

Utilization of (3*S*,3'*S*)-astaxanthin acyl esters in pigmentation of rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The deposition of natural, optically active, astaxanthin fatty acid esters in rainbow trout (*Oncorhynchus mykiss*) was studied. Mono-esterified and di-esterified (3*S*,3'*S*) astaxanthin were purified from the green microalga *Haematococcus pluvialis* and incorporated into extruded diets and compared with diets containing synthetic racemic astaxanthin (Carophyll Pink) and a total carotenoid extract from the alga. All sources of astaxanthin achieved >4 mg kg⁻¹ in the white muscle after 6 weeks feeding. No significant difference ($P > 0.05$) between the deposition of astaxanthin or total carotenoid for the different diets was observed. Other xanthophylls, namely lutein, zeaxanthin and idoxanthin were found in the white muscle of rainbow trout fed all diets and together accounted for 10–14% of total carotenoid. Astaxanthin was deposited in the white muscle in the stereochemical form administered in the diet, i.e. racemic astaxanthin for Carophyll Pink and ~100% (3*S*,3'*S*)-astaxanthin for the algal sources. In contrast, epimerization of (3*S*,3'*S*) astaxanthin from the alga was observed for the astaxanthin esters deposited in the skin of rainbow trout, with a ratio close to 1.0:2.0:1.0 (3*S*,3'*S*:3*R*,3'*S*:3*R*,3'*R*).

KEY WORDS: astaxanthin, *Haematococcus pluvialis*, *Oncorhynchus mykiss*, pigmentation, rainbow trout

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Introduction

The carotenoids astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione) and canthaxanthin (β,β -carotene-4,4'-dione) are used to pigment the muscle of farmed salmonids. In salmonids, 4-ketocarotenoids such as astaxanthin are absorbed much more effectively than 3-hydroxycarotenoids [e.g. zeaxanthin (3,3'-dihydroxy- β,β -carotene) and lutein (3,3'-dihydroxy- β,ϵ -carotene)] which are themselves extensively used as pigments in animals (e.g. egg yolk pigmentation). The utilization of astaxanthin and canthaxanthin in salmonids is generally low (typified by low retention efficiencies of 3–20% in salmonid pigmentation trials; Foss *et al.* 1984; Choubert & Storebakken 1989; Smith *et al.* 1992). This is a major concern as a result of the high cost of these pigments in feeds (Nickell & Bromage 1997). More recently, Bjerkeng *et al.* (1997) have reported higher retentions (24–32%) for astaxanthin in rainbow trout (*Oncorhynchus mykiss*).

Once absorbed across the intestinal wall of the fish, carotenoids are transported by serum lipoproteins. In farmed *O. mykiss*, astaxanthin and canthaxanthin are uniformly distributed amongst all lipoprotein fractions (Choubert *et al.* 1992, 1994). In wild fish, however, considerable variation in the carotenoid content and composition of lipoproteins has been observed (e.g. in spent chum salmon; Ando *et al.* 1986).

Astaxanthin binds (with a weak bathochromic shift in its absorption spectrum) to actomyosin of the myofibrils in salmon. *In vitro* studies have examined the relationship between the structure of the carotenoid molecule and its ability to bind to actomyosin. For example, whilst the free astaxanthin binds tightly to actomyosin, mono-esterified astaxanthin only binds weakly to actomyosin, whereas the diester does not bind at all (Henmi *et al.* 1987). Esterified astaxanthin is also found in the skin of salmonids and can account for up to 20% of total carotenoid in the fish (Schiedt *et al.* 1988a,b).

The microalga *Haematococcus pluvialis* Flotow is a well-documented natural source of astaxanthin (e.g. Donkin 1976; Boussiba & Vonshak 1991; Grung *et al.* 1992; Harker *et al.* 1996). The use of this microalga and a number of other natural sources (e.g. Antarctic krill and the yeast *Xanthophyllomyces dendrorhous* – formerly *Phaffia rhodozyma*) for pigmentation in aquaculture (and poultry) has been proposed for many years, although it is only recently that products incorporating such sources of carotenoids have become commercially available (e.g. Spencer 1989; Burbrick 1991; Johnson & An 1991). *Haematococcus* has a complex life cycle (Elliot 1934) and astaxanthin biosynthesis and subsequent accumulation is generally associated with, but not exclusive to (W. Hartley & A.J. Young, unpublished data) the formation of aplanospores or cysts when the alga is subject to growth-limiting conditions, e.g. depletion of nitrogen in the growth medium and/or exposure to high irradiances. The astaxanthin-rich cysts are surrounded by a thick sporopollenin cell wall that renders them resistant to chemical attack (Good & Chapman 1979; Burczyk 1987). Indeed, a characteristic of these cysts is their resistance to acetolysis (VanWinkle Swift & Rickoll 1997). A study by Sommer *et al.* (1991) highlighted the key issue of variable utilization of this carotenoid from *Haematococcus*. Intact *Haematococcus* cysts failed to achieve desired levels of pigmentation in *O. mykiss* whilst processed cysts performed much better (Sommer *et al.* 1991).

In addition to this physical barrier of a very tough and resistant cell wall, astaxanthin from *Haematococcus* differs in two important aspects from commercially available, chemically manufactured, products. First, algal astaxanthin is esterified (primarily as monoesters; Renstrøm & Liaaen-Jensen 1981; Grung *et al.* 1992). This may be an important factor, limiting the uptake of the carotenoid as it has been demonstrated that synthetic astaxanthin di-palmitate was very poorly utilized in salmonids compared to unesterified astaxanthin (Foss *et al.* 1987). However, the lipid solubility of astaxanthin acyl esters would be expected to be better than that of crystalline astaxanthin. Hydrolysis of the astaxanthin esters must, however, take place before uptake as no esters are found in plasma or in the white muscle of salmonids (Schiedt 1998). The astaxanthin esters found in the skin of salmonids are the result of re-esterification of the free carotenoid with fatty acids endogenous to the fish (Foss *et al.* 1987).

Secondly, the configuration of algal astaxanthin is predominantly (>99%) the (3*S*,3'*S*) form (Renstrøm *et al.* 1981) whereas the synthetic products (Carophyll Pink: F. Hoffman-La Roche Ltd, Basel, Switzerland and Lucantin Pink: BASF Ltd, Ludwigshafen, Germany) are a racemic mixture of the

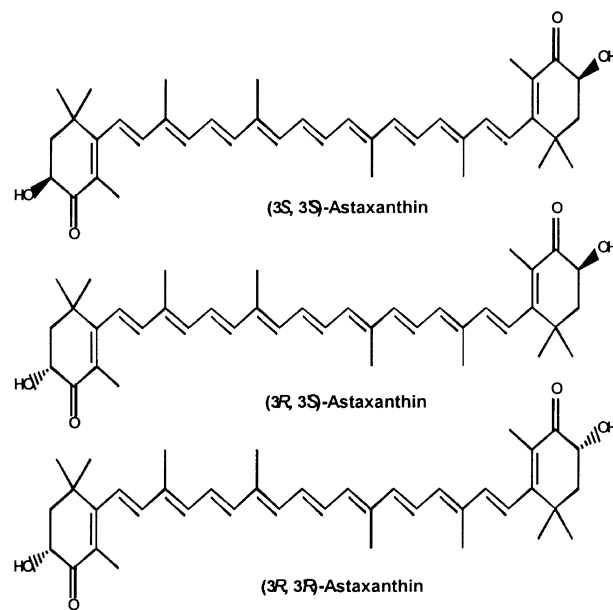


Figure 1 Structures of (3*S*,3'*S*)-astaxanthin (1); (3*R*,3'*S*)-astaxanthin (2); (3*R*,3'*R*)-astaxanthin (3).

three enantiomers (3*R*,3'*R*, 3*S*,3'*S*, 3*R*,3'*S*; in the ratio 1:1:2; Fig. 1). Foss *et al.* (1987) demonstrated that all three forms of unesterified astaxanthin are equally utilized, suggesting that the algal form may not be discriminated against on the basis of chirality by salmonids.

The main aim of this study was to evaluate the relative retention of algal carotenoids for pigmentation in aquaculture. The efficacy of natural astaxanthin acyl esters isolated from the microalga *Haematococcus pluvialis* in pigmenting the white muscle and skin of rainbow trout was studied.

Materials and methods

Experimental design

A total of 500 rainbow trout (*O. mykiss*) were maintained in 12 tanks for a total of 10 weeks (including 4 weeks acclimation) at 15 ± 0.5 °C with a flow rate of 1000 L h⁻¹ under a 12 h light:12 h dark photoperiod. Each tank measured 1.0 × 0.5 × 1.0 m with a capacity of 400 L. A total of 20 fish, with an initial mean weight of 140 ± 2 g were sampled following the acclimation period in order to determine their baseline pigment content and composition. A sample of 180 rainbow trout (15 fish per tank) with a final mean weight of $\sim 250 \pm 3$ g was harvested after 6 weeks feeding. Triplicate tanks were set up for each of the four diets (see below).

Diets

Four pigmented diets were used in this study; Diet 1 – Carophyll Pink; Diet 2 – Astaxanthin monoesters isolated from *Haematococcus*; Diet 3 – Astaxanthin diesters isolated from *Haematococcus*; Diet 4 – total carotenoid extract from *Haematococcus*. The target level for inclusion in all four diets was 40 mg kg⁻¹ astaxanthin (free or as esters). Details for the preparation of diets 1–4 are given below. Fish were hand fed twice daily (1.3–1.8% b.w.) and all food was eaten within 2 min, thus minimizing the leaching of pigment products (S. Lagocki, unpublished data). During the acclimation period (4 weeks) the fish were fed to apparent satiation

Table 1 Basal diet formulation and proximate composition

Ingredients	Inclusion (g kg ⁻¹)
LT-94 fishmeal ¹	613.0
Wheat gluten	56.0
Wheat	216.0
Fish oil	100.0
Vitamin premix ²	8.0
Mineral premix ²	7.0
Total	1000
Proximate composition ³	
Diet (%)	Basal
Protein	50.1
Oil	19.9
Moisture	4.0
Ash	10.7

¹Norsildmel, Bergen, Norway.

²Vitamins/mineral premixes as formulated by Trouw Aquaculture Ltd (Wincham, UK). A total of 1008 g of supplementary oil (Cod Liver Oil, Seven seas Ltd, Hull, UK) added to 12 kg of basal formulation to provide a carrier for the astaxanthin sources and to increase final oil level to a commercial level of 26% (w/w).

³Proximate composition by near infrared spectral analysis and before topcoating of supplementary oil.

using a non-pigmented expanded feed (Standard Expanded 40, Trouw Aquaculture, Wincham, UK). Fish were weighed prior to the study and after the 6-week feeding period. The formulation of the basal diet and proximate composition is shown in Table 1. The basal feed was produced as a single 150 kg extruded batch with a pellet size of 4 mm (Nutreco, ARC, Stavanger, Norway).

Diet 1 was prepared by heating (35 °C) 6 g of Carophyll Pink in 100 mL of distilled water. This was mixed with pure cod liver oil (84 g kg⁻¹ basal diet; Seven Seas Ltd, Hull, UK) and ethoxyquin (90 mg kg⁻¹). The emulsion raised the basal oil content from 19 to 26% (w/w). The resulting astaxanthin emulsion was then slowly added (10 min) to the basal diet (12 kg) whilst being gently mixed using a commercial mixer (Minimix 150, Belle Engineering Ltd, Sheen, UK). The diet was carefully blended for a further 30 min to ensure homogenous distribution of pigment products. There was no evidence of residual oil on the mixing bowl after 30 min of gentle mixing.

Diets 2–4 were prepared by direct mixing of the algal carotenoid extract with cod liver oil (no water was added to these products). Topcoating of the algal products were identical to the method outlined for Carophyll Pink. The four diets were stored in airtight opaque containers at room temperature prior to use. The actual inclusion levels (Table 2) were determined by HPLC (see below) following extraction of the feeds with Maxatase (Schüep & Schierle 1985). The stability of each diet was determined and losses of carotenoid were <1.0% for all algal diets during the experimental period and ~3% for Carophyll Pink.

Fillet colour analysis

The perceived colour of the fillets was determined on fresh unskinned fillets using the SalmoFan (F. Hoffman-La Roche

Table 2 Carotenoid content and composition of the extruded diets

Carotenoid	Dietary inclusion rate (mg kg ⁻¹)			
	Diet 1 ¹	Diet 2 ²	Diet 3 ³	Diet 4 ⁴
Astaxanthin	38.30	0.74	–	2.04
Astaxanthin monoesters	–	38.83	–	25.98
Astaxanthin diesters	–	–	36.91	9.57
Lutein	–	0.26	–	1.74
Canthaxanthin	–	–	–	3.06
β-Carotene	–	0.70	1.29	2.17
Total carotenoid	38.30	42.90	38.20	44.27

¹Carophyll Pink (F. Hoffman-La Roche, Basel, Switzerland).

²Astaxanthin monoesters isolated from *Haematococcus* (see Materials and methods).

³Astaxanthin diesters isolated from *Haematococcus*.

⁴Total carotenoid extract from *Haematococcus*.

Ltd, Basel, Switzerland). Three individuals made separate determinations for each fillet under natural daylight.

Carotenoid retention

The deposition efficiency of total carotenoid and of astaxanthin was determined after 6 weeks feeding using Equation (1) (Choubert & Luquet 1982). A factor of 0.6 was used to represent the ratio of muscle to total fish weight (determined from 20 randomly selected fish).

$$\text{NAR} = \frac{[(W_f \times C_f) - (W_i \times C_i)] \times 0.6 \times 100}{\text{FI} \times C_d} \quad (1)$$

where W_i = mean initial biomass, W_f = mean final biomass, C_f = mean final muscle astaxanthin/carotenoid concentration, C_i = mean initial muscle astaxanthin/carotenoid concentration, FI = mean total feed intake per treatment and C_d = analysed astaxanthin concentration in the diet.

Tissue preparation

Fish were anaesthetized with phenoxy-2-ethanol (200 mg mL⁻¹; Sigma, Poole, UK) and weighed. The fish were killed by a blow to the head, cut open and their viscera excised and discarded. The fish were then filleted with one fillet being used as a source of white muscle for carotenoid analysis and the other used for colour analysis by the SalmoFan.

A sample (~2 g) of white muscle was removed from immediately below the dorsal fin of every fish and accurately weighed into a centrifuge tube (25 mL). This muscle sample was found to be representative of the whole fillet for rainbow trout of the age and size range encountered in this study (S. Lagocki & S. Davis, unpublished data). For each gram of tissue, distilled water (1.0 mL) and methanol (1.0 mL; containing 500 mg L⁻¹ butylated hydroxytoluene) were added to each tube. The samples were homogenized for 30 s. Chloroform (3 mL for every g tissue) was added and the sample homogenized for a further 30 s. Samples were then incubated in the dark for 10 min at 20 °C after which the sample was homogenized for a further 30 s. The samples were then centrifuged 2010 × *g* for 10 min at 16 °C. An aliquot of hypo-phase (3.0 mL) was transferred to an amber vial, gently blown down under a stream of nitrogen and stored at -20 °C prior to analysis.

Carotenoids were extracted from the skin of the rainbow trout using the method described by Schiedt *et al.* (1995). Samples were filtered (Acrodisk GHP, 13 mm, 0.45 µm; Pall Gelman Laboratory, Portsmouth, UK) before HPLC analysis.

Isolation of astaxanthin acyl esters

Encysted cells of *H. pluvialis* with an astaxanthin content of 1–2% (w/dw) were first disrupted by homogenization in acetone in the presence of glass beads (2.5–3.5 mm) using a tissue homogenizer (Mickle Laboratory Engineering Co. Ltd, Gomshall, Surrey, UK). The extract was filtered and dried under vacuum. The astaxanthin monoester (Rf 0.47) and diester (Rf 0.79) fractions were isolated using thin layer chromatography (TLC) on Kieselgel 60 (Merck, Poole, UK) using acetone/hexane (3/7, v/v) as the developing solvent. The TLC plates were coated with 2.5% (v/v) citric acid (in methanol) prior to use in order to improve band resolution. Carotenoids were quantified in *n*-hexane using the published extinction coefficient (A (1%/1 cm) = 2100) for all-*trans* astaxanthin (Britton 1995).

Cleavage of astaxanthin esters by anaerobic saponification

Anaerobic saponification of astaxanthin esters was performed at an analytical scale on the astaxanthin esters recovered from the skin of rainbow trout. The method used was based on that originally developed by Schiedt *et al.* (1993). A known amount (typically 10–100 µg) of carotenoid esters was dissolved in dichloromethane (maximum 3 mL) and mixed. Sodium methoxide (1.0 mL; prepared by dissolving 1.5 g of sodium in 100 mL of methanol) per mL of astaxanthin solution was added to a side arm flask and the entire system thoroughly flushed with oxygen-free nitrogen in order to remove all traces of oxygen. The sodium methoxide and astaxanthin solutions were frozen with liquid nitrogen and placed under vacuum. The solutions were thawed at room temperature, in order to de-gas them. The entire system was flushed again with oxygen-free nitrogen. The freeze/thaw cycle was repeated at least three times. The sodium methoxide solution was carefully mixed with the astaxanthin solution under high vacuum with constant stirring for 15 min at room temperature. The reaction mixture was then acidified with 1 mL of 1 N H₂SO₄ mL⁻¹ of sodium methoxide, removed and diluted with ethanol and water. Astaxanthin was then extracted with hexane:diethyl ether (1:1 v/v; freshly redistilled), washed twice with water and finally dried under oxygen-free nitrogen.

Analysis of astaxanthin

The carotenoid content and composition of the white muscle and skin of rainbow trout was determined by HPLC using a modification of the method developed by Schüep &

Schierle (1985). A Lichrosorb silica 60 rapid analysis column (50 × 4.6 mm, 5 µm particles; Phenomenex, Macclesfield, UK) and security guard cartridge were first acidified with 1% (v/v) H₃PO₄ in methanol (Kirkland & Dilks 1973) to prevent peak tailing. The solvent system (*n*-hexane:acetone, 86:14 v/v) was delivered at a flow rate of 1.2 mL min⁻¹ at 20 °C using an Agilent (Stockport, UK) 1100 series Binary pump. Samples were injected in 20 µL of the eluting solvent using an Agilent 1100 series autosampler. Carotenoids were detected at 470 and 450 nm using an Agilent 1100 series diode-array detector and integrated (at the λ_{max}) using Chemstation 6.1 software (Agilent). The *t*_R of the main carotenoids were: all-*trans* astaxanthin (3.9 min); 9-*cis* astaxanthin (4.5 min), 13-*cis* astaxanthin (4.9 min), idoxanthin (5.9 min); lutein (7.9 min) and zeaxanthin (8.7 min). F. Hoffman-La Roche (Basel, Switzerland) kindly provided the carotenoid standards, including racemic idoxanthin and the individual chiral forms and several mono-*cis* isomers of astaxanthin.

Analysis of chiral forms of astaxanthin

The analysis of the chiral forms of astaxanthin was achieved by derivatization of the unesterified carotenoid to produce their dicamphanates. Astaxanthin was dissolved in dry pyridine (0.5 mL) and reacted with 50 mg of (-)-camphanoyl chloride (Sigma, Poole, UK) for 10 min at room temperature. The resulting dicamphanates were extracted by partition into diethyl ether. Analysis of dicamphanates was by normal-phase HPLC (Lichrosorb SI60; 250 × 4.6 mm, 5 µm particles; detection at 491 nm). The solvent system used was *n*-hexane/ethyl acetate (75/25, v/v) at a flow rate of 0.5 mL min⁻¹. *T*_R = (3*R*,3'*R*) 18.5 min; (3*R*,3'*S*) 'meso' 19.8 min; (3*S*,3'*S*) 21.3 min.

Statistical analysis

The data are presented as the mean ± SEM per tank (*n* = 3) for each diet. The analysis of variance was calculated using the *t*-test and one-way ANOVA test on Minitab v.10 (Minitab Ltd, Coventry, UK).

Results

Diet content and composition

The carotenoid content and composition of each of the four diets used in this study is given in Table 2. Diet 1 was all-*trans* racemic astaxanthin. In diet 2 astaxanthin was supplied as isolated (3*S*,3'*S*) astaxanthin monoesters. Diet 3 was isolated (3*S*,3'*S*) astaxanthin diesters, and diet 4 was a total carotenoid extract from *Haematococcus*. In contrast to the other three diets, diet 4 contained significant amounts of carotenoids other than astaxanthin (Table 2). The basal diet formulation also contained carotenoids, including lutein (at very low levels) but not astaxanthin. The target inclusion rate for each diet was 40 mg kg⁻¹, but some difference was noted in the total carotenoid and the total astaxanthin content of the four diets (Table 2). The astaxanthin diesters (diet 3) had the lowest rate of inclusion at 38.2 ppm, whilst the algal carotenoid extract (diet 4) had the highest at 44.3 ppm.

Growth performance and feed utilization

No significance differences (*P* > 0.05) were observed with respect to growth performance and feed utilization (Table 3).

Fillet colour

No significance difference (*P* > 0.05) in white muscle colouration (assessed using the SalmoFan) was observed for fish fed any of the four diets. Median values of 26 (diets 3, 4) and 27 (diets 1, 2) were obtained, with a range of 22–29.

Carotenoid deposition

The deposition of total carotenoid and of astaxanthin in the white muscle of trout was similar for all four diets used in this study, with levels of pigmentation of 4–6 mg kg⁻¹ being achieved (Fig. 2). The major carotenoid deposited was all-*trans* astaxanthin, together with trace amounts of *cis*-isomers (see below). There was no significant difference (*P* > 0.05) between the pigmentation achieved by the different diets, either in terms of total carotenoid or astaxanthin deposited.

Table 3 Growth performance and feed utilization of rainbow trout after 6-week feeding of the experimental diets (mean ± SEM; *n* = 3 tanks per diet). See Table 2 for details

Growth data	Diet 1 ¹	Diet 2 ²	Diet 3 ³	Diet 4 ⁴
Initial mean weight	140.37 ± 1.87	141.92 ± 1.98	142.03 ± 1.51	139.60 ± 1.19
Final mean weight	246.70 ± 0.08	249.03 ± 0.28	252.06 ± 3.78	251.32 ± 4.97
Feed : gain ratio	1.02 ± 0.03	1.02 ± 0.03	0.99 ± 0.02	1.0 ± 0.03

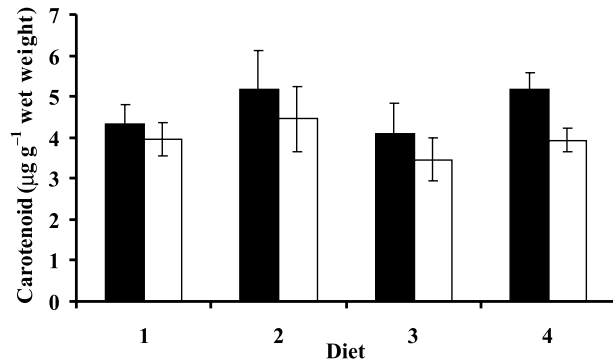


Figure 2 Total carotenoid (■) and astaxanthin content (□) of muscle of rainbow trout. Diet 1: Carophyll Pink; Diet 2: Astaxanthin monoesters; Diet 3: Astaxanthin diesters; Diet 4: Total carotenoid extract from *Haematococcus*. See Table 2 for details of carotenoid composition of the diets. Values are mean \pm SEM ($n = 3$ tanks per diet).

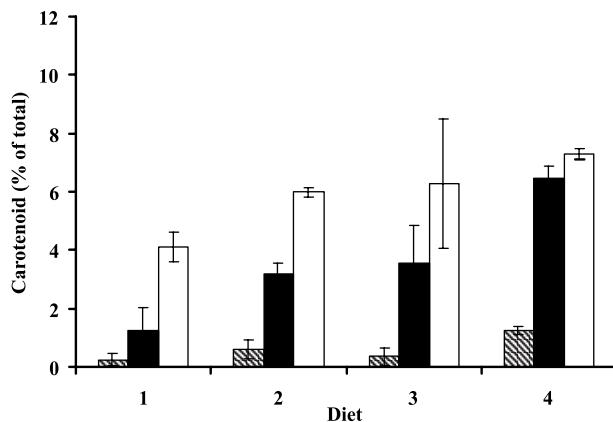


Figure 3 Carotenoid composition (% of total carotenoid) of muscle of rainbow trout: idoxanthin (▨); lutein (■); zeaxanthin (□). Diet 1: Carophyll Pink; Diet 2: Astaxanthin monoesters; Diet 3: Astaxanthin diesters; Diet 4: Total carotenoid extract from *Haematococcus*. See Table 2 for details of carotenoid composition of the diets. Values are mean \pm SEM ($n = 3$ tanks per diet).

In addition to astaxanthin, trace amounts of lutein, zeaxanthin and idoxanthin (3,3',4'-trihydroxy- β , β -caroten-4-one) were found in all fish examined, including those fed synthetic all-*trans* astaxanthin (Fig. 3). No significant difference ($P > 0.05$) was found between the different diets for the levels of these xanthophylls deposited in the white muscle of rainbow trout.

The net apparent retention (NAR) was calculated for both total carotenoid and for astaxanthin because of the presence of xanthophylls in the diets derived from *Haematococcus* (Table 4). The NAR for total carotenoid was in the range 14–16%, whilst the value for astaxanthin was 12–15%. No significant difference in the retention of dietary carotenoids was observed between the four diets ($P > 0.05$).

Geometrical isomers

Both 9-*cis* and 13-*cis* astaxanthin were detected in the white muscle of rainbow trout. Whilst 9-*cis* astaxanthin was found in fish fed all four diets, the presence of 13-*cis* astaxanthin was restricted to those fish fed with algal astaxanthin (diets 2–4). Levels of these isomers were small and accounted for <4% of total carotenoid in the white muscle of rainbow trout. Both the 9-*cis* and 13-*cis* isomers were naturally found in the diets incorporating astaxanthin extracted from *Haematococcus*, and Carophyll Pink (extracted using the Maxatase method; Schüep & Schierle 1985).

Astaxanthin chirality

The chirality of the astaxanthin deposited in the white muscle and skin of fish fed each of the four test diets is shown in Table 5. The chirality of the unesterified astaxanthin accumulated in the white muscle of rainbow trout was the same as the original dietary source, so that all three algal diets deposited the (3*S*,3'*S*) form whilst the synthetic product was deposited as a racemic mixture.

Diet	Net apparent retention of	
	Total dietary carotenoid ¹	Dietary astaxanthin ²
Diet 1: Carophyll Pink	14.8 \pm 2.5	13.6 \pm 1.9
Diet 2: Astaxanthin monoesters	15.8 \pm 4.6	14.9 \pm 4.1
Diet 3: Astaxanthin diesters	14.0 \pm 3.8	12.4 \pm 2.8
Diet 4: Total carotenoid extract	15.7 \pm 1.6	14.1 \pm 1.3

¹Determined for total carotenoid content of the diets (Table 2) and white muscle.

²Determined for astaxanthin content (free plus esters) of the diets (Table 2) and white muscle.

Values are percentage \pm SEM.

Table 4 Net apparent retention of dietary carotenoid and dietary astaxanthin after 6 weeks feeding (mean \pm SEM; $n = 3$ tanks per diet). See Materials and methods for details

Table 5 Chirality of astaxanthin in the dietary supplements, the white muscle of rainbow trout, and the skin of rainbow trout after 6 weeks feeding. The chirality of the astaxanthin esters in the skin was determined following anaerobic saponification (see Materials and methods)

Carotenoid	Chirality of astaxanthin (3 <i>S</i> ,3' <i>S</i> :3 <i>R</i> ,3' <i>R</i>)		
	Feeds	White muscle	Skin
Diet 1: Carophyll Pink	1.00:2.00:1.00	0.55:1.00:0.54	0.53:1.00:0.58
Diet 2: Astaxanthin monoesters	1.00:0.08:0.06	1.00:0.20:0.10	0.66:1.00:0.55
Diet 3: Astaxanthin diesters	1.00:0.07:0.06	1.00:0.40:0.15	0.59:1.00:0.49
Diet 4: Total carotenoid extract	1.00:0.09:0.05	1.00:0.13:0.06	0.85:1.00:0.60

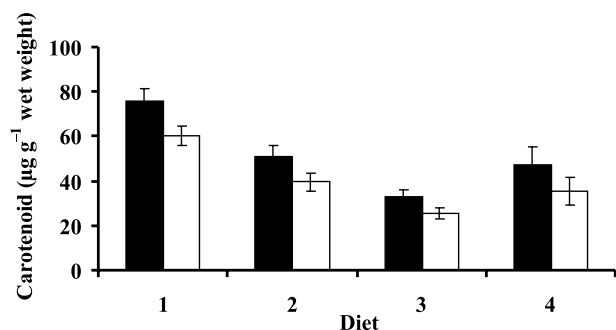


Figure 4 Astaxanthin (■) and lutein (□) content of the skin of rainbow trout. Diet 1: Carophyll Pink; Diet 2: Astaxanthin monoesters; Diet 3: Astaxanthin diesters; Diet 4: Total carotenoid extract from *Haematococcus*. See Table 2 for details of carotenoid composition of the diets. Values are mean ± SEM ($n = 3$ tanks per diet).

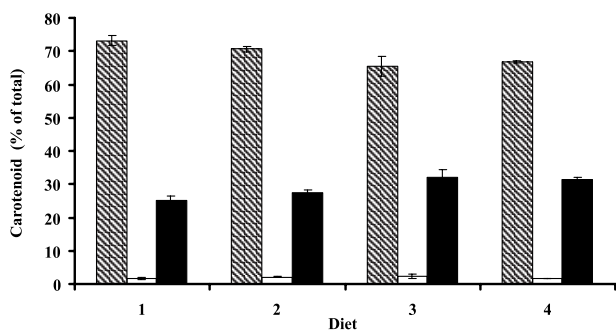


Figure 5 Carotenoid composition of the skin of rainbow trout: astaxanthin esters (▨); free astaxanthin (□); lutein esters (■). Diet 1: Carophyll Pink; Diet 2: Astaxanthin monoesters; Diet 3: Astaxanthin diesters; Diet 4: Total carotenoid extract from *Haematococcus*. See Table 2 for details of carotenoid composition of the diets. Values are mean ± SEM ($n = 3$ tanks per diet).

In contrast, *in vivo* racemization of astaxanthin was observed for the deposition of astaxanthin esters in the skin of rainbow trout. For all three algal-derived diets (diets 2–4) a nearly racemic ratio of 1:2:1 (3*S*,3'*S*:3*R*,3'*S*:3*R*,3'*R*) was observed suggesting a high degree of epimerization of

(3*S*,3'*S*)-astaxanthin from the algal diets. The methodologies used (including anaerobic saponification of the astaxanthin esters) were first validated using appropriate standards as well as using the natural esters from *Haematococcus*. These methods did not, themselves, introduce any artefacts.

Discussion

A number of studies have demonstrated that microalgae represent a viable source of natural pigments for use in aquaculture (e.g. Sommer *et al.* 1991, 1992; Choubert & Heinrich 1993; Gouveia *et al.* 1998). The results from these studies have also highlighted a number of issues concerning the utilization of carotenoids derived from natural sources. The majority of natural sources of astaxanthin, including shrimp or krill waste and microalgae, exist as an esterified form. Often this is a complex mixture of mono- and diesters of the carotenoid, with only trace levels of unesterified astaxanthin present. This study has demonstrated that rainbow trout can effectively utilize natural astaxanthin acyl esters, as esters predominate in the diet of wild fish.

Importantly, this study also revealed that both the isolated *Haematococcus* mono- and diesters of astaxanthin were as equally well utilized as the synthetic unesterified astaxanthin. Similarly the 'cell-free' total carotenoid extract from *Haematococcus* was effective in pigmenting rainbow trout (Figs 1 & 2). These results are in contradiction of the findings of Schiedt & Leuenberger (1981) and Schiedt *et al.* (1985) who reported that synthetic astaxanthin di-palmitate was largely ineffective in pigmenting salmonids when compared with the unesterified form of the carotenoid. However, an earlier study by Simpson & Kamata (1979) reported that higher levels of pigmentation could be achieved using an esterified rather than an unesterified source of astaxanthin. Choubert & Heinrich (1993) reported very low carotenoid retention rates (1.5%) for rainbow trout fed with *Haematococcus* and suggested that cleavage of the astaxanthin esters may be a limiting step for the deposition of astaxanthin. The present

study suggests that this is not the case and that the algal cell wall is the main limiting factor. Sommer *et al.* (1991, 1992) also reported a lower efficiency for astaxanthin from cells of *Haematococcus* compared with synthetic astaxanthin. The fatty acid composition of the astaxanthin esters from the alga is similar to the composition of plant and green algal membranes and consists primarily of C18:1 (Renström & Liaaen-Jensen 1981; J. Bowen, C. Soutar & A.J. Young, unpublished data), although some discrimination against different astaxanthin esters may be possible. In the current study astaxanthin from all diets was equally utilized, suggesting that esterification *per se* is not a major factor in limiting pigmentation by *Haematococcus*.

Salmonids show a clear dose–response pattern for pigmentation because of astaxanthin inclusion in their diet up to ~50 ppm. At inclusion levels above this a decreasing dose–response is observed (Schiedt 1998). Small differences in the actual inclusion rates for the different diets used in this study (Table 2) may therefore be expected to exert an effect on the actual levels of pigmentation achieved with the rainbow trout. However, no significant difference was observed in the levels of total carotenoid or astaxanthin deposited in the white muscle (Fig. 2) or in the dietary retention rates (Table 4).

The presence of geometrical isomers of astaxanthin in the white muscle of salmonids was observed in this study and has been previously reported. Bjerkeng *et al.* (1997) detected the 9-*cis*, 13-*cis* and 15-*cis* forms in the white muscle of rainbow trout fed with synthetic astaxanthin. More recently, Østerlie *et al.* (1999) demonstrated the selective distribution of geometrical isomers of astaxanthin in the faeces, blood, liver and muscle of rainbow trout. The apparent digestibility coefficients (ADC) were significantly lower for a mixture of *cis/trans* isomers of astaxanthin than for all-*trans* astaxanthin (Bjerkeng *et al.* 1997) so that trout fed the all-*trans* form had a higher overall carotenoid content (Østerlie *et al.* 1999). The ADCs were in the order all-*trans* > 13-*cis* > 9-*cis*. Isomerization of all-*trans* astaxanthin may take place in the gastrointestinal tract and also following uptake (Bjerkeng *et al.* 1997). It might also be expected that hydrolysis of the astaxanthin esters in the intestinal wall may also promote *trans-cis* isomerization. Whilst the levels of *cis*-isomers were higher in the diets derived from *Haematococcus* (diets 2–4) levels were still low (<4% total carotenoid) and this did not appear to affect retention. In the present study, the 15-*cis* form was not detected in the white muscle of rainbow trout.

Wild *Salmo* and *Oncorhynchus* species possess the three enantiomers of astaxanthin in a typical ratio of 78–85:2–6:12–17 (3*S*,3'*S*):(3*R*,3'*S*):(3*R*,3'*R*) (Schiedt 1998). In con-

trast, synthetic astaxanthin (Carophyll Pink) has a ratio of 1:2:1 (i.e. a ratio of 1:1 for the racemate and *meso* forms; Bernhard 1990). Salmonids do not discriminate between the three enantiomers of unesterified astaxanthin and all are deposited equally in the muscle. This was observed in the present study as both racemic (diet 1) and (3*S*,3'*S*)-astaxanthin (diets 2–4) was deposited unchanged. Differences in the ratio of the (3*S*,3'*S*):(3*R*,3'*R*) forms have, however, been seen between the skin and posterior kidney of rainbow trout (Østerlie *et al.* 1999). It has also been reported that astaxanthin esters are utilized differently from the unesterified carotenoid (Schiedt *et al.* 1985). Whilst all three configurations of unesterified carotenoid are equally well utilized, astaxanthin di-palmitate was utilized in the order racemic > (3*R*,3'*R*) > (3*S*,3'*S*).

In contrast to the situation in muscle and in the plasma, astaxanthin is deposited in the skin of salmonids, including *O. mykiss*, in an esterified form. Re-esterification, with fatty acids endogenous to the fish (mainly 20:5 and 18:1; Schiedt *et al.* 1985) has, however, been found to be unspecific in relation to the enantiomeric form of the carotenoid molecule (Schiedt *et al.* 1985). Specific enrichment of the (3*R*,3'*R*) form has been observed in the skin when the di-palmitates of these enantiomers have been used. Racemic astaxanthin di-palmitate was deposited in the ratio 0.4:1.0:1.0 (3*S*,3'*S*):(3*R*,3'*S*):(3*R*,3'*R*) suggesting discrimination against the (3*S*,3'*S*) form (Schiedt *et al.* 1985, 1988a,b). *In vivo* racemization of ³H-(3*S*,3'*S*)-astaxanthin has also been observed in the shrimp *Penaeus japonicus* although it was not determined whether this occurred during or after absorption (Schiedt *et al.* 1991, 1993).

In the present study, whilst no epimerization of astaxanthin in the white muscle of rainbow trout was observed, the deposition of algal astaxanthin in the skin was accompanied by epimerization of (3*S*,3'*S*)-astaxanthin (Table 5), with both the (3*R*,3'*S*, 3*R*,3'*R*) forms being deposited. Epimerization of other carotenoids is observed in salmonids but this is generally attributed to the process of reductive metabolism of astaxanthin or uptake of other dietary sources of carotenoids (Schiedt 1998). For example, the configuration of lutein in the white muscle of rainbow trout fed (3*R*,3'*R*) or (3*S*,3'*S*)-astaxanthin di-palmitate was (3*R*,3'*R*,6'*R*), whilst in the skin 3'-epilutein was the main form (Schiedt *et al.* 1985; Schiedt 1998). Stereo-selective attack by ester hydrolyases in the intestinal wall of the fish cannot be ruled out.

The detection of idoxanthin in fish fed natural or synthetic astaxanthin confirms the observations of Schiedt *et al.* (1988a,b) for Atlantic salmon and Bjerkeng *et al.* (1997) for rainbow trout. Although both zeaxanthin and lutein were

found in all pigmented rainbow trout, levels were too low to permit identification of their configurations. It is therefore not possible to determine whether their presence in the white muscle of rainbow trout was the result of reductive metabolism of astaxanthin (as proposed by Matsuno 1991; reviewed by Schiedt 1998) or simply because of enhanced absorption from the basal diet (which naturally contains these two xanthophylls). The zeaxanthin deposited in the fish fed with algal astaxanthin would, however, be expected to be (3*R*,3'*R*) as the chirality at C(3) is unaffected (Schiedt 1998).

The results from this study suggest that the use of algal sources of pigments, is not limited by the esterification of astaxanthin, but rather it is the extent to which the cyst or cell wall is broken or cracked that is probably the key factor limiting utilization and retention of carotenoid in salmonids. We have observed that the presence of an intact cell wall in old, large spray-dried cysts (> 3% astaxanthin) can completely inhibit deposition of astaxanthin from *Haematococcus* in the white muscle of rainbow trout (J. Bowen & A.J. Young, unpublished data). Processing of these cells can be achieved by a number of methods, including cryogenic grinding (Bubrick 1991), homogenization (Mendes-Pinto *et al.* 2001) and pressure treatment (J. Bowen & A.J. Young, unpublished data). An important consideration therefore is the overall efficiency of such processing in terms of uniformity of treatment and subsequent bioavailability of astaxanthin. The accumulation of the sporopollenin cell wall in cysts of *Haematococcus* depends on the age of the cell, so that (in general) older cells (which contain more astaxanthin) are larger and possess a thicker cell wall. These cells, in turn, require exposure to harsher conditions in order to rupture the cell wall (J. Bowen & A.J. Young, unpublished data).

The data from this study demonstrate that natural astaxanthin fatty acid esters are as effectively utilized by rainbow trout as synthetic unesterified astaxanthin. The deposition and retention of the mono- and diesters of astaxanthin were the same as that achieved by synthetic unesterified astaxanthin. In addition, the stability of algal astaxanthin esters (either isolated as in this study or especially within broken algal cells; J. Bowen & A.J. Young, unpublished data) in extruded diets was also improved compared with that of unesterified astaxanthin. These results have implications for the utilization of other natural sources of astaxanthin esters, especially shrimp or krill waste (usually characterized, however, by a low level of astaxanthin). It suggests that the utilization of algal astaxanthin sources (from algae such as *Haematococcus* and *Chlorella*) may be most limited by the algal cell wall.

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