Processing of astaxanthin-rich *Haematococcus* cells for dietary inclusion and optimal pigmentation in Rainbow trout, *Onchorynchus mykiss* L.

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Abstract

A range of physical cell disruption techniques have been evaluated to aid the processing of astaxanthin-rich haematocysts of *Haematoccus pluvialis* for inclusion in salmonid feeds. Cell disruption by a scaleable pressure treatment system was shown to be effective in breaking open the haematocysts without altering the content or isomeric composition of carotenoids in the algal cells. Storage of disrupted cells was optimal at -20°C in the dark under nitrogen. Disrupted cells were spray-dried, incorporated into commercial diets and fed to Rainbow trout (*Onchorynchus mykiss* L). A marketable level of pigmentation in fish muscle was achieved after 10 weeks dietary supplementation. The geometric and optimal isomer composition of the astaxanthin deposited in the muscle was nearly identical to that seen in *Haematococcus*. Changes were observed in the chirality of the astaxanthin deposited in the skin in comparison to that isolated from both the white muscle and the alga.

Introduction

Wild salmonids (trout and salmon) achieve natural flesh pigmentation by the ingestion of crustaceans that contain astaxanthin (Liaaen-Jensen 1998). In intensive aquaculture, salmonid feeds are commonly supplemented with the carotenoids astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) and canthaxanthin (β,β-carotene-4,4'-dione; e.g., Ambati *et al.* 2014). A wide range of natural sources of astaxanthin have been used as alternatives to the chemically synthesised products. The pigmentation options and strategy for the application of natural pigments in diets for farmed salmon and trout were reviewed by Davies (2005). Natural sources include *H. pluvialis* (e.g., Bowen *et al.* 2002), krill (Mori *et al.* 1989); shrimp wastes

(Saito & Regier 1971); crayfish oil extracts (Peterson *et al.* 1966); red crab wastes and oil extracts (Spinelli & Mahnken 1978), the yeast *Xanthophyllomyces dendrorhous* (Johnson *et al.* 1977) and the marine bacterium *Paracoccus carotinifaciens* (which additionally contains high levels of adonirubin: 3-hydroxy-β,β-carotene-4,4'-dione, EFSA 2007; Kurina *et al.* 2015). Of these, both *H. pluvialis* and *P. carotinifaciens* can be regarded as the most suitable natural sources of astaxanthin as they possess the (3*S*,3'*S*) form of the carotenoid, as found in wild salmonids (Bowen *et al.* 2002; ESFA, 2007).

The freshwater microalga *Haematococcus pluvialis* (Flowtow) is well know for its ability to accumulate high levels (> 3.0% of cell dry weight) of the carotenoid astaxanthin (Lorenz & Cysewski 2000; Ambati *et al.* 2014). The accumulation of astaxanthin in this species, is normally a result of growth-limiting conditions, typically deprivation of nutrients such as nitrogen and / or exposure to high irradiances (Boussiba & Vonshak 1991; Harker *et al.* 1996). Astaxanthin is normally accumulated in a non-motile life stage of the alga (haematocysts). A characteristic of the astaxanthin-rich haematocyst is the presence of a tough and resilient sporopollenin cell wall (Burczyk 1987). This cell wall is regarded as one of the main limiting factors concerning the subsequent bioavailability of algal astaxanthin when used to pigment salmonids (Good & Chapman 1979; Mendes-Pinto *et al.* 2001) and other commercial species (Gomes *et al.* 2002). A particular characteristic of these cysts is their resistance to acetolysis (VanWinkle Swift & Rickoll 1997). Sommer *et al.* (1991) highlighted the key issue of variable utilisation of astaxanthin from *Haematococcus* preparations: intact haematocysts failed to achieve desired levels of

pigmentation in Rainbow trout (*Oncorhynchus mykiss*) whilst processed cysts performed much better.

The main aims of this study were to first evaluate a range of novel methodologies to process the astaxanthin-rich heamatocysts of *H. pluvialis* and then determine the subsequent efficacy of pigmentation of these processed products in Rainbow trout, representing a commercially valuable salmonid species.

Materials and methods

Algal material

Strain 34/7 of *Haematococcus pluvialis* (Flotow) was obtained from the Culture Collection of Algae and Protozoa (Windermere,UK). The alga was cultivated in batch mode in modified Bold's Basal Medium (BBM; Nichols & Bold 1964) outdoors (Liverpool, UK) in 65L tubular photobioreactors. Cells were harvested when the astaxanthin content has reached at least 1% of cell dry weight, typically after approximately 20 days cultivation.

Carotenoid analysis

A simple, small-scale, rapid mechanical technique was employed to extract carotenoids from algal cells and products. An appropriate volume of algal culture (typically 5mL) was centrifuged using a Mistral 1000 centrifuge at 1200 x *g* for 5 min. The supernatant was discarded and the cells re-suspended in distilled water. The cells were then centrifuged for a further 5 min at 1200 x g, the supernatant was discarded and the cells were re-suspended in redistilled acetone. The cells were

transferred to a bijou bottle, two-thirds full with 0.5cm diameter glass beads. To ensure complete recovery the centrifuge tube was rinsed with redistilled acetone which was then added to the bijou bottle. The cells were then homogenised for 5 min using a tissue homogeniser (Cavey Laboratory Engineering Co. Ltd, Guilford, UK), and then filtered through absorbent cotton wool to remove all cell debris. The filtrate was dried under oxygen-free nitrogen and stored at –20^{o}C until analysis (typically within 48 hours). A further technique developed by the Cyanotech Corporation (Hawaii, USA; Lorenz 2001) was also used for samples of NatuRose™. An aliquot (10-15mg) of NatuRose™ powder was weighed into a 50mL centrifuge tube. Glass beads (5g) were added together with 2mL of acetone and vortexed vigorously for 5 min after which a further 6mL of acetone was added to the tube and vortexed briefly (15 sec). The sample was then centrifuged for 5 min at 3600 x g, the supernatant removed and collected in a 50mL volumetric flask. Acetone (1mL) was added to the pellet and vortexed for 5 min. A further 6mL of acetone was then added, vortexed and re-centrifuged. The supernatant was recovered and the extraction repeated until a colourless pellet was achieved. All supernatant fractions were collected in the volumetric and after the final extraction the volume was made up to 50mL with acetone. The combined extract was then mixed gently and an aliquot re-centrifuged to remove any particulate matter. These two techniques were only suitable for small sample volumes (up to 10mL). Solvent extracts were dried under a steady-stream of N_2 and stored at -20^{o}C prior to analysis by UV/Vis spectroscopy or HPLC (typically within 24 hours).

UV/Vis spectroscopy and normal phase HPLC was used for identification of carotenoids in both the algal material and fish tissues by comparison to and co-elution with known standards using previously published methodology (Bowen *et al.* 2002; White *et al.* 2003).

Algal cell disruption

For the evaluation of scale-up potential, four different cell disruption techniques were examined comprising two different types of homogeniser, an ultrasonic disruptor and a pressure fracturing system. The two homogenisers used were a Polytron PT3100 (Phillip Harris Ltd., UK) and a Silverson SL2T (Silverson, Chesham, UK). The Polytron homogeniser tested was equipped with two different aggregates; HT62-628 and HT62-638. Both aggregates were tested using algal cells with a total carotenoid content of 1% DW at a range of available speeds (up to 28,000 rpm) for up to 30 min. The Silverson homogeniser was equipped with three different homogenising screens for various particle sizes; fine, medium and a square hole high sheer screen for larger particles. All three screens were tested using algal cells with a total carotenoid content of 1% DW at a range of different speeds (up to 9000 rpm) for up to 30 min. The ultrasonic disruptor (US0/200 Ultrasonic Homogniser; Phillip Harris Ltd., UK) was tested using algal cells with a total carotenoid content of 1% DW at a range of different amplitude settings (up to 100%) for up to 30 min. The Stansted Cell Disruptor 0512W-116 (Stansted Fluid Power Ltd., Harlow, UK) allowed algal cells to be subjected to pressures of up to 100,000 psi. This was initially tested with cells with a total carotenoid content of 1% DW over a range of different pressures and after a number of passes of algal material through the

disruptor. Prior to disruption, ethoxyquin (Sigma, Poole, UK) was added to the cell suspension to a final concentration of 0.3% (w/w of total carotenoids) to minimise oxidation.

A simple solvent-leaching method (avoiding any physical extraction) was employed in order to evaluate the release of astaxanthin from carotenoid-rich algal cells (described in Mendes-Pinto *et al.* 2001). The 'potential bioavailability' of the carotenoids leached from the material tested was expressed as the maximum carotenoid extracted using solvent extraction as a function of the total carotenoid content of the algal cells as determined through small-scale mechanical extraction (see above).

Spray drying

Following cell disruption, all algal materials were spray-dried prior to incorporation into diets (see below). Drying was achieved using an SD-05 Spray Drier (Lab-Plant Ltd, Filey, UK). A range of conditions were tested until a fine, free-flowing powder was collected with a low residual moisture content. The drying conditions used were as follows: Inlet temperature, 220°C; Outlet temperature, 140°C; Air flow, $62 m^2 hr^{-1}$; Pump flow, $175 mL hr^{-1}$; Compressor pressure, 0.4 – 0.7 bar. After drying, products were tested for the presence of astacene (as an indicator of oxidative damage to astaxanthin) but was not detected in any sample. The residual moisture content was less than 1% (w/w). The carotenoid content and composition of the two products was analysed before diet production (see below). The stability of carotenoids in the products was assessed over a period of four months using HPLC as described above.

Rainbow trout feed trial design

Four identical experimental diets were used in this study, differing only in the source of astaxanthin incorporated. All diets were typical of commercial feeds used for production size Rainbow trout: Diet 1 – Carophyll Pink; Diet 2 – NatuRose™; Diet 3 – Product One (spray-dried *H. pluvialis* produced by pressure fracture at 5,000psi), Diet 4– Product Two (spray-dried *H. pluvialis* produced by pressure fracture at 20,000psi). 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 (Skretting, ARC, Stavanger, Norway). Diet 1 was prepared by heating Carophyll Pink® (DSM Products) to 35°C in distilled water to melt the gelatin coating. The resulting mixture was added to pure cod liver oil (1.008g/12kg of basal diet; Seven Seas Ltd, Hull, UK). The mixture was then mixed vigorously to form an emulsion which was then added slowly (over a 10 min period) to the basal diet whilst being mixed using a commercial mixer (Minimx 150). The resulting diet was then mixed for a further 30 min to ensure homogenous distribution of the astaxanthin. Diets 2-4 were prepared by direct mixing of the algal supplements with the cod liver oil without the use of water. The target astaxanthin level for the feed trail was 50ppm. The actual astaxanthin inclusion levels of the four experimental diets are shown in Table 2. A total of 360 female rainbow trout (with a mean initial weight of 96g) were divided equally between 12 tanks (30 fish per tank) and fed the experimental diets (triplicate tanks per diet) for a period of 10 weeks. The trial was terminated at 10 weeks once the fish were deemed to be of marketable weight (i.e., at least 250g). The Rainbow trout in each tank were pooled into groups of five fish

(i.e., six pooled groups per tank). The fillets taken from each group were pooled, chopped and mixed. A larger sample of at least 5g was then taken from the pool for analysis. This ensured that white epaxial muscle from all experimental fish was homogeneously included in the analysis of carotenoid content and composition. Digestibility Coefficients were determined from faecal recovery of rainbow trout by a separate procedure. Rainbow trout were lightly anaesthetized by use of MS222 and faecal material expressed by gentle pressure applied to the hind abdominal area. Material obtained was pooled from each triplicate group of trout for each respective treatment and dried, grounds and stored for carotenoid analysis and the determination of yttrium oxide as an inert marker. Yttrium oxide was measured by ICP-MS following the protocol of White *et al.* (2003). The Apparent Digestibility Coefficient (ADC) for astaxanthin was calculated using equation 1 utilising yttrium as a non-absorbable marker in the feed:

$$ADC\ (\%) = 100 - [100 \times (d^{yt}/f^{yt}) \times (f^{ax}/d^{ax})] \quad \ldots(eq.\ 1)$$

Where:

d^{yt} = yttrium concentration in the diet (mg kg^{-1}); f^{yt} = yttrium concentration in the faeces (mg kg^{-1}); f^{ax} = astaxanthin concentration in the faeces (mg kg^{-1}); d^{ax} = astaxanthin concentration in the diet (mg kg^{-1}).

The Net apparent retention (NAR) of astaxanthin was determined from both initial and final flesh astaxanthin concentrations. A ratio of 0.61 was applied to the biomass to compensate for the muscle to live weight ratio (equation 2).

NAR (%) = $[(0.61 \times W^f \times F^{ax}) - (0.61 \times W^i \times I^{ax})] / \Sigma f \times d^{ax}$ …(eq. 2)

Where: W^f = final biomass; W^i = initial biomass; I^{ax} = mean initial flesh astaxanthin concentration (mg kg^{-1}); F^{ax} = mean final flesh astaxanthin concentration (mg kg^{-1}); Σf = total feed fed; d^{ax} = dietary astaxanthin concentration (mg kg^{-1})

The Specific Growth rate (SGR) was calculated as shown in equation 3 and expressed as the percentage increase in body weight per day.

SGR (%) = $((\ln W_2 - \ln W_1)/\text{days}) \times 100$ …(eq.3)

Where: W_1 = initial mean weight of fish, W_2 = final mean weight of fish, Days = number of days of the trial.

The Food Conversion ratio (FCR) was determined as FCR = (total feed intake/live weight gain).

Results

Cell disruption and carotenoid extraction

The two homogenisers tested in this study had very little effect (determined by light microscopy) on cell wall integrity of the encysted cells or subsequent potential bioavailabiity (see below), even at the highest speeds and the longest time period (data not shown). The homogenisers also resulted in a substantial increase in the temperature of the cell suspension. Similarly, the ultrasonic disruptor had little effect on the cell wall integrity and caused a substantial increase in temperature of the cell suspension. As carotenoids such as astaxanthin are heat-labile neither of these techniques were tested for larger scale processing or feed trials.

The pressure disruptor yielded two fractured cell products: (a) treatment at 5,000psi (i.e., one pass through the disruptor) resulted in rupture or cracking of the

cell wall; (b) pressures > 20,000psi resulted in the production of a product lacking any discernible cell structure (Fig. 1). Additional passes through the disruptor at pressures up to 5,000psi did not increase disruption to the cells. Some heat build-up was seen with the pressure disruptor so to minimise damage to the carotenoids both the undisrupted culture and the processed cells were kept at 0-4°C. Neither astacene (3,3'-dihydroxy-2,3,2',3'-tetradehydro-β,β-carotene-4,4'-dione e) nor semi-astacene (3,3'-dihydroxy-2,3-didehydro-β,β-carotene-4,4'-dione) could be detected in these disrupted products (both are indicators of oxidation) and astaxanthin yields were unaffected by the processing.

The potential bioavailabilty of carotenoids in intact and processed algal cells was assessed by measuring the release of pigments into acetone over 24 hours (Table 2). The leaching of carotenoids in all three algal products (NatuRose™, Products One and Two) was tri-phasic with an initial rapid release (over 30 min) of the majority of astaxanthin released. The levels of carotenoid released peaked at 16 hours, after which levels declined slightly (probably as a result of oxidative degradation). In all three products ca. 93% of carotenoid was released in 24 hours. The carotenoid composition of the leached pigment extract was identical to that of the starting material indicating that there was no preferential extraction of any individual carotenoids. By comparison the amount of pigment leached by intact (unprocessed) cells of *H. pluvialis* (taken from the same batch as Products One and Two) over 24 hours was ca. 10%. The leaching of pigment from older, more heavily encysted cells possessing a higher astaxanthin content (3% DW) was lower at ca. 0.4%.

Carotenoid Content and Composition

Although the products were seen to have slightly different total carotenoid contents (1.38 and 1.48% DW, respectively) the only difference between the carotenoid composition of the three algal products was the absence of adonirubin in Products One and Two (Fig. 2; Table 3). The chirality and geometric isomer composition of astaxanthin was determined for all four dietary products using semi-preparative TLC, pooled and hydrolysed using anaerobic saponification. The main chiral form of astaxanthin in all four products was the (3*S*,3′*S*) isomer. Cell disruption and subsequent spray drying of algal cells did not affect the chirality of astaxanthin. (Table 2). The geometric isomer composition of astaxanthin isolated from the three algal products was similar (Table 2) and was unaltered from that seen in the alga prior to processing (data not shown).

Product Stability

The stability of astaxanthin in Products One and Two (following spray drying) was determined over 16 weeks for a range of storage conditions: in the dark at +4°C and -20°C in an atmosphere of either air or oxygen-free nitrogen with or without the addition of 0.3% (w/w) ethoxyquin. The depletion of carotenoid levels in these two products during storage was bi-phasic (Fig. 3). During the first four weeks the rate of pigment loss was steep with both products loosing between 12-25% of the total carotenoid in that time (ca. 0.60 – 1.16% d^{-1} and ca. 0.02 – 0.05% d^{-1} for Products One and Two, respectively). Over the remaining 12 weeks, the rate of loss slowed to ca. 0.02 – 0.04% d^{-1} and ca. 0.75 – 1.00% d^{-1} for Products One and Two, respectively. In both products the pattern of pigment loss followed third order kinetics. The astaxanthin content was most stable when processed cells stored at -20°C under

nitrogen (ca. 20% loss of pigment over 16 weeks). Spray drying itself did not result in any mechanical damage to the algal cells and the carotenoid content and composition of algal materials was not adversely affected by the conditions used (data not shown). There was no significant difference ($p>0.05$) between the overall stability of the two products on storage.

Rainbow trout feed trials

All four experimental diets used in this study achieved a level of white epaxial muscle pigmentation in the range 3-5$\mu g g^{-1}$ after ten weeks feeding (Fig. 4). No significant difference ($p>0.05$) was observed between the total carotenoid content of the white epaxial muscle for the four diets tested. Rainbow trout fed Carophyll Pink® achieved the highest level of flesh pigmentation (3.91$\mu g\ g^{-1}$ total carotenoid, 3.24$\mu g\ g^{-1}$ astaxanthin), followed by NatuRose™ (3.83$\mu g\ g^{-1}$ total carotenoid, 3.24$\mu g\ g^{-1}$ astaxanthin), Product One (3.69$\mu g\ g^{-1}$ total carotenoid, 3.06$\mu g\ g^{-1}$ astaxanthin) and Product Two (3.46$\mu g\ g^{-1}$ total carotenoid, 2.87$\mu g\ g^{-1}$ astaxanthin). In all cases, following 10 weeks feeding, the main carotenoid isolated from white muscle was free, unesterified, astaxanthin (typically accounting for 83-87% of the total carotenoid content). The other carotenoids detected in the white epaxial muscle included lutein (β,ε-carotene-3,3'-diol), zeaxanthin (β,β-carotene-3,3'-diol), idoxanthin (3,3',4'-trihydroxy-β,β-caroten-4-one) and (in those fish fed an algal astaxanthin supplement in diets 2-4) canthaxanthin (Fig. 5). The final mean muscle astaxanthin concentrations in rainbow trout were significantly higher ($P<0.05$) in fish fed Carophyll Pink (2.8 ± 0.2$\mu g\ g^{-1}$) compared with those fed Product One (2.3 ± 0.1$\mu g\ g^{-1}$) and Product Two (2.3 ± 0.1$\mu g\ g^{-1}$), but not significantly higher than those

fish fed NatuRose™ (2.5 ± 0.2μg g^{-1}). In confirmation of this, the mean NAR values for Carophyll Pink (7.2 ± 0.4%) were also significantly higher than those noted in fish fed Product One (4.2 ± 0.1%) and Product Two (3.9 ± 0.1%), although were not significantly higher than the mean retention values recorded in fish fed NatuRose™ (5.0 ± 0.4% - see Table 5). When compared to Carophyll Pink® the pigmenting efficacy of the three algal supplements was 98% for the NatuRose™, 94% for Product One and 88% for Product Two. The net apparent retention (NAR) of both total dietary carotenoid and dietary astaxanthin was not significant different ($p>0.05$) for all four groups of fish at the end of the trial (Table 4). The NAR for Product Two was significantly lower ($p<0.05$) than those calculated for the other astaxanthin sources.

Discussion

In recent years there has been considerable interest in exploring the potential of natural sources of carotenoids, especially astaxanthin, for commercial pigmentation in animal feeds (see Ambati *et al.* 2014). *H. pluvialis* has been identified as one of the most promising natural sources but pigmentation of salmonid tissues does not occur when the astaxanthin-rich, haematocysts are intact (unpublished data; Sommer *et al.* 1991, Mendes-Pinto *et al.* 2001). Similarly, the leaching of astaxanthin into acetone from intact haematocysts (as a measure of potential carotenoid bioavailability) is negligible (0.41% of total astaxanthin released after 24 hours; data not shown) showing that processing of haematocysts is required to break or fracture the cell in order to aid the release of astaxanthin. We have evaluated several processing techniques that have a capability for scale-up to commercial operation. Of these, the pressure-fracture method has proven to be a successful approach to

produce a bioavailable natural astaxanthin product. This technique showed clear evidence of physical disruption to the sporopollenin cell wall of haematocysts without altering the astaxanthin content or composition.

Carotenoids such as astaxanthin are generally subject to degradation and artefact formation when exposed to oxygen, light, high temperatures and, in the case of astaxanthin, alkalinity. Astacene (an indicator of poor handling of astaxanthin; Schiedt & Liaaen-Jensen 1995) was not detected in any processed materials produced in this study. Similarly, pressure disruption had little or no effect on the geometric isomer composition of algal samples (e.g., when compared to NatuRose™; Table 2). The generation of *Z*-isomers is often an indicator of sample maltreatment (Schiedt and Liaaen-Jensen 1995) and their absence here indicates that both disruption and spray-drying had negligible effect on the algal carotenoids. In order to maximise the shelf life of a processed, disrupted, algal product rich in astaxanthin, storage in the dark at -20^{o}C with the exogenous antioxidant ethoxyquin (0.3% w/w) was shown to be effective. By contrast, astaxanthin is much more stable in dried, intact, haematocysts, presumably due to minimal oxidation of the carotenoid within the haematocyct (see also Mendes-Pinto *et al.* 2001). The disruption of the cells to pressure treatment was identical with both younger, smaller haematocysts (~1% DW carotenoid) and with older, larger and more heavily encysted cells possessing a higher total carotenoid content (~2.5% DW; data not shown). The chirality of astaxanthin in all algal products was unaffected by processing and was in agreement with other published studies with the main form in the alga being (3*S,*3′*S*). (Renstrøm *et al.* 1981; Grung *et al.* 1992).

A marketable level of white epaxial muscle pigmentation (i.e., 3-5$\mu g g^{-1}$; Meyers 1994) was achieved by all four experimental diets used in this study. We observed NAR values in the range 11-15% for astaxanthin (12-16% for total carotenoids; Table 4). The NAR of astaxanthin in salmonids is most commonly reported as 3-20% (Choubert & Storebakken 1989; Smith *et al.* 1992) but has been reported to reach 24-32% in some instances (Bjerkeng *et al.* 1997). The ADCs for astaxanthin in this study (Table 4) were in keeping with previously reported values of between 60-80% (Choubert & Storebakken 1989; Bjerkeng *et al.* 1997). The lack of significant difference in ADC values between dietary treatments suggests that if rupturing of the cell wall of *H. pluvialis* is adequate then the intestinal absorption of astaxanthin is not limited. There is no additional benefit in terms of pigmentation from further disruption of the algal cell. In Atlantic salmon the average ADC for unesterified astaxanthin (as in Carophyll Pink) was higher than that of astaxanthin dipalmitate (64% compared to 47%) although this difference was not significant (Storebakken *et al.* 1987). Furthermore, Foss *et al.* (1987) reported higher digestibility values for unesterified astaxanthin fed to rainbow trout (91-97%) compared to astaxanthin dipalmitate (42-67%). We have previously shown that extracts of astaxanthin *mono*- and *bis*-esters isolated from *H. pluvialis* are effective in pigmenting the white muscle of *O. mykiss* (Bowen *et al.* 2002). The cell preparations used in the present study (i.e., in diets 2, 3 and 4) all contain a complex mixture of astaxanthin bis-esters as the main form of the carotenoid (Fig. 2). We have previously shown that purified free-astaxanthin, mono- and bis-esters of astaxanthin make a similar contribution to pigmentation to *O. mykiss* (Bowen *et al.* 2002). Similarly, use of a cell-free solvent

extract of astaxanthin (prepared from the same batch of NatuRose™used here) showed that complete removal of the cell wall does not improve the NAR for algal astaxanthin (14.12% and 14.47%, for cell-free and NatuRose™, respectively). Cells subject to higher pressure treatment (Product Two) yielded a pigmentation product with a much lower NAR than the other supplements (Table 4). As the carotenoid composition was similar to the other algal products tested It is not known caused this, but it suggests that the processing itself had reduced the availability of the astaxanthin, probably by altering its local environment.

Muscle astaxanthin levels achieved after 10 weeks feeding (Fig. 4) are in keeping with values previously reported in other feed trials of similar duration and feed carotenoid inclusion level (Sommer *et al.* 1991, 1992). Using nearly double the feed inclusion levels (100mg kg^{-1}) used in the current study, Choubert & Heinrich (1993) achieved levels of flesh pigmentation of 6.2mg carotenoid kg^{-1} in rainbow trout fed diets supplemented with *H. pluvialis*. In contrast to previous studies (Sommer *et al.* 1991, 1992; Choubert & Heinrich 1993), no significant difference in flesh pigmentation were recorded between fish fed the commercial synthetic and algal sources of astaxanthin and flesh retention values for astaxanthin were similar. However, pigmentation efficiency for algal Products One and Two was significantly lower than the synthetic source. This is not unexpected as a reduced efficiency for the uptake and deposition of esterified sources of astaxanthin (as in Products One and Two) compared to 'free' astaxanthin (the synthetic product) has been shown before (e.g., White *et al*. 2003).

The chirality of astaxanthin isolated from the white muscle almost mirrors that

seen in the original supplements (cf. Tables 2 and 5), with little or no epimerisation evident. No epimerization of astaxanthin was seen in the skin of fish fed with Carophyll Pink® but in fish fed three algal products significant changes were observed in the chirality of the astaxanthin deposited in the skin in comparison to that isolated from both the white muscle and the products (in line with observations from Bowen *et al.* 2002). Astaxanthin isolated from the skin of *O. mykiss* fed algal astaxanthin supplements display an increase in the relative amounts of (3*R*,3′*S*)-astaxanthin and (3*R*,3′*R*)-astaxanthin present. Little difference was observed between the geometric isomer composition of the astaxanthin isolated from any of the supplements or from the white muscle and skin of pigmented fish; the main *cis* isomers isolated were the 9-*cis* and 13-*cis* forms (Table 5).

Overall, the results demonstrate that it is not necessary to either fully disrupt, or, extract the carotenoids from haeamatocycts, to achieve an effective level of pigmentation in salmonids. For example, the higher pressures applied during processing of haeamatocysts (i.e., Product Two at 20,000psi compared to 5000psi used to produce Product One) had a noticeable effect on cell integrity but the resulting enhanced disruption did not affect the 'potential bioavailability' of the product in terms of the release of carotenoids (Table 3) or subsequent deposition in the white muscle of *O. mykiss* in the feed trials. (Fig. 4). Although higher pressures cause a higher degree of cell disruption (as illustrated in Fig. 1) they do not necessarily improve the potential bioavailability or the utilisation of algal astaxanthin by *O. mykiss* (Tables 3- 4). Whilst there was no significant difference in the white muscle pigmentation levels achieved by either pressure-disrupted product (Fig. 4)

the NAR for Product Two (produced at higher pressure) was significantly lower than that for Product One (Table 4). For haematocysts of *H. pluvialis* possessing 1.5% DW astaxanthin a pressure of 5,000psi resulted in sufficient levels of cellular disruption to achieve target levels of white muscle pigmentation in *O. mykiss* (Fig. 4). Astaxanthin levels, cell size and the thickness of the sporopollenin cell wall increase with the age of a culture of *H. pluvialis* (Elliot 1934; Burcyzk 1987) so further optimisation of the pressure settings may be required for older cultures. The Stansted Cell Disruptor used in this study operates at pressures up to 40,000psi and is also available at much larger scale to process commercially-viable volumes of algal culture.

In conclusion, pigmentation in *O. mykiss* by pressure-disrupted haematocysts of *H. pluvialis* was achieved at a commercially acceptable dietary inclusion level and was equal to the pigmenting efficiency achieved by existing commercial products (namely Carophyll Pink and and NatuRose™). The technique used to disrupt cells is suitable for commercial-scale algal production. The cost benefit analysis of prior processing of algal and yeast derived natural single cell type sources of astaxanthin will have important implications for sustainable aquaculture development and efficacy of these products for the aquafeed industry, retailer and ultimately the consumer.

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Figure legends

Figure 1 Light microscope image of haematocysts of *H. pluvialis* (A) and subjected to a pressure treatment of (B) 5000psi (Product One), (C) 20,000psi (Product Two). AND/OR include Electron micrograph of (C) Product One (5000psi), (D) Product Two (20,000psi).

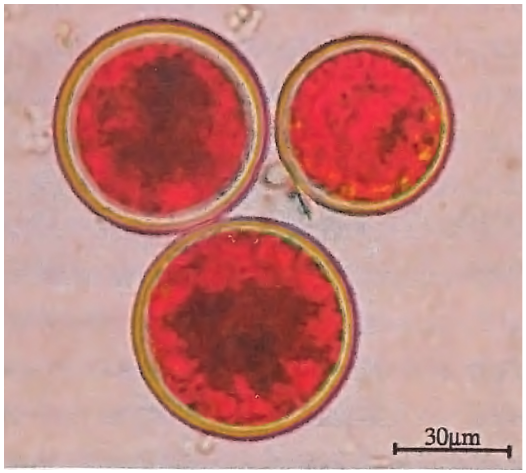
Figure 2 Carotenoid content of the four astaxanthin diets used during trial five (mean ±SE, *n* = 3). Diet: 1 Carophyll Pink®; 2 NatuRose™; 3 Product One (5,000psi); 4 Product Two (20,000psi). Carotenoids:  free astaxanthin;  astaxanthin monoesters;  astaxanthin bisesters;  canthaxanthin;  lutein;  β-carotene;  adonirubin.

Figure 3 Mean retention of carotenoids in (A) Product One and (B) Product Two (±SE, *n* = 3) . Storage conditions: ———— = -20°C +N_2; ..... = -20°C -N_2; - - - - - - - - - - - - = +4°C +N_2; ..... +4°C -N_2.

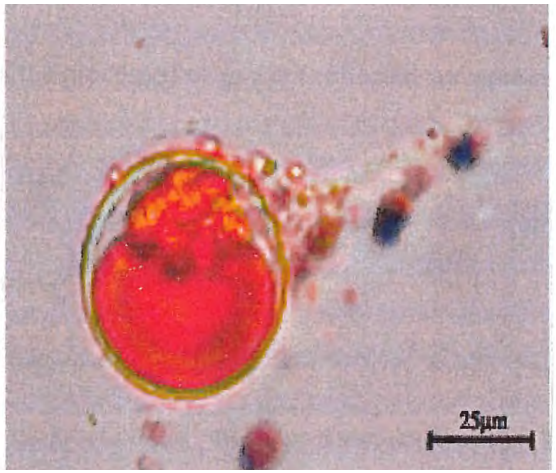
Figure 4 Total carotenoid  and astaxanthin  contents of white muscle of *O. mykiss* after 10 weeks feeding of the four experimental diets (mean ±SE, *n* = 3). Diet:

1 Carophyll Pink®; 2 NatuRose™; 3 Product One (5,000psi); 4 Product Two (20,000psi).

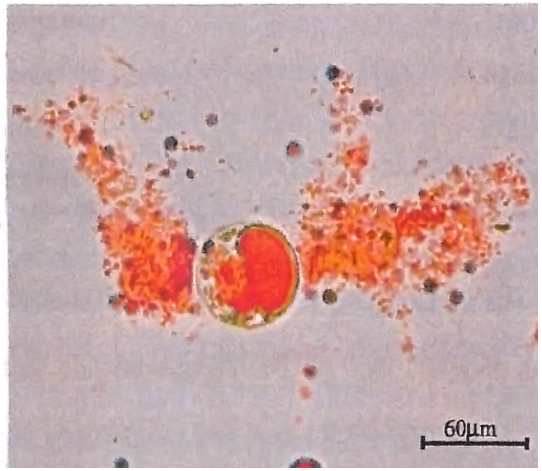
Figure 5 Carotenoid composition of the white muscle of *O. mykiss* after 10 weeks feeding of the four experimental diets (mean ±SE, *n* = 3). Diet: 1 Carophyll Pink®; 2 NatuRose™; 3 Product One (5,000psi); 4 Product Two (20,000psi). Carotenoids:  free astaxanthin;  lutein;  zeaxanthin;  idoxanthin;  canthaxanthin;  others.



A



B



C

Fig. 1 A-C

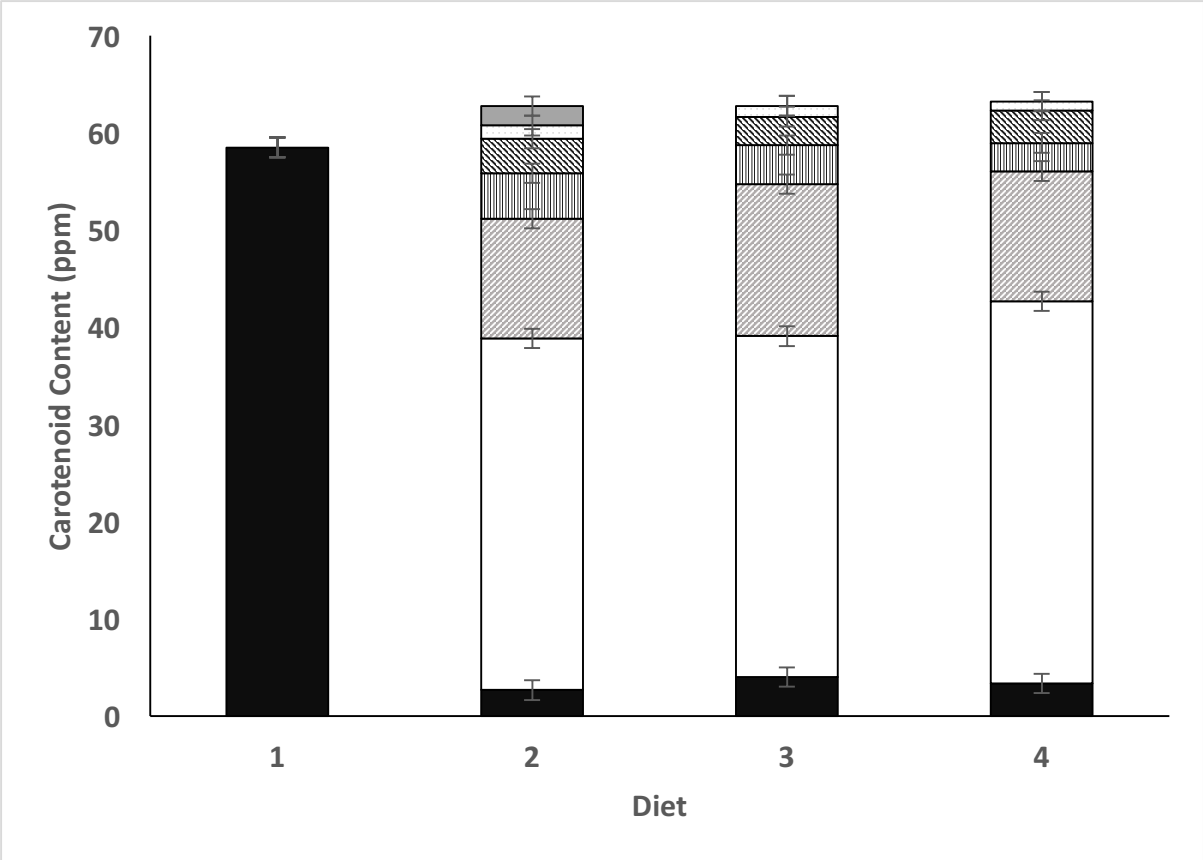
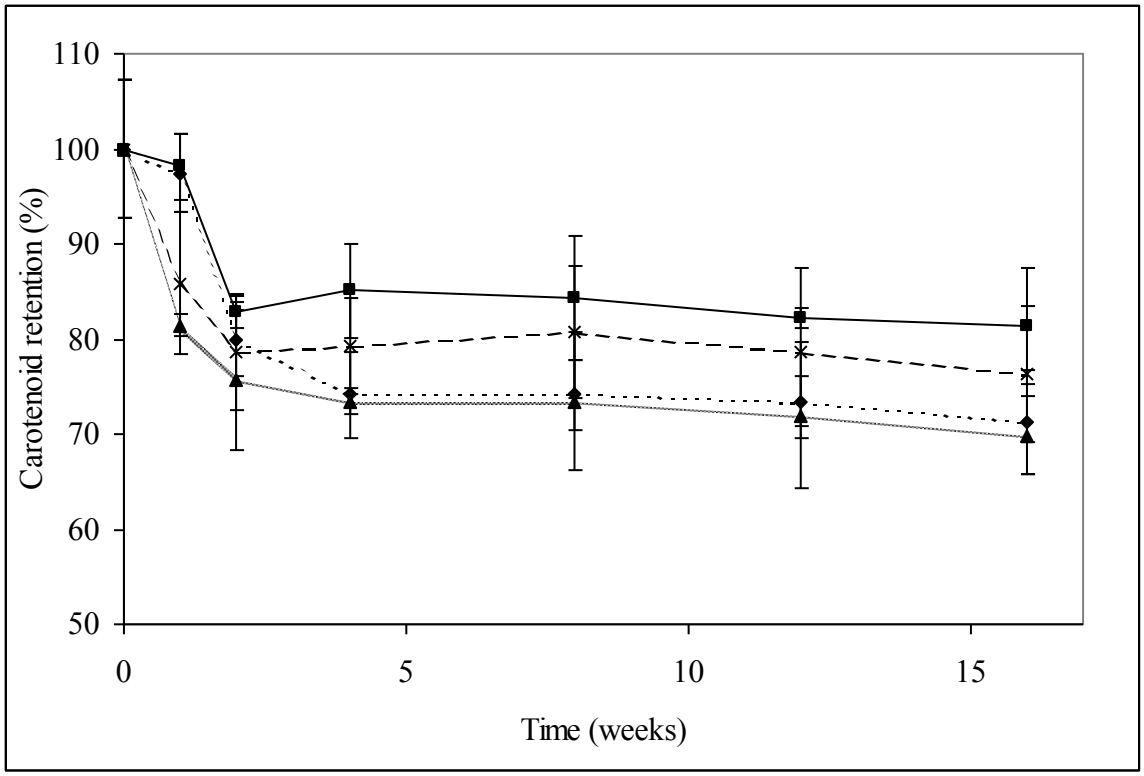
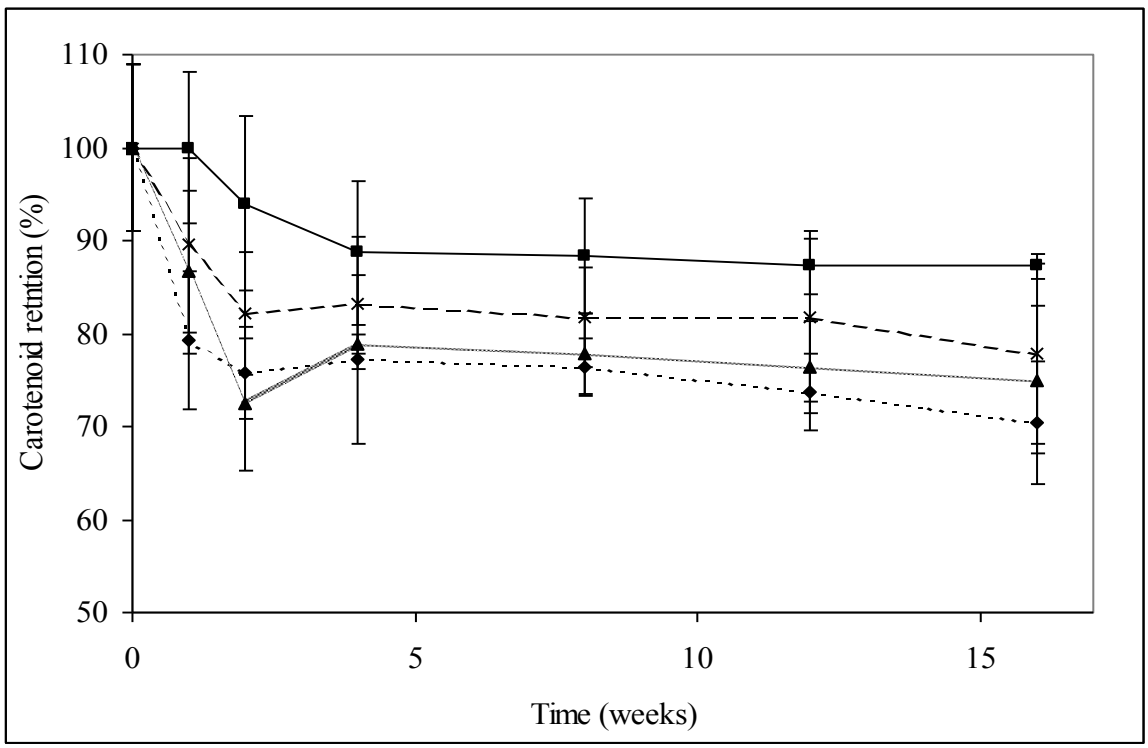


Figure 2


110
100
90
80
70
60
50
Carotenoid retention (%)
0
5
10
15
Time (weeks)

A.


110
100
90
80
70
60
50
Carotenoid retntion (%)
0
5
10
15
Time (weeks)

B.

Fig. 3 A-B:

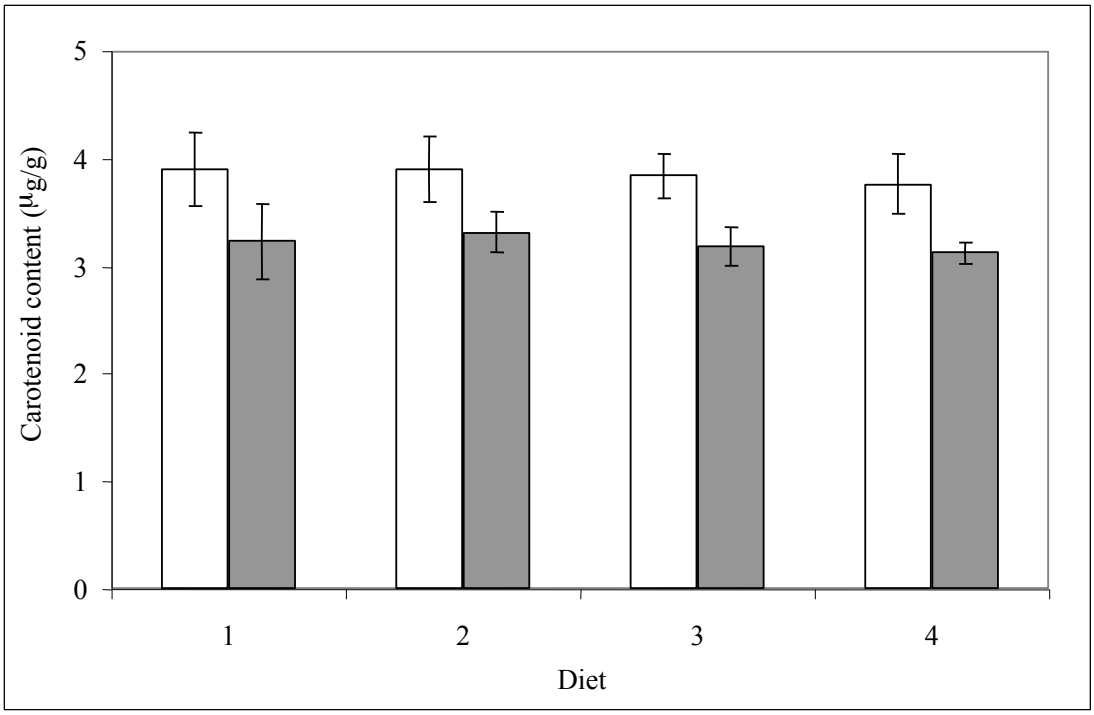


Fig. 4:

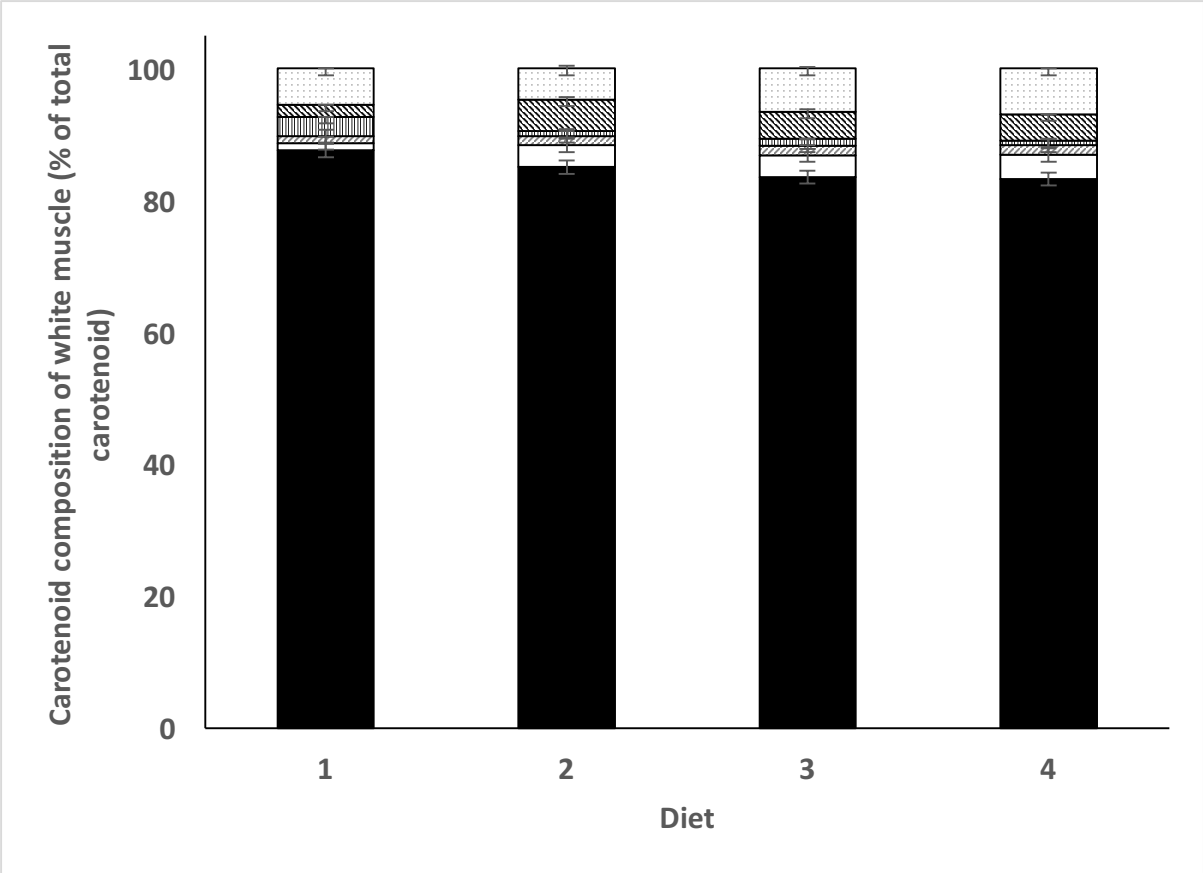

Carotenoid composition of white muscle (% of total carotenoid)
100
80
60
40
20
0
1
2
3
4
Diet

Figure 5