the carotenoids, torularhodin [27] and torulene [28] with small amount of β-carotene [13], γ-carotene [17]. Lutein [24] and canthaxanthin [11] were isolated from the fish.

The red yeast, *P. rhodozyma* was found to contain (3R, 3'R)-astaxanthin [8] as a major pigment[54]. Johnson *et al*[53] studied the possiblity of *P. rhodozyma* as a dietary pigment source for salmonids and crustaceans. However, they could not obtain fully successful coloration by using these yeast for rainbow trout and crustacean. Later Johnson *et al*[15] conducted more detaile experiments with the red yeast, in which they treated *P. rhodozyma* in different ways ; parcially and fully-digested *P. rhodozyma* with extracellular enzyme, whole *P. rhodozyma*, mechanically broken yeast, and *P. rhodozyma* oil. The fish fed with fully digested yeast showed the highest pigmentation and almost twice the amount of astaxanthin was deposited after 6 weeks feeding than any of the other diets tested. While, the fish fed with parcially digested yeast picked up only a small amount of astaxanthin. Longer enzyme treatment of the yeast probably more effectively remove the cell wall than did short treatment with enzyme or mechanical disruption of the cell. They concluded that the red yeast, *P. rhodozyma* could be an excellent astaxanthin source for salmonids with the proper modification of cell wall.

The green algae, *Haematococcus pluvialis* was found to contain astaxanthin[55] and its absolute configuration is (3S, 3'S)-form[56]. Brinchmann[40] noted that *H. pluvialis* could be an excellent astaxanthin source for fish. Nakazoe and Hata[57] treated *H. pluvialis* with the enzyme, cellulase. They could get some pigmentation in sea bream but it was not fully successful. Recently Sommer *et al*[16] studied the effect of supplementing trout diet with *H. pluvialis*. They found that homogenized (broken) spores of the green algae, *H. pluvialis* resulted in a significant deposition of total carotenoid and astaxanthin, as well as visual enhancement of the flesh coloration in trout and no significant differences were seen in growth. The intact (whole) spores of *H. pluvialis* gave poor pigmentation. They summarized that *Haematococcus* can be an effective pigment source for rainbow trout and that algae spores could be used to color trout flesh and skin.

Choubert[14] fed rainbow trout with *Spirulina* which contained the carotenoids, β-carotene [13], cryptoxanthin [18], echinenone [20], mxyoxanthophyll [26], zeaxanthin [30], and β-carotene-5, 6-epoxide [15], at the level of 2.5, 5, 10 and 20% in the diet for 56 days. The incorporation of *Spirulina* algae in the diet caused a yellow-brown pigmentation to the fish. He sug-

gested that a supplementary feeding of *Spirulina* algae to trout, for pigmentation purpose only, is not applicable in intensive fish culture.

The application of plant sources for the pigmentation of salmonids has not been successful. The flower, *A. aestivalis*, the red yest, *P. rhodozyma*, and the green algae, *H. pluvialis* could be a good pigment source for salmonids. It would be appear that a major problem in the industrialization of algae or red yeast is the methods of cell rupture. The French press or Braun tissue disintegrator are good for small samples but are not applicable to large samples. The drying of cells would also cause them to rupture but would probably also result in pigment loss. Chemical methods (e.g. HCL digestion) are generally destructive to carotenoid and some other nutrients. As described by Johnson *et al*[15], the application of extracellurar enzyme will be a useful method for the utilization of these yeast and algae. The flower, *Adonis aestivalis* may be a potential source of astaxanthin since the petal can be dried without significant loss of the pigment. It remains to be seen whether this "weed" can be cultivated and harvested in a non-labor-intensive operation. It also must be determine whether a low-alkaloid plant results from cultivation.

Pure Carotenoid Preparation

The literature contains conflicting reports on the use of crystalline carotenoids. Steven[58] fed trout β-carotene [13] and astacene [4], which were dissolved in arachis oil and injected into earthworms. He found no incorporation of these pigments in the skin or fins of fish. Schiedt *et al*[59] also reported that β-carotene was not incorporated into the flesh of rainbow trout. Simpson and Kamata[60] found the deposition of astacene in rainbow trout. Peterson *et al*[1] and Lee *et al*[46] fed lutein [24] to rainbow trout and they reported that lutein was quickly absorbed in the fish without modification but resulted in an undesirable yellowisk coloration.

Canthaxanthin

Canthaxanthin (β,β-carotene-4,4'-dione) [11] is a naturally occurring oxycarotenoid extensively distributed in nature, especially in marine organisms. It has been found in bacteria, algae, daphnids, hydra, brine shrimp, prawn and lobsters. Canthaxanthin was first synthesized from β-carotene[61], followed by complete synthesis by Isler and Schudel[62]. It has been synthesized on a commercial scale for more than a decade and is now available as a gelatin-stabilized micro-dispersed granule. This granulate makes it possible to incorporate canthaxanthin in any diet at controllable levels without significant influence of the dietary quality and is now widly used for the pigmentation of salmonids.

Sice Deufel's first work in 1965[17], a number of investigators studied the application of synthetic canthaxanthin for salmonid culture[18,31,63,64]. These authors reported that canthaxanthin could rapidly be taken up by trout and salmon.

Deufel[17] fed rainbow trout 4 mg% of canthaxanthin for 24 weeks and described the trout as having a salmon-like coloration in flesh and skin. He noted that carotenoids, especially ketocarotenoids, provided beneficial effects on spawning (fertilization, sexual maturity and embryonic development). Steel[31] reported that a fat-dispersible crystallin canthaxanthin did not result in good pigmentation, while a water-dispersible canthaxanthin produced trout rated significantly higher in color than control fish. Schimidt and Baker[18] fed a beadlet form of stabilized canthaxanthin to rainbow trout for 31 weeks. At the end of the test periods the fish had a flesh color similar to that of red coho salmon. These authors reported that the induced pigmentation may be more stable to processing than the natural pigmentation of coho salmon. Other researchers[2-4,65] reported good coloration of salmonids with the feeding of canthaxanthin. Saito and Regier[3] notice that the hue of the flesh of salmonid is slightly different when astaxanthin or canthaxanthin is fed and canthaxanthin gives a slightly more orange color than a salmon color.

Zeaxanthin

Zeaxanthin (β,β-carotene-3,3'-diol) [30] has been fed to a number of fish and crustaceans to establish the bioconversion of zeaxanthin to astaxanthin. It has been reported that zeaxanthin is converted to astaxanthin in fancy red carp, golden yellow carp, goldfish, and prawn and intencification of color was noted on feeding of zeaxanthin[66,67]. Schiedt *et al*[59] reported that astaxanthin and canthaxanthin were well incorporated in trout but zeaxanthin was absorbed very poorly in the flesh and skin of trout. Salmonids cannot convert zeaxanthin to astaxanthin[68]. Thus the application of zeaxanthin for the pigmentation purpos has no benefit.

Astaxanthin

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) [5] is the most commonly occurring carotenoid in aquatic organisms. Since it was first isolated from the lobster shell by Kuhn and Lederer[69], it has been found in a number of organisms. The pink to red coloration of many echinoderms and crustaceans, the skin of several fish, and the muscle of salmonids fish consist partly and wholy of astaxanthin and its esters[70-73].

Astaxanthin was first synthesized from canthaxanthin[74]. The first total synthesis of (3S, 3'S)-astaxanthin was successfully completed by Kienzel and Mayer[75], in which the C9 compound

(4R, 6R)-4-hydroxy-2, 2, 6-trimethyl-cyclohexanone was used as a starting compound. Three optical isomers of astaxanthin ((3S, 3'S)-[6], (3S, 3'R)-[7] and (3R, 3'R)-astaxanthin [8] were synthesized by Widmer *et al*[76,77] and Zell *et al*[78]. Recently, Widmer[79] reported that 6-oxo-isopherone is an ideal starting material for the synthesis of numerous carotenoids pocessing cyclic end groups, including some arene- and cyclopentane-analogs.

Torrissen and Braekkan[80] and Simpson and Kamata[60] reported on the feeding of pure preparation of astaxanthin, its mono- and diester to rainbow trout. These reports taken together showed that the skin preferentially takes up the ester and deposited all fed forms as the ester. Astaxanthin is found only in the flesh as the free form, and astaxanthin is the effecient form for pigmenting the flesh.

A number of studies have been conducted using synthetic astaxanthin and astaxanthin dipalmitate. Schiedt *et al*[59] studied absorption and pigmenting effeciency of astaxanthin [5], canthaxanthin [11], zeaxanthin [30] and astaxanthin dipalmitate [9] in rainbow trout. They reported that astaxanthin was the best pigmenter for salmonid followed by canthaxanthin and astaxanthin dipalmitate. Strebakken *et al*[23] and Foss *et al*[22] reported the similar results in which astaxanthin was more efficiently utilized than canthaxanthin, which in turn was more effectively absorbed than astaxanthin dipalmitate for flesh pigmentation of Atlantic salmon. It was also reported that there was no significant difference in the carotenoid concentration in the skin of the fish fed the astaxanthin, canthaxanthin and astaxanthin dipalmitate. Mori *et al*[81], however, found that there was practically no difference between krill astaxanthin diester and synthetic free astaxanthin in their absorption and deposition by coho salmon. They suggested that this different utilization of astaxanthin and astaxanthin dipalmitate by fish may be ascribed to differences in hydrolyzability of astaxanthin diester in intestine due to species specificity of fish tested.

Several studies have shown that rainbow trout utilyzed dietary astaxanthin 1.3-1.5 times more efficiently than canthaxanthin[21,22,24,82-84]. Foss *et al*[22] and Torrissen[84] indicated that the two carotenoids appeared to have a synergic effect when they were fed in a mixture. According to Foss *et al*[22] who fed rainbow trout and sea trout with the diets containing astaxanthin plus canthaxanthin or astaxanthin dipalmitate plus canthaxanthin, astaxanthin and canthaxanthin were deposited to the same extent when fed in a mixture. . While astaxanthin dipalmitate was deposited in the flesh as free astaxanthin but more slowly than canthaxanthin, resulting in enhanced canthaxanthin deposition.

Since CD spectroscopy and HPLC analysis were employed in carotenoid chemistry, absolute configuration of astaxanthin from various origins has been studied. Renstrom *et al*[56] reported that astaxanthin in the green alge, *H. pluvialis*, has (3S, 3'S)-configuration. Veeman *et al*[85] isolated (3S, 3'S)-astaxanthin [6] from the spider mite, *Schizonbia sycophanta*. Renstrom *et al*[86]

and Kamata and Simpson[51] reported the presence of (3S, 3'S)-astaxanthin in the flower, *Adonis annua* and *A. aestivalis*, respectively. While (3R, 3'R)-astaxanthin [8] was found in the yeast, *P. rhodozyma*[87]. The co-occurrence of three optical isomers of astaxanthin were first reported by Ronneberg *et al*[88] in lobster eggs. Since then the optical isomers of astaxanthin has been reported from various organisms such as lobster, *Hommarus gammarus*[89], the shrimp, *Pandalus borealis*[90]. In salmonids, such as *Salmo trutta*, *S. salar* and *Oncorhynchus* spp, all three optical isomers of astaxanthin were isolated as the ratio of 78-85% (3S, 3'S)-, 2-6% (3S, 3'R)- and 12-17% (3R, 3')-astaxanthin[91].

Vecchi and Muller[92] first succeeded in the separation of the (3RS, 3'RS)-all-*trans*-astaxanthin and its *cis*-isomers by HPLC based on the resolution of racemic astaxanthin as the diastereomeric diester of di-(-)-camphaenic acid. Matsuno *et al*[93] later, developed a HPLC system for the separation of three optical isomers of astaxanthin without conversion to diastereomeric di-(-)-camphanates, and reported the occurrence of enantiomeric and meso-astaxanthin in 37 species of aquatic animals.

Johnson *et al*[53] first reported that the differences in absolute configuration of astaxanthin affect the absorption of pigment in lobster.

Foss *et al*[21] investigated the efficiency of individual optical isomers of astaxanthin in trout, and found the same utilization was present in all three astaxanthin isomers. Schiedt *et al*[59] carried out the similar experiment to Foss *et al*[21]. They found equal absorption and deposition of "racemic" and (3S, 3'S)-astaxanthin and a slightly increased deposition of (3R, 3'R)-astaxanthin compared with (3S, 3'S)-astaxanthin. They also noted that (3R, 3'R)-astaxanthin dipalmitate was utilized better than the (3R, 3'S)-astaxanthin if adoministered as a single isomers, as well as from a "racemic" mixture of the three isomers. Foss *et al*[22] also reported an increased level of (3R, 3'R)-astaxanthin and a reduced level of (3S, 3'S)-astaxanthin in the flesh of both rainbow trout and sea trout fed with racemic astaxanthin in the flesh of both rainbow trout and sea trout with racemic astaxanthin dipalmitate.

Factoros affecting absorption of carotenoids

It has been known that a various factors such as pigment sourse, diet composition, physiological state, fish size, state of sexual maturation and genetic background, affect absorption and deposition of carotenoids in the flesh of salmonids. The cost of dietary supplementation with synthetic carotenoids is relatively expensive, and reach to 10-15% of total feed cost. In spite of relatively high cost of carotenoids, less work has been done on factors affecting carotenoid absorption and deposition in the flesh. Several authors studied factors affecting carotenoids deposition

in salmonids[23,82,94-97].

The effect of pigment source, pigment level in the diet and the digestibility (availability) of astaxanthin on the pigmenting cultured salmonids have already been discussed. It was also mentioned some technological treatments such as, chemical, mechanical and enzymatic treatment, can effectively improve the pigment availability from various materials, such as crustacean waste, yeast and algae. Other factors affect the pigmentation of salmonids are briefly discussed.

Dietary lipid level and lipid quality

An important physical property of carotenoids is their solubility in lipid and the studies on carotenoid absorption indicated that fat promotes carotenoid absorption[98,99]. Abdual-Malak *et al*[65], Spinelli[8], Seuraman *et al*[100] and Torrissen[82] reported an increased pigment deposition with increasing lipid content in the diet.

The effect of lipid quality on absorption of pigment in salmonids has been studied by Torrissen[82]. He found no significant difference in pigmentation between the fish fed a capelin oil 'high' in free fatty acids and those fed an oil lower in free fatty acids. No other study on the effect of lipid quality on pigmentation has not been reported.

Effect of vitamin E

The effect of vitamin E on pigment absorption is not clear. Both vitamin E and carotenoids can act as an antioxidant and quench singlet oxygen effeciently[101]. It is, therfore thought the exclusion of vitamin E from the diet would decrease the amount of astaxanthin or canthaxanthin available for muscle deposition. However, Torissen[82] found no significant effect of exclusion of vitamin E. On the other hand, Pozo *et al*[95] reported an increasing level of α-tocopherol in the diet of trout increased the deposition of canthaxanthin in the flesh. Further study will be required to determine the effect of vitamin E on pigmentation of salmonids.

Effect of fish size

Several authors have reported that 'big' fish (weight over 200g) fish had a higher pigment level than small fish[5,10,23,72,102,103]. Abdul-Malak *et al*[65] found that rainbow trout less than 150g deposited little canthaxanthin in the flesh. A similar result was reported by Arai *et al*[10] in which less than 80g fish absorbed little pigment but over 180g fish showed excellent pigmentation. It is known that salmon increase pigmenting capacity with increasing size and there is a liner rela-

tion between fish weight and fish pigmentation after they have reached a certain size.

Water temperature

The influence of water temperature on fish growth has been reviewed by Brett[104]. The rate of carotenoid deposition in salmonids is affected by growth rate. Storebakken[23] studied pigmentation of Atlantic salmon, rainbow trout and sea trout at four different stations, where temperature avarage from 7.2 to 10.1℃ and found no correlation between water temperature and pigment retention. No and Storebakken[97] studied the effect of water temperature (5℃ and 15℃) on pigmentation of rainbow trout. They found a higher growth rate, flesh percentage in the body, fat content and a higher carotenoid content in fish reared in 15℃ than 5℃ for 6 weeks. It was, however, found that the fish contained the same amount of astaxanthin when the fish reached to the same size reared at 5℃. They concluded that this difference in concentration of astaxanthin was due to the different fish size and water temperature did not affect the absorption of pigment in the flesh.

Metabolism of Carotenoids in Salmonids

Carotenoid pigments, mainly xanthophylls, are found in tissue, principally gonads, skin, muscle and liver of many fish species[71]. It is generally assumed that fish, like other animals, are not able to synthesize carotenoids *de novo*, but many fish have the metabolic capacity to modify dietary carotenoids structually. Carotenoid metabolism in fish can take two major pathways[105], one is oxidative and another is reductive pathway. In the oxidative pathway β-carotene [13], lutein [24] and zeaxanthin [30] are converted into astaxanthin [5]. On the other hand, astaxanthin, for example, will be transformed step-by-step into zeaxanthin and β-carotene in the reductive pathway. In salmonid species, it has been believed that salmonids cannot metabolize any carotenoids into astaxanthin *de nove* because of the lack of ability to oxidize carotenoids at C-3, 3', 4 and 4' positions to astaxanthin[106]. Recently, the reductive metabolism of astaxanthin and canthaxanthin have been reported in some marine and freshwater fish including salmonids.

A possible mechanism of absorption and transformation of carotenoids in fish was proposed by Hata[107], in which carotenoids esters were absorbed from the intestine where they were hydrolyzed to free form and fatty acids, then transferred into the skin where they were re-esterified and stored. Kamata *et al*[108] supported his proposal by analyzing fatty acid composition of dietary astaxanthin diester and fish astaxanthin diester. After feeding of astaxanthin diester to rainbow tout, astaxanthin diester was isolated from the fish and fatty acids were analyzed. They

found the totally different fatty acid profiles between dietary astaxanthin diester and fish astaxanthin diester. They suggested that dietary astaxanthin diester was hydrolyzed then re-esterified in the skin and stored as fish astaxanthin diester.

The hydrolysis of carotenoid esters in intestine was reported by Mori *et al*[81] and Green and Selivonchick[109]. Mori *et al*[81] found that carotenoid in the intestinal tract of coho salmon fed with krill oil was composed exclusively of free astaxanthin which suggests that fish should have hydrolyzed almost all the esterified astaxanthin to free astaxanthin in the intestine. Greene and Selivonchick[109] also reported that pink salmon hydrolyzed almost all esters in the intestine with non specific lipase.

Schiedt *et al*[59], Foss *et al*[22] and Strebakken *et al*[23] analyzed the distribution of the three optical isomers of astaxanthin in the skin of rainbow trout fed racemic astaxanthin dipalmitate and found a slight preferred accumulation of the (3R, 3'R) - and (3S, 3'R) -isomers. Kamata *et al*[108], however, reported there was a slight preference for (3S, 3'S) -astaxanthin in the skin of rainbow trout fed with racemic astaxanthin. The similar distribution of the three optical isomers of astaxanthin in the skin of rainbow trout was reported by Bjerkeng *et al*[110], in which a preferred accumulation of the (3S, 3'S) and (3S, 3'R) isomers related to that of the (3R, 3'R) isomer was found. Schiedt *et al*[59] and Foss *et al*[22] suggested that the different distribution of optical isomers of astaxanthin in the skin of trout would be because of that the esterase responsible for the hydrolysis of astaxanthin dipalmitate was more efficient in using (3R, 3'R) -astaxanthin esters than (3S, 3'S) -astaxanthin esters as a substrate. Arai *et al*[10] and Mori *et al*[81], however, reported that hydrolase has no stereospecificity on the hydlolization of optical isomers of astaxanthin based on the finding of the ratio of optical isomers of all-*trans*-astaxanthin extracted from the intestinal tract, serum and flesh of coho salmon were practically identical with that of dietary astaxanthin diester.

A number of investigators have reported that no epimerization took place at C-3 or C-3' of astaxanthin in the flesh of salmonids, and the isomeric composition of the isolated astaxanthin corresponded closely to that of the feeds[21,22,59,108,110-112].

Kitahara[113] first reported the reductive metabolism of astaxanthin [5] to zeaxanthin [30] in chum salmon (*Oncorhynchus keta*) during anadromous migration. He proposed the following metabolic pathway of astaxanthin to zeaxanthin based on the finding of the increased amount of β-adonixanthin [2], β-carotene-triol [14] as well as increased zeaxanthin content during anadromous migration;

Astaxanthin [5] ⟶ β-adonixanthin [2] ⟶ β-carotene-triol [14] ⟶ zeaxanthin [30]

Schiedt *et al*[59,114,115,116] have studied the metabolism of astaxanthin by feeding of (3S, 3'S) -astaxanthin-$^3H_2$ to rainbow trout. They isolated radioactive adonixanthin [2], zeaxanthin

mono- and diester, zeaxanthin-5,6-epoxide [3] deepoxyneoxanthin [19] along with astaxanthin in the skin of the trout. They comfirmed the following metabolic pathway would take place in the skin of trout;

Astaxanthin [5] ⟶ β-adonixanthin [2] ⟶ zeaxanthin [30]
⟶ antheraxanthin [3] ⟶ deepoxyneoxanthin [19]

They also suggested the conversion of astaxanthin to zeaxanthin from configurational analysis of both astaxanthin and zeaxanthin isolated from rainbow trout. (3S, 3'S)-[33] and meso-zeaxanthin [32], so far considered as unnatural, along with (3R, 3'R)-zeaxanthin [31] were isolated from the skin of trout fed with racemic astaxanthin. They concluded that (3S, 3'S)- and meso-zeaxanthin were considered as metabolites of the different configurational astaxanthin isomers based on the fact that astaxanthin itself is not epimerized at C-3 and C-3' during absorption nor the elimination of oxygen does not alter the configuration at C-3 and C-3'. Katsuyama *et al*[117] fed three stereoisomers of astaxanthin diester to rainbow trout separatly and found that the dietary astaxanthin dieater, irrespective of its absolute configuration, were mostly absorbed and accumulated in the skin keeping their configurations, and partially matabolized to zeaxanthin in the skin but not in the muscle. They suggested the conversion from 3S to 3R-configuration was carried out *de novo* and vice versa in the skin based on the isolation of all three stereoisomers of zeaxanthin from the skin of the test groups. Ando and Hatano[118,119] presumed that the reductive metabolism of astaxanthin to zeaxanthin took place in the muscle of both male and female chum salmon based on the contents and indivisual composition of carotenoids in the muscle, serum and ovaries during spawning migration.

Schiedt *et al*[114,116] also proposed the conversion of astaxanthin in Atlantic salmon, (*Salmo salar*) based on the finding of an increased amount of (3S, 3'S)- and meso-zeaxanthin and idoxanthin in the skin after feeding with racemic astaxanthin. The possible metabolic pathway of astaxanthin to zeaxanthin is shown in Fig-1.

Schiedt *et al*[59,114] suggested the conversion of canthaxanthin [11] into β-carotene [13] through (4-hydroxy-echinenone [21]), echinenone [20] and (4-hydroxy-β-carotene [16]). and phoenicoxanthin [1] to cryptoxanthin [18] through 3-hydroxy-4-keto-β-carotene [10] by feeding of canthaxanthin and phoenicoxanthin, respectively. They concluded that salmonid fish can eliminate 4-oxo groups but not 3-hydroxy groups.

It is generally accepted that salmonid cannot oxidize any carotenoids into astaxanthin. Guillou *et al*[120], however, reported the possibility of oxidative metabolic pathway from either canthaxanthin or zeaxanthin to astaxanthin through phoenicoxanthin in the liver of matured female rainbow trout based on the isolation of ^{3}H-astaxanthin and ^{3}H-phenicoxanthin after forced-feeding of ^{3}H-zeaxanthin or ^{3}H-canthaxanthin. They sugested that large matured female rainbow trout are

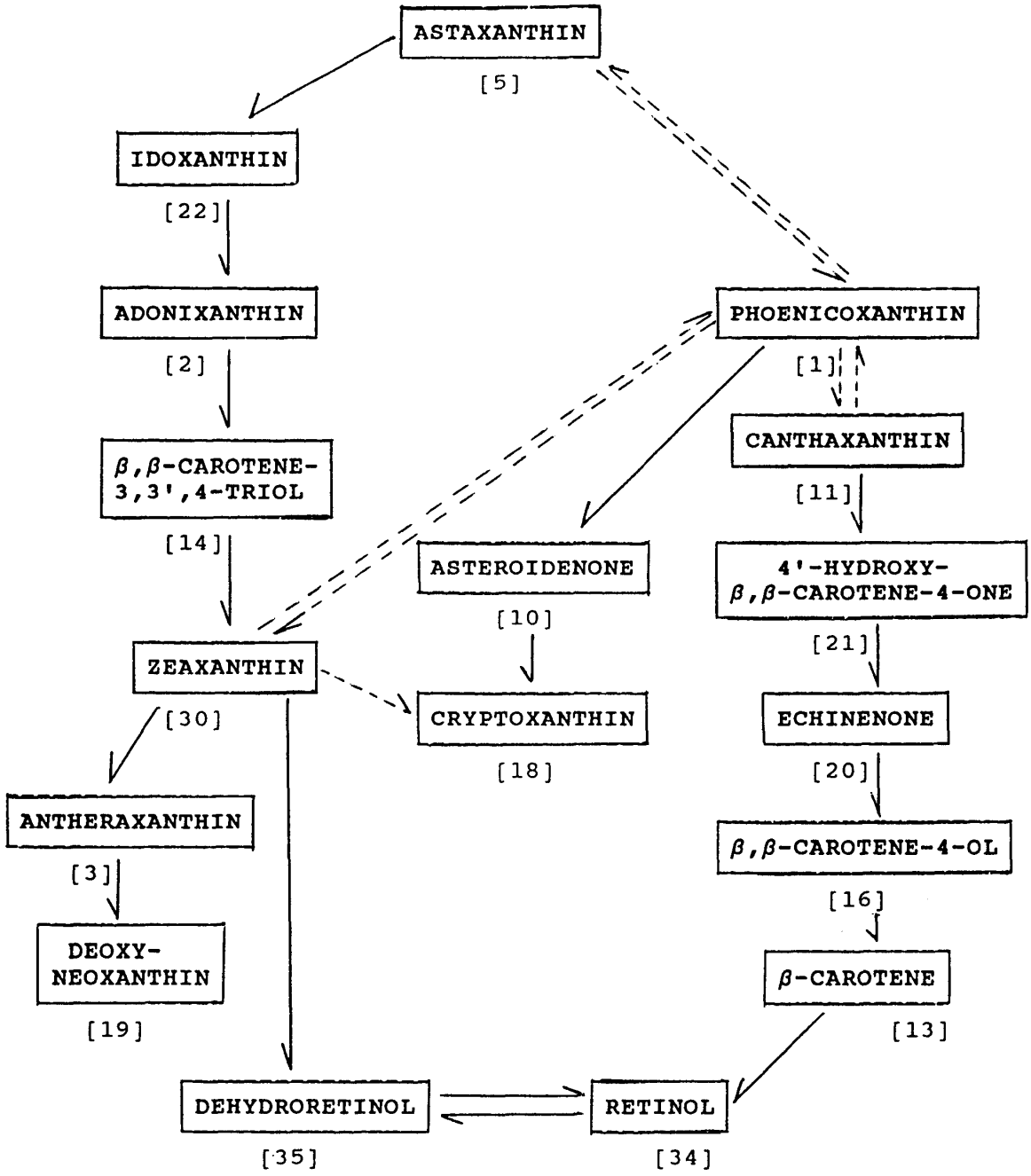


Fig. 1 The possible metabolism of astaxanthin in salmonid fish

able to oxidize small quntities of canthaxanthin or zeaxanthin to astaxanthin in the liver. Guillou *et al*[120], also reported that reduction of astaxanthin to phoenicoxanthin and zeaxanthin to cryptoxanthin, in which the removal of hydroxy groups from C-3 and C-3' positions was required. However, phoenicoxanthin has never been detected as metabolite in reductive reaction of astaxanthin in salmonids nor the elimination of hydroxy groups from C-3 and C-3' position in salmonid fish. The further experiments will be required to confirm the metabolism of carotenoids in salmonids fish.

Carotenoids as Vitamin A Precusor

Carotenoids likely have many roles to play in fish physiology. Other than well-known pigmentation (protection and sexual dichroism) and pro-vitamin A functions, the functions of carotenoids in fish have still remained obscure. It is generally accepted that carotenoid pigments can act as protective agents to prevent cells from damage caused by photodynamic action[121]. In this reaction, carotenoid protect against photosentitized oxidation by quenching singlet ecited oxygen or by acting as a filter pigment. Only a few metabolic functions have been ascribed to these pigments.

In early studies[17,122], it was reported that carotenoids had some functions during embryonic development. Tacon[101] summarized the proposed functions during embryonic development as follows: Xanthophylls

1. act as fertilization hormones and enhance chemotaxis of spermatozoa.
2. enhance growth, maturation rate and fecundity
3. reduce the mortality rate during embryonic development
4. increase the ability to tolerate various stringent environmental conditions, such as low oxygene concentration, elevated water temperature, elevated ammonia level and the harmful effects of light
5. perform a respiratory function under condition of limited oxygen.

However, the studies of functions in fish usually have not been substantiated with adequate scientific data. There are still some questions on the role of carotenoids during embryonic development. For example, Quatz[123] reported that there was no chemotactic effect of canthaxanthin on spermatozoa, nor any effect of astaxanthin on the rate of fertilization of rainbow trout eggs. Torrissen[124] also found no effect egg pigmentation on survival during embryonic stage. Further research will be required to establish the true nutritional significance of carotenoids during egg and larvae development in fish.

Several carotenoids are capable of acting as provitamin A precursors. It has been conclu-

sively established that a number of carotenoids with at least one unsubstituted β-ionone ring and fully cojugated isoprenoid side chain can serve as the precursor of retinol in fish.

Grangaud *et al*[125] first demonstrated that astaxanthin is converted into retinol in the liver of retinol-depleted freshwater fish *Gambusia holbrooki*, in 1962. They proposed the metabolic transformation of astaxanthin to retinol [34] via β-carotene.

Gross and Budowski[126] reported the conversion of some carotenoids, astaxanthin [5], canthaxanthin [11] and isozeaxanthin [23] into retinol [34] and 3-dehydroretinol [35] via β-carotene [13] in guppies (*Lebistes reticulatus*). Goswami and Barua[127] reported that freshwater fish, *Channa gachua*, are able to convert β-carotene into either retinol or retinoic acid [36], but not into dehydroretinol. The conversion of lutein [24] to dehydroretinol [35] via 3-hydroxy-retinol [37] in some freshwater fish was reported by Barua *et al*[128-130], and Goswami and Bhattcharjes[131]. Goswami[132] also studied the metabolism of cryptoxanthin [18] in some fresh water fish *Channa gachu*, *Labeo boga* (retinol-rich) and *Heteropneustres fossilis* (dehydroretinol-rich) and he suggested that retinol-rich fresh water fish can convert cryptoxanthin into retinol.

Matsuno[133] reported that some freshwater fish such as tilapia, black bass, ayu and marine fish yellowtail could convert astaxanthin [5], zeaxanthin [30], lutein [24] and tunaxanthin [29] to retinol [34] and 3-dehydroretinol [35].

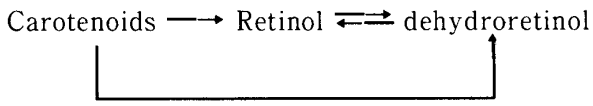
Katsuyama and Matsuno[133] fed β-carotene, canthaxanthin, astaxanthin, zeaxanthin and tunaxanthin to tilapia, *Tilapia nilotica*. They proposed two type of metabolic pathways of carotenoids into vitamin A : one is that carotenoids are first converted into retinol then to dehydroretinol and another is that carotenoids are directly transformed into dehydroretinol. β-carotene and canthaxanthin are converted to retinol first then to dehydroretinol. On the other hand, dihydroxy carotenoids such as astaxanthin, zeaxanthin, lutein and tunaxanthin are directly converted into dehydroretinol.

Schiedt *et al*[59,135] studied the conversion of astaxanthin, zeaxanthin and canthaxanthin into vitamin A in vitamin A-depleted rainbow trout using labeled 15, 15'-$^{3}H_2$-carotenoids and confirmed that astaxanthin, zeaxanthin and canthaxanthin can be vitamin A precursors, (both retinol and dehydroretinol) in rainbow trout. Schiedt *et al*[59] proposed that retinol is the intermediate precusor of dehydroretinol from the fact that retinol showed always a higher specific radio-activity than dehydroretinol. Their proposal was confirmed by the administration of labelled 6, 7-$^{14}C_2$-retyenyl acetate. The degree of incorporation in the vitamin A fraction depends not only on the size and age of the fish but also on their vitamin A-status. Schiedt *et al*[59] detected a higher radioactivity in the vitamin A fraction in the liver of vitamin A-depleted fish after feeding of labeled astaxanthin but not in the fish with saturated vitamin A status. Al-Kahalifa and Simpson[136] reported the bioconversion of astaxanthin into vitamin A in the fish fed a vitamin A

and carotenoids-devoid diet for 35 days. They also found that astaxanthin was not converted into vitamin A in vitamin A-sufficient trout although astaxanthin was converted into zeaxanthin.

Guillou *et al*[137], examined the bioconversion of astaxanthin into vitamin A in mature rainbow trout. They detected much higher level of dehydroretinol in the gonads of trout fed astaxanthin compared to canthaxanthin and control groups. This increased dehydroretinol was only detected in gonads and not in other tissues including the liver. They concluded that astaxanthin was bioconverted directly to dehydroretinol in gonads without beeing first converted into retinol form.

In conclusion, salmonid fish are able to convert dietary carotenoids, such as astaxanthin, canthaxanthin, zeaxanthin and β-caroten, to vitamin A (both retinol and dehydroretinol). It seems likely that two possible metabolic pathways of carotenoids into vitamin A are present in salmonid fish.



Summaries

In salmonid culture, a number of investigators have tested a various materials including crustacean waste products, yeast, algae, flower extract and synthetic carotenoids to produce natural pink coloration to meet consummer's expectation. The use of crustacean waste has benefit not only an economic point of view but also an environmental pollution problem. However, the usage of these materials was not fully successful mainly due to the low concentration and poor digestibility of astaxanthin and high content of minerals caused nutritionaly inbalanced fish feed. One of useful methods to utilize these crustacean waste is application of oil extract. Indeed krill oil extract has become effecient pigment source not only for salmonids but also for other cultured fish such as sea bream.

The astaxanthin-rich red yeast and green algae will be useful as a pigment source for cultured fish when the proper procedur for rupturing cell wall of these organisms is developed.

Synthetic astaxanthin and canthaxanthin are the two most effective and widly applied pigment for salmonid culture. However these synthetic carotenoids are rather expensive. The combination of these synthetic pigments and other natural products such as krill oil or red yeast will be helpful to deduce the feed cost.

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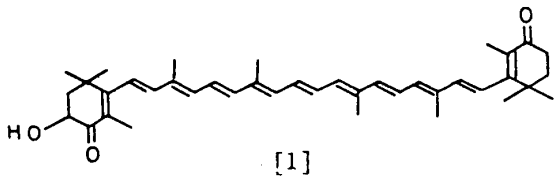
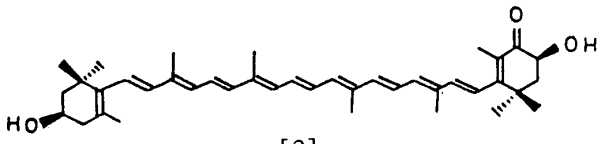
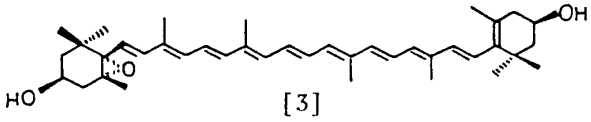


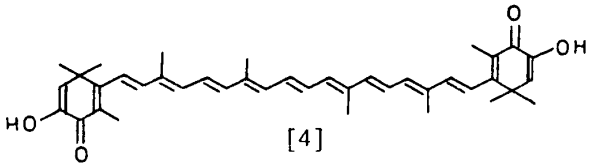
[1]
Adonirubin
(Phoenicoxanthin)



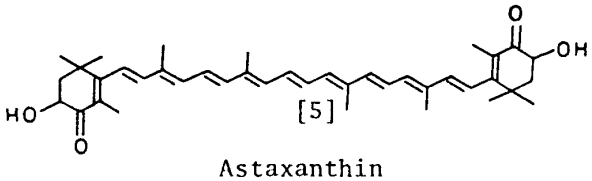
[2]
Adonixanthin
(Doradexanthin)



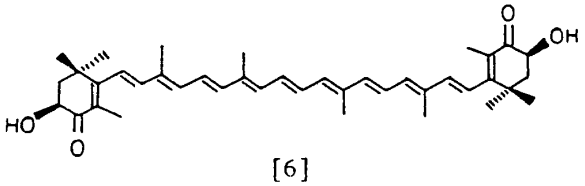
[3]
Antheraxanthin
(Zeaxanthin-5,6-epoxide)



[4]
Astacene



[5]
Astaxanthin



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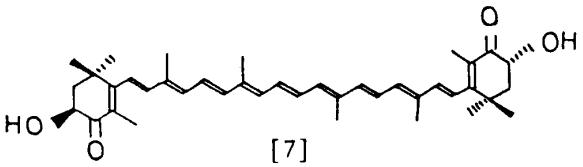
(3S,3'S)-Astaxanthin



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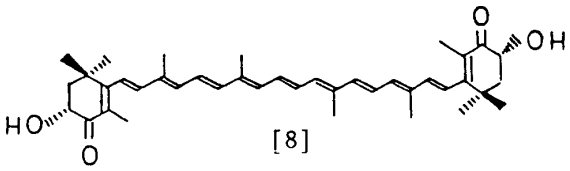
(3S,3'R)-Astaxanthin



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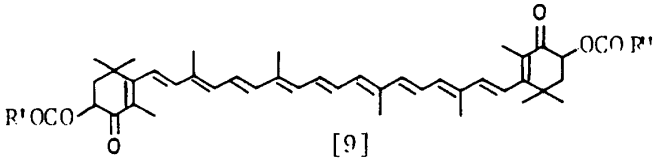
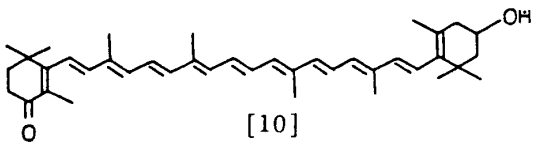
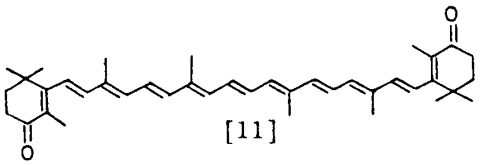
(3R,3'R)-Astaxanthin



[9]
Astaxanthin diester

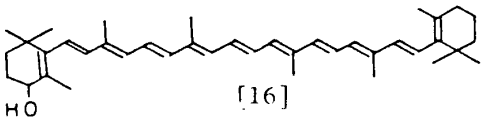


[10]
Asteroidenone
(3'-Hydroxy-echinenone)



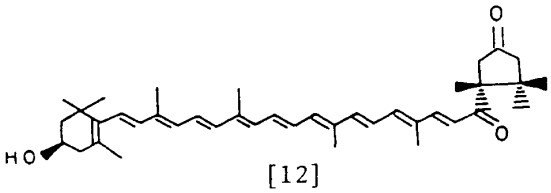
[11]

Canthaxanthin



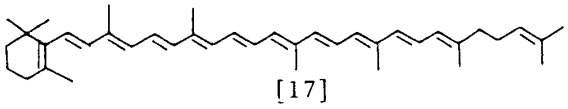
[16]

4-Hydroxy-β-carotene



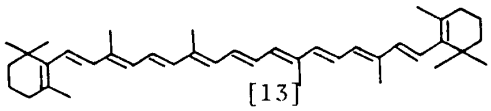
[12]

Capsanthin



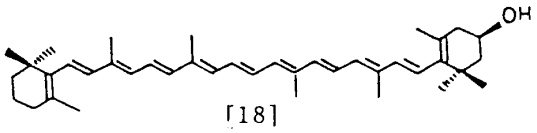
[17]

γ-Carotene



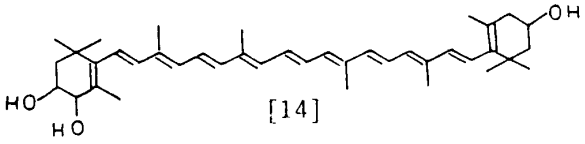
[13]

β-Carotene



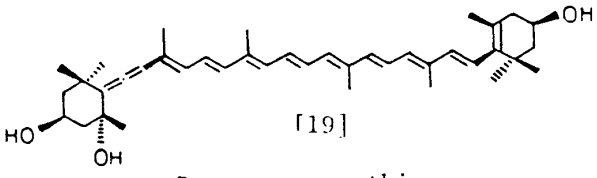
[18]

Cryptoxanthin



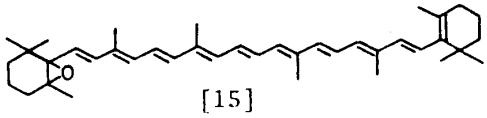
[14]

3,4,3'-Trihydroxy-β-carotene



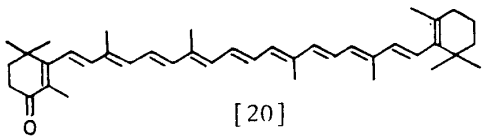
[19]

Deepoxyneoxanthin



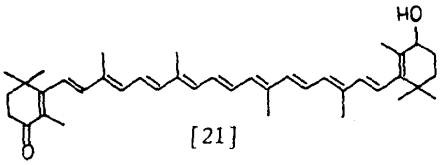
[15]

5,6-Epoxy-β-carotene



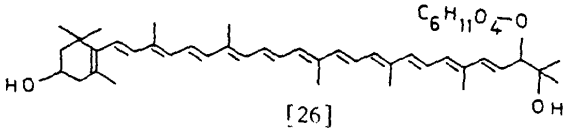
[20]

Echinenone



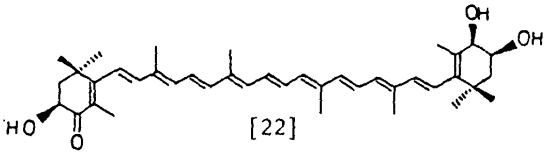
[21]

4-Hydroxy-echinenone



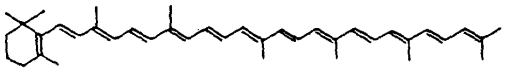
[26]

Myxoxanthophill



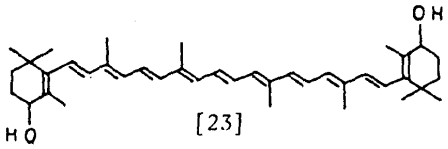
[22]

Idoxanthin



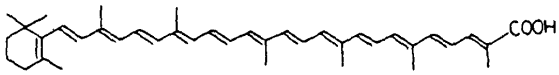
[27]

Torularhodine



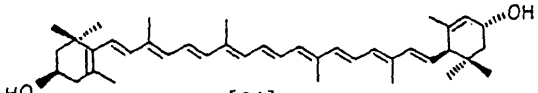
[23]

Isozeaxanthin



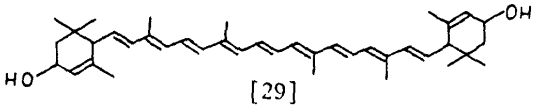
[28]

Torulene



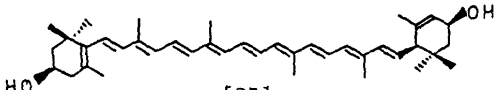
[24]

Lutein



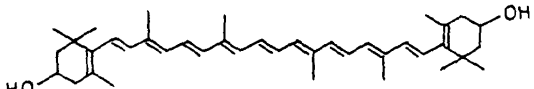
[29]

Tunaxanthin



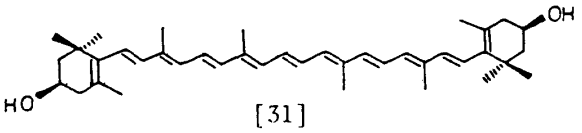
[25]

epi-Lutein



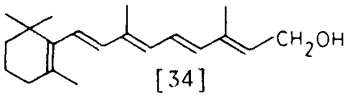
[30]

Zeaxanthin



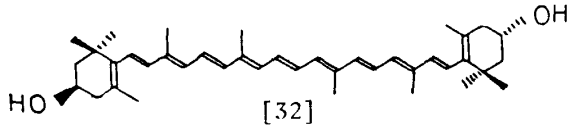
[31]

(3R,3'R)-Zeaxanthin



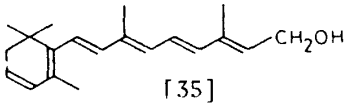
[34]

Retinol



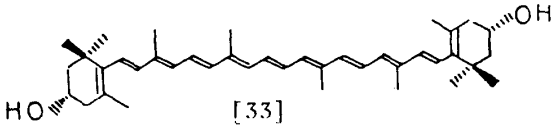
[32]

(3R,3'S)-Zeaxanthin



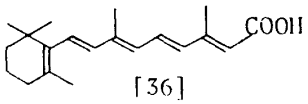
[35]

Dehydroretinol



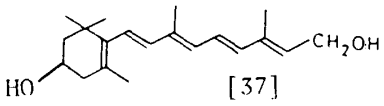
[33]

(3S,3'S)-Zeaxanthin



[36]

Retinoic Acid



[37]

3-Hydroxy-retinol