Evaluation of *Haematococcus pluvialis* as a natural dietary source of the carotenoid astaxanthin for rainbow trout flesh pigmentation

By

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Abstract
Stephen Lagocki “Evaluation of Haematococcus pluvialis as a natural dietary source of astaxanthin carotenoid for rainbow trout flesh pigmentation”

The efficacy of the lipid soluble carotenoid astaxanthin (biosynthesised as a secondary metabolite by the microalga Haematococcus pluvialis) as a source of pigment to enhance the aesthetic appeal of the flesh of farmed salmonid species has been investigated, using rainbow trout (Oncorhynchus mykiss) as a model. Accretion of astaxanthin within rainbow trout epaxial white muscle was primarily shown to be dependent on dietary inclusion and the type of diet used as a pigment carrier. Different dietary inclusion rates demonstrated a dose response. Cold pressed low oil and commercially extruded high oil diets containing similar astaxanthin concentration were tested, fish receiving the latter obtained higher growth rates and retained higher concentrations of astaxanthin within their flesh as compared to those receiving the former. Assessment of intact encysted H. pluvialis cells and processed H. pluvialis cells which had been mechanically treated to ruptured their cell wall revealed that the astaxanthin retained within the cells is only bioavailable to rainbow trout when the cell wall has been breached. The carotenoid profile of H. pluvialis confirmed that its astaxanthin was in three forms, i.e. free (5.9%), mono-esterified (82.3%) and di-esterified (11.8 %) to fatty acid moieties. Assessment of H. pluvialis purified mono and di-esterified astaxanthin extracts fed to rainbow trout demonstrated that astaxanthin mono-esters were more bioavailable than di-esters. Image analysis confirmed the major colour component of pigmented rainbow trout fillets is red and the distribution of astaxanthin within fillets varies greatly, with the head region having the lightest hue, which gradually increases towards the tail region. The addition of 2-palmitoylaminoproprionic acid (Corbinol) as a feed additive to diets containing synthetic astaxanthin had no effect on increasing the deposition of astaxanthin in the epaxial white muscle. Nevertheless, none of the in vivo trials incorporating H. pluvialis cells or H. pluvialis derived astaxanthin extracts demonstrated any detrimental effects on rainbow trout, in terms of feed utilisation, growth performance or health. Also, the protection afforded to astaxanthin by ruptured H. pluvialis cysts was demonstrated (during in vitro experiments) to provide increased stability as compared to synthetic astaxanthin, with respect to diet storage, exposure to emulated sunlight and loss in a turbulent water column. The present studies contribute to the awareness of Haematococcus pluvialis as a source of natural astaxanthin for salmonid species, by highlighting both negative and positive aspects with respect to its use in the aquaculture industry.
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Finally and most importantly my greatest thanks is saved for my mother and father, and for Melanie. To my parents for their unconditional support (financial and moral), their excellent and constructive advice, for making those 600 mile round trips when I needed them most and for always believing in my ability in everything I want to achieve. To Melanie who without complaint has endured all those fishy smells, the endless discussions on astaxanthin and proof reading, also her boundless encouragement and for supporting me through the worst and best of times.
Author’s Declaration

At no time during the registration for the degree of doctor of philosophy has the author been registered at any other university award.

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All laboratory and experimental procedures incorporating the use of rainbow trout (*Oncorhynchus mykiss*) were undertaken in accordance with the Animals (Scientific Procedures) Act 1986 under Home Office license (Project License PPL 30/00290)

Relevant scientific conference/meetings were attended at which work was always presented and an external institution (Liverpool John Moores University, Liverpool, Lancashire, UK) visited for consultation purposes.

Several reports were prepared for the industrial sponsors and several papers prepared for (peer reviewed) publication.

Conference/Meetings


1st EU Meeting (1997) Liverpool John Moores University, Lancashire, UK.


Industrial Reports


Academic Reports


Publications


xxiv
Manuscripts in preparation

Lagocki, S., Davies, S.J. and Brown, M.T. (2001). Pigmentation efficacy of *Haematococcus pluvialis* derived astaxanthin compared to a synthetic source in test diets for rainbow trout (*Oncorhynchus mykiss*). Completed

Lagocki, S., Jones, E. and Serwata, R. (2001). Rheology of synthetic and natural sources of astaxanthin mixed with cod liver oil, for application of carotenoids to experimental expanded salmonid diets. Completed

Lagocki, S., Russell P. and Davies, S.J. (2001). Application of image analysis to observe the distribution of astaxanthin in the fillets of rainbow trout (*Oncorhynchus mykiss*) fed synthetic astaxanthin. Completed

Russell P. and Lagocki, S. (2001). The use of image analysis to compare the colour of rainbow trout (*Oncorhynchus mykiss*) fillets fed pigmented (with synthetic astaxanthin) and non-pigmented experimental diets.

Author Signed ..................................................

Date ..........................................................
CHAPTER 1

General introduction
1.1 Colour and its perception

Man has been enchanted by the concept of colour since the dawn of civilisation, some 180,000 years before the birth of Christ (McLaren, 1986). Archaeological evidence dates the first use of colour additives to enhance the aesthetic appeal of food and beverages to 1500 BC, as documented by the ancient Egyptians (Borzelleca and Hallagan, 1992). Man’s fascination with colour has led scientists over the last over 150 years to synthesise over 4 million novel dyes, of which some 10,000 are commercially manufactured (McLaren, 1986).

Colour is the sensation produced by the human eye when light of different wavelengths falls on retinal cells that are sensitive to various frequencies (wavelengths). The frequencies of the electromagnetic waves that induce the sensation of colour are collectively termed the visible spectrum, and range from $400 \times 10^{12}$ Hz (producing red light with a wavelength 700 nm) to $750 \times 10^{12}$ Hz (producing violet light with a wavelength 400 nm) (Goodman and Denny, 1985). Although the visible spectrum is continuous, by convention it is divided into seven major colours (red, orange, yellow, green, blue, indigo and violet) being listed in order of increasing wavelength (Table 1.1).

<table>
<thead>
<tr>
<th>Colour</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Violet</td>
<td>400 - 440</td>
</tr>
<tr>
<td>Indigo</td>
<td>440 - 450</td>
</tr>
<tr>
<td>Blue</td>
<td>450 - 500</td>
</tr>
<tr>
<td>Green</td>
<td>500 - 553</td>
</tr>
<tr>
<td>Yellow</td>
<td>555 - 605</td>
</tr>
<tr>
<td>Orange</td>
<td>605 - 675</td>
</tr>
<tr>
<td>Red</td>
<td>675 - 700</td>
</tr>
</tbody>
</table>

In terms of light, an equal mixture of all of the seven major colours produces white light, whereas an equal mixture of these colours in the form of pigments, dyes or filters results in the colour being black.
The perceived colour of dyes, pigments and filters is due to them selectively absorbing certain wavelengths and reflecting or transmitting the rest (McLaren, 1986). The true colour of an object is only perceived by the human eye when illuminated by white light, for example trout fillets only appear to have a reddish hue when illuminated correctly (with white light). In comparison, if the same trout fillets were illuminated by green light the fillets are perceived as having a green hue (Storebakken, unpublished data). This is because the pigment (astaxanthin) present in the flesh absorbs all of the white light except red which is reflected. Hence, colour perception is a subtractive process, since the final colour is that remaining after the absorption of all the others. So when all the light is absorbed the object appears black, whereas, conjoining light of different colours is an additive process. Combining the various coloured lights from the whole spectrum results in the production of white light. Thus, different colours are produced by either mixing the major colour components in different ratios or by omitting one or more of the components from the mixture.

1.1.1 Colour differences

The perceived colour of an object may be affected by a variety of factors, including: the observer, the size of the object, its background, the ambient temperature, the angle at which its viewed, and its aforementioned illumination (section 1.1). Colour vision in humans arises from the eye possessing three types of photosensitive cone cells which are sensitive to light of different wavelengths either, red, blue or green (Green, et al., 1990a). The sensitivity of the human eye in detecting colour can vary greatly, at worst an individual can be colour blind due to the absence of, or shortage of one type of cone cell. Even in the best case scenario where an individual is deemed to have 'normal' colour vision, the sensitivity of their eyes will be slightly different to others in this group as there is usually some bias towards red or blue (Minolta, 1998). This problem is exacerbated by age, where an individual's eyesight as a whole tends to deteriorate, in the context of colour vision, resulting in decreased sensitivity which manifests itself in a reduced ability to
distinguish between different shades. Differences in the size of an object can induce the ‘area effect’, which has the effect of a colour, covering a large area, to appear brighter and more vivid than the same colour covering a smaller area (Minolta, 1998). When judging the colour of an object, background colour is equally important as it may induce the ‘contrast effect’. This is where an object placed in front of a bright background will appear more dull than if it were placed in front of dark background (Minolta, 1998). The ambient temperature may affect the colour of an object; as the ambient temperature increases so does the temperature of the colour compound. The temperature change may directly affect the chromophore causing the object to either alter by one or more shades, or completely change colour; this phenomenon is referred to as thermochromism (Minolta, 1998). The angle at which an object is viewed is also important because particular colouring materials, for example metallic paints, have directional characteristics such that if the viewing angle is altered they can appear brighter or darker; this is also applicable to illumination (Minolta, 1998). Hence, when determining the colour of objects such as rainbow trout fillets, the conditions of illumination, temperature, background colour, viewing angle and those performing the assessment must remain constant. Whilst colour is a matter of perception and subjective interpretation differences can easily be identified, but expressing them in an accurate communicable manner is difficult. This is because colour does not have an independent existence in spatial terms (it being dependent upon light for its perception) and it can be likened to other sensations, for instance sound (Wotley, pers comm.). Sound is measured numerically in terms of decibels and similarly colour is measurable in terms of its hue, lightness and chroma. Hue refers to the seven colours of the visible spectrum (section 1.1) which are in fact a continuum, where red merges into orange which merges into yellow, merging into green then into blue on to indigo, violet and finally back to red, forming the colour wheel. Lightness describes the fraction of light either reflected or transmitted by an object and has two extremes, the first where an object appears white if all the light is reflected or transmitted, the second where no light is
reflected or transmitted and the object appears black (McLaren, 1986). In this context, colours can be separated into bright or dark, which allows the introduction of achromatic sensations (colours not found on the colour wheel) including white, grey and black and is perpendicular to hue. Chroma is often called saturation, which describes the dullness or vividness of a colour (McLaren, 1986). As hue and lightness are perpendicular to each other and the colour wheel lies at the mid-point of the two extremes of lightness, the centre of the colour wheel would be grey. Moving out in any direction from the grey centre, the chromicity of the colours is said to increase as they become more vivid (Minolta, 1998). These three functions of colour can be used as co-ordinates, where hue, lightness and chroma are the x, y and z co-ordinates, respectively. So, theoretically having three co-ordinates implies that colour can be depicted as a three dimensional object. The Commission Internationale de l'Eclairage (Paris, France) merged the colour wheel with the concept that colour can have three co-ordinates (Cartesian) and in 1976 formed the L*a*b* colour space, represented as a spherical colour solid or 'globe' (plate 1.1) (McLaren, 1986).

1.1.2 The structure of coloured compounds

Not all chemical compounds are coloured, and colouration is not dependent on the state of matter, as a manifold of coloured compounds have been documented as solids, liquids and gases. The first hypothesis correlating the colour of a compound to its chemical structure was proffered in 1868 by Graebe and Lieberman who stated that all dyes were decolourised on reduction, so were associated with unsaturation (McLaren, 1986). In 1876, Witt expanded this hypothesis by assuming all coloured organic compounds had to contain one or more active groups that provided the colour (these groups he termed chromophores), of which less than twenty five have been determined (McLaren, 1986). Thus, a chromophore is any chemical group within a compound that absorbs a specific wavelength, causing the molecule to reflect a characteristic colour.
Plate 1.1 A representation of colour as a solid "globe" (colour solid) for L* a* b* colour space where:

- **L*** = Lightness.
- **a*** = 1st Chromaticity co-ordinate (direction).
- **b*** = 2nd Chromaticity co-ordinate (direction).
- +**a*** = Red direction.
- −**a*** = Green direction.
- +**b*** = Yellow direction.
- −**b*** = Blue direction.
However, light can be described as having two forms. The first as photons, thought of as being uncharged particles of energy that have a constant velocity ($3 \times 10^8$ ms$^{-1}$) and zero mass (Goodman and Denny, 1985). The second, as waves that consist of magnetic and electric vectors (fields) mutually perpendicular to each other, both of which are perpendicular to the linear direction of propagation of the sinusoidally oscillating wave (Freifelder, 1982). As a chromophore absorbs electromagnetic waves in the ultra-violet and visible regions, the molecule becomes excited. This energy is normally converted to kinetic energy and released as heat radiation as the excited molecule collides with others (Freifelder, 1982). As the majority of an atom’s volume is occupied by negatively charged electrons that are spatially arranged in different orbits, the absorbed radiation has greatest effect on these atomic constituents. The electrons within a chromophoric molecule can absorb enough energy for an electron to move from one orbit (ground state) to another orbit (excited state) of higher energy (McLaren, 1986). The energy of the excited electron is expelled, as it can no longer be maintained in the higher energy orbital and so it returns to its ground state. As it returns to its ground state, the excited electron liberates all its excess energy as electromagnetic radiation in the infra red (heat) and visible range (the colour of the light being dependent on the wavelength emitted). All coloured organic compounds (including carotenoids) contain a conjugate carbon-carbon double bond system with at least six double bonds. Carbon-carbon double bonds are formed when two carbon atoms, each containing a sp$^2$ hybrid orbital (produced by the hybridisation of a 2p orbital and a 2s orbital) are specifically orientated, forming a strong $\sigma$ bond due to sp$^2$-sp$^2$ overlap (McMurry, 1988). As the two sp$^2$ hybrids approach each other, so do the unhybridised 2p orbitals on each carbon which also overlap creating a $\pi$ bond, resulting in four electrons being shared, constituting a carbon-carbon double bond. In a conjugated system containing alternate carbon-carbon double bonds and carbon-carbon single bonds, the $\pi$ orbitals overlap the single bonded carbons reducing the length of the $\sigma$ bond and reducing the level of excitation energy required. When the conjugated system reaches six double bonded
carbons, a bathochromic shift occurs with the absorption peak extending into the visible region, and the compound appears coloured (McMurry, 1988).

1.2 Carotenoid pigments

The term carotenoid was derived from carotene, the name given by Wackenroder in 1831, to the yellow-orange crystalline pigment he isolated from carrots (*Daucus carota*) (Christiansen, 1996). Carotenoids are one of the most prevalent and structurally diverse groups of naturally occurring pigments, their colours essentially range from deep red to light yellow, yet have been associated with a variety of greens, blues, purples and browns (Britton *et al.*, 1995). To date over 600 structurally unique carotenoids have been elucidated, several of which (namely β-carotene, canthaxanthin and astaxanthin) have gained immense commercial interest (Armstrong, 1999; Springate and Nickell, 2000).

They have been detected in the majority of taxonomical kingdoms, including Prokaryotae, Fungi, Protista, Plantae and Animalia, in which they perform many vital functions, primarily photo-protection, photosynthesis and nutrition (Armstrong, 1999). Due to their prevalence, the natural global production of carotenoids is estimated to be in excess of 100 million tonnes per annum, representing an output of more than 3 tonnes every second, of which two pigments, fucoxanthin (found in brown seaweed and microalgae) and peridinin (found in dinoflagellates) constitute over 20 % of total production (Britton, 1995). Even though vast quantities of carotenoids are continually produced by innumerable species of plants, algae, fungi and micro-organisms, all animals species such as the salmonids rely on a dietary intake of carotenoids for a variety of functions (Latscha, 1990). This is because they are unable to synthesise carotenoids de novo (Shiedt, 1990).
1.2.1 Structure

1.2.2.1 Terpenes and terpenoids

Many natural products belong to a large class of compounds called the terpenes (Wade, 1999). Terpenes are simple lipids that are immensely diverse and account for thousands of organic compounds, all of which, regardless of their structural differences, follow the isoprene rule (McMurry, 1988). The isoprene rule was formulated by Otto Wallach in 1887 who discovered terpenes to have a common structural feature, ‘they all emanated from the head to tail conjoining of simple five carbon isoprene (2-methyl-1,3-butadiene) units’, (Fig. 1.1a) the head being the branched end and the tail the unbranched ethyl group (Fig. 1.1b), making it an asymmetrical molecule (Wade, 1999; Wise and Croteau, 1999). Chemically the isoprene unit is described as being allylic as its structural system contains a saturated carbon atom (α-carbon) adjacent to a carbon-carbon double bond (Fig. 1.1c), which provides structural stability due to an electron shift that causes the π-electrons to delocalize (Goodman and Denny, 1985). The classification of a terpene is based upon the number of carbons it contains in units of ten, as all terpenes consist of two or more isoprene units covalently bonded together, with the simplest compounds being classified as the monoterpenes (Table 1.2) (Wade, 1999).

Table 1.2 The Classification of terpene compounds by the number of constituent isoprene units (McMurry, 1988).

<table>
<thead>
<tr>
<th>Carbon atoms</th>
<th>Isoprene units</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2</td>
<td>Monoterpenes</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>Sesquiterpenes</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>Diterpenes</td>
</tr>
<tr>
<td>25</td>
<td>5</td>
<td>Sesterterpenes</td>
</tr>
<tr>
<td>30</td>
<td>6</td>
<td>Triterpenes</td>
</tr>
<tr>
<td>40</td>
<td>8</td>
<td>Tetraterpenes</td>
</tr>
</tbody>
</table>

Terpenoids are a family of compounds whose carbon skeletons are not exclusively composed of C₅ isoprene units.
Fig. 1.1 a, the chemical structure of an isoprene unit: b, the asymmetric molecular structure of isoprene showing the head and tail regions: c, the IUPAC numbering of the carbons within the isoprene unit: d, the regular (tail – head) conjoining of two isoprene units: e, the irregular (tail – tail) conjoining of two isoprene units: f, the regular (head – head) conjoining of two isoprene units: g, the asymmetric molecular structure of isopentyl-diphosphate (IPP) (Latscha, 1990).
The structure of these compounds during biosynthesis have been rearranged and the addition or loss of one or more carbon atoms caused further alteration to the carbon skeleton (Wade, 1999). The terpenoids embody a large group of a functionally divergent group of compounds that are known to act as pheromones, steroidal hormones, pollinator attractants, phytotoxins and pigments (Hohn, 1999).

The largest group of terpenoid pigments are the carotenoids. Fundamentally, most naturally arising carotenoids are hydrophobic tetraterpenoids comprising of a C_{40} methyl-branched hydrocarbon spine, educed from the successive 1'-4 homoallylic-allylic condensations of eight isoprene units (Armstrong, 1999). Yet, chemically the head to tail (regular) bonding of isoprene (Fig. 1.1d) units is not discrete, as irregular adducts may be formed due to either tail to tail bonding (Fig.1.1e) or head to head bonding (Fig. 1.1f) (Latscha, 1990). Not all the carotenoids are classed as terpenoids; lycopene and β-carotene are exceptions as they are true terpenes containing eight isoprene units (C_{40}) and are classified as a tetraterpene. Thermodynamically, the formation of terpenoids from the bonding of two or more isoprene units is favourable. However, in terms of biosynthesis, isoprene is not the direct precursor of natural terpenoids, this role is taken by its biologically active phosphorylated derivative, isopentyl-diphosphate (IPP) (Latscha, 1990). As with isoprene, IPP also has an asymmetric molecular structure IPP (Fig. 1.1g).

1.2.2.2 Carotenoid chemistry

Structurally, acyclic lycopene (C_{40} H_{56}) may be regarded as the prototype for all carotenoids, because from its basic structure, lycopene potentially (Fig. 1.2) may undergo one or more chemical reactions involving hydrogenation, dehydrogenation, cyclisation, insertion of oxygen in various forms (Table 1.3), double bond migration, methyl migration, carbon chain elongation and shortening, to create all the known carotenoids (Goodwin, 1980; Latscha, 1990; Schiedt, 1990; Weedon and Moss, 1995). Depending on their degree of substitution, cyclisation, rearrangement, elongation and elimination, carotenoids are
either classed as carotenoids or xanthophylls, where the carotenoids are pure hydrocarbons and
the xanthophylls contain one or more oxygenated functional groups (Table 1.3) (Britton et al., 1995). These structural changes usually result in the newly formed carotenoids having
several different chemical characteristics to lycopene. An obvious example of this is
colour, which arises due to the presence of a conjugated double bond system within the
carbon back bone of all carotenoids, which acts as a chromophore.

Table 1.3 The most frequently observed functional groups containing oxygen found in
carotenoids (Latscha, 1990; Weedon and Moss, 1995).

<table>
<thead>
<tr>
<th>Group</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>—OH</td>
<td>Hydroxy</td>
</tr>
<tr>
<td>—CHO</td>
<td>Aldehyde</td>
</tr>
<tr>
<td>—OCH₃</td>
<td>Methoxy</td>
</tr>
<tr>
<td>—COOH</td>
<td>Carboxy</td>
</tr>
<tr>
<td>—COOCH₃</td>
<td>Carbomethoxy</td>
</tr>
<tr>
<td>—COOH₃</td>
<td>Acetate</td>
</tr>
<tr>
<td>—OC₆H₁₁O₄</td>
<td>Glycosyloxy</td>
</tr>
<tr>
<td>—C=O</td>
<td>Carbonyl</td>
</tr>
<tr>
<td>—C₂O</td>
<td>Epoxy</td>
</tr>
</tbody>
</table>

Thus, modifying the number of conjugated double bonds within the chromophore may
induce a shift in absorption causing the carotenoid to appear a different colour (Table 1.4).
Fig. 1.2 The molecular structure of acyclic lycopene (Eugster, 1995).
Table 1.4 An extremely simplified guide to the correlation between the number of conjugated double bonds within a carotenoid, the range in which its maximum absorption ($\lambda_{\text{max}}$) should occur and its potential colour (Goodman and Denny, 1985; McLaren, 1986; Latscha, 1990).

<table>
<thead>
<tr>
<th>Conjugated double bonds</th>
<th>Potential $\lambda_{\text{max}}$ (nm)</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>400 - 425</td>
<td>Yellow</td>
</tr>
<tr>
<td>8</td>
<td>420 - 450</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>440 - 470</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>455 - 485</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>470 - 500</td>
<td>Orange</td>
</tr>
<tr>
<td>12</td>
<td>485 - 515</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>500 - 525</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>520 - 540</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>535 - 550</td>
<td>Red</td>
</tr>
</tbody>
</table>

The generality that carotenoids containing seven conjugated double bonds are yellow and those containing fifteen are red is quite simplistic even though the delocalised electron system created on formation of the conjugated double bond complex is responsible for colouration. Nevertheless, the delocalised electron system is readily influenced by various substituents including cyclisation, addition of functional groups and the insertion of double bonds into the carbon back bone, or cyclic end groups (Goodwin, 1980). The substituents probably cause steric hindrance by partially disrupting the delocalised electron system within the conjugated double bond complex, causing the absorption maxima ($\lambda_{\text{max}}$) to shift.

The change in absorption maxima ($\Delta \lambda_{\text{max}}$) is possibly a function of steric hindrance, where different substituents have varying effects on $\lambda_{\text{max}}$, some of which are listed in Table 1.5. Obviously, the number of substituents present has an additive effect, where the aggregate effect may result in the carotenoid having a different colour than expected (Latscha, 1990).

Hence, the characteristic absorption spectra and colour of any carotenoid is a consequence of its conjugated polyene structure (that constitutes its chromophore) and its substituents (Schiedt et al., 1989).
Table 1.5 The influence of several substituents on the principal absorption maxima ($\lambda_{\text{max}}$) of carotenoids (Goodwin, 1980; Latscha, 1990; Weedon and Moss, 1995).

<table>
<thead>
<tr>
<th>Substituent</th>
<th>Position</th>
<th>$\Delta \lambda$ nm $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1^{\text{st}}$ Carbonyl group</td>
<td>Backbone</td>
<td>+28</td>
</tr>
<tr>
<td>$2^{\text{nd}}$ Carbonyl group</td>
<td>Backbone</td>
<td>+1-7</td>
</tr>
<tr>
<td>$1^{\text{st}}$ Carbonyl group</td>
<td>Ring</td>
<td>+7</td>
</tr>
<tr>
<td>$2^{\text{nd}}$ Carbonyl group</td>
<td>Ring</td>
<td>+5-9</td>
</tr>
<tr>
<td>Double bond</td>
<td>Backbone</td>
<td>+7-35</td>
</tr>
<tr>
<td>Double bond</td>
<td>Ring</td>
<td>+5-9</td>
</tr>
<tr>
<td>Cyclisation</td>
<td>N/A $^a$</td>
<td>-24</td>
</tr>
<tr>
<td>5, 6 Epoxide</td>
<td>N/A $^ab$</td>
<td>-8</td>
</tr>
<tr>
<td>5, 8 Epoxide</td>
<td>N/A $^ab$</td>
<td>-20</td>
</tr>
<tr>
<td>$\text{Trans } \rightarrow \text{Cis}$</td>
<td>N/A $^ab$</td>
<td>-(2-5)</td>
</tr>
<tr>
<td>Normal $\rightarrow$ Retro</td>
<td>N/A $^ab$</td>
<td>-10</td>
</tr>
</tbody>
</table>

$^a$ Not applicable; $^b$ May occur in the backbone and a ring simultaneously; $^c$ Change in wavelength.

Carotenoids may contain cyclic regions within their structures; cyclisation of the carbon back bone into a ring structure only occurs at the end of the polyene chain, so carotenoids can only form up to two cyclic regions, one at either end. Those carotenoids such as astaxanthin that contain two carbon rings are described as bicyclic, whereas those that only contain one ring are described as monocyclic, for example $\gamma$-carotene. The majority of carotenoids contain cyclic regions that can be one of four types (cyclopentyl, cyclohexyl, methylene cyclohexyl and phenyl) where the cyclopentyl group is a five membered ring and the cyclohexyl group, methylene cyclohexyl group and phenyl group are all six membered rings (Table 1.6) (Weedon and Moss, 1995). Although structurally different, all four cyclic groups contain 9 carbon atoms (Table 1.6). Comparatively, carotenoids containing six membered rings are more abundant than those that are acyclic or those containing five membered rings. The abundance of cyclic carotenoids has led to the differences in their cyclic structures being designated with lower case Greek letters (Table 1.6) which are used in their IUPAC nomenclature (Weedon and Moss, 1995). IUPAC (International Union of Pure and Applied Chemistry) was founded in 1892 to develop a detailed nomenclature system for organic compounds that follow several rules (IUPAC rules), being accepted world wide as the standard nomenclature system (Wade, 1999).
Table 1.6. The IUPAC designations for carotenoid cyclised and non-cyclised end groups (Goodwin, 1980; Latscha, 1990; Weedon and Moss, 1995).

<table>
<thead>
<tr>
<th>Group</th>
<th>Prefix(^a)</th>
<th>Structure</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyclic</td>
<td>(\psi) (psi)</td>
<td><img src="image" alt="Structure" /></td>
<td>(C_5H_{17} \cdot R)</td>
</tr>
<tr>
<td>Cyclopentyl</td>
<td>(\kappa) (kappa)</td>
<td><img src="image" alt="Structure" /></td>
<td>(C_5H_{17} \cdot R)</td>
</tr>
<tr>
<td>Cyclohexyl</td>
<td>(\beta) (beta)</td>
<td><img src="image" alt="Structure" /></td>
<td>(C_5H_{15} \cdot R)</td>
</tr>
<tr>
<td>Cyclohexyl</td>
<td>(\epsilon) (epsilon)</td>
<td><img src="image" alt="Structure" /></td>
<td>(C_5H_{15} \cdot R)</td>
</tr>
<tr>
<td>Methylene cyclohexyl</td>
<td>(\gamma) (gamma)</td>
<td><img src="image" alt="Structure" /></td>
<td>(C_5H_{15} \cdot R)</td>
</tr>
<tr>
<td>Phenyl</td>
<td>(\chi) (chi)</td>
<td><img src="image" alt="Structure" /></td>
<td>(C_6H_{11} \cdot R)</td>
</tr>
<tr>
<td>Phenyl</td>
<td>(\phi) (phi)</td>
<td><img src="image" alt="Structure" /></td>
<td>(C_6H_{11} \cdot R)</td>
</tr>
</tbody>
</table>

* Used as part of the carotenoid's name, being based on the stem name 'carotene'.
1.3 Astaxanthin

1.3.1 Structure

Astaxanthin has long been associated with the colour change (from blue/black to red/orange) observed when lobsters are cooked, such that the name astaxanthin was derived from the Latin taxonomical classification for the edible lobster *Astacus gammarus* (Roche, 1999a). Astaxanthin is an oxygenated carotenoid classified as a xanthophyll, and like most other naturally occurring carotenoids it contains 40 carbons (C_{40}H_{52}O_{4}) and is cyclised at either end of the carbon back bone (Fig. 1.3). Both cyclic regions are composed of cyclohexyl six membered rings in the β configuration more commonly known as β-ionone rings. Astaxanthin is formed (like all carotenoids) from the irregular condensation (tail to tail) of two asymmetric C_{20} precursor grenarly-grenerly pyrophosphate (GGPP) molecules (Latscha, 1990). The carbon double bond between C5 and C6 within the β-ionone rings is formally conjugated with the polyene chain, which favours co-planarity between the two components, resulting in a planar molecule (Fig. 1.3) (Britton, 1995). Following the IUPAC rules of nomenclature, the carbon atoms within astaxanthin are numbered from within the cyclic region as they contain the closest substituent groups, which include a hydroxyl group (-OH) on their third carbons (C3 and C3’) and an carbonyl group (-C=O) on their fourth carbons (C4 and C4’) (Fig. 1.3) (Wade, 1999). However, astaxanthin has a symmetrical structure with the same groups on the same carbons in each β-ionone ring. To reflect its symmetrical structure, the carbons within the back bone are numbered in ascending order from left right upto the point of symmetry after which they are numbered in descending order with the addition of a prime symbol (Fig. 1.3). The addition of all these structural features determined the IUPAC name for astaxanthin to be 3,3’-dihydroxy-β,β-carotene-4,4’-dione. The hydroxyl groups at C3 and C3’ in the β-ionone rings induce chirality into the astaxanthin molecule, as the hydroxyl group may be above or below the plane of the molecule (Bjerkeng, 1992; Jansen *et al*., 1994, Turujman *et al*., 1997).
Fig. 1.3 The symmetrical molecular structure of astaxanthin (C₄₀H₅₂O₄), showing the IUPAC numbering of the carbon atoms (Weedon and Moss, 1995).
This results in astaxanthin having three configurational isomers, consisting of two enantiomers (3R, 3’R and 3S, 3S’) and a mesoform (3R, 3’S or 3S, 3’R) (Bjerke, 1997). If both hydroxyl groups are above the plane of the molecule it is denoted as 3S, 3’S (Fig. 1.4a), but if both are below the plane of the molecule it is denoted 3R, 3’R (Fig. 1.4b) (Foss et al., 1984). If one hydroxyl group is below and the other is above the plane of the molecule or vice versa they are denoted 3R, 3’S (Fig. 1.4c) or 3S, 3’R (Fig. 1.4d) (Foss et al., 1984). The stem name carotene implies that all the double bonds within a molecule are in the *trans* configuration, but the double bonds at C9, C11, C13 and C15 may be in the *cis* configuration (Fig. 1.5). Due to the double bonds at positions 9 and 13 in the polyene chain being tri-substituted *i.e.* bonded to three other atoms or groups other than hydrogen, it is convention to use the E, Z system to designate *cis* and *trans* double bonds (Wade, 1999). The E, Z system is based on a series of sequence rules that assign priority to the substituent groups, where the higher the atomic number of the group the higher its priority (McMurry, 1988). If the two highest priority group are on the same side of the carbon double bond (*cis* configuration) it is designated as a Z isomer, from the German ‘zusammen’ meaning together (Fig. 1.6a) (Wade, 1999). Whereas if the two highest priority groups are on opposite sides of the carbon double bond (*trans* configuration) then it is designated as an E isomer, again from the German ‘entgegen’ meaning opposite (Fig. 1.6b) (Wade, 1999). In terms of astaxanthin, in nature only one double bond within the molecule may be in the Z configuration, as they form kinks in the carbon chain (Fig. 1.5) that may result in greater steric hindrance, particularly between the hydrogen and the methyl group at C9 and C13 (Fig. 1.7) (Britton, 1995). Thus, increased steric hindrance causes the Z isomers to be thermodynamically less stable than their all E isomer counterparts, which are more readily found in nature (Britton, 1995). Astaxanthin may be converted between its E and Z isomers, but this has no effect on the chiral centres, such that Z isomers also have three configurational isomers, consisting of two enantiomers (3R, SR’ and 3S, 3S’) and a mesoform (3R, 3’S or 3S, 3’R) (Abu-Lafi and Turujman, 1997).
Fig 1.4 The configurational isomers of astaxanthin: a, 3S, 3'S enantiomer; b, 3R, 3'R enantiomer; c, 3R, 3'S mesoform (identical); d, 3S, 3'R mesoform (Abu-Lafi and Turujman, 1997).
Fig. 1.5 The molecular structures of the trans (E) and cis (Z) isomers of astaxanthin (Abu-Lafi and Turujman, 1997).
Fig 1.6 The priority of different groups around a carbon double bond for determining their E, Z designation: a, Z isomer of 1-chloro-1-propene; b, E isomer of 1-chloro-1-propene; c, the cis and trans confirmations of single bonds in a polyene chain (Wade, 1999).
Fig. 1.7 Sterically (a) hindered and (b) unhindered cis carbon double bonds within polyene chains (Britton, 1995).
1.3.2 Production of synthetic astaxanthin

In 1985 Hoffmann-La Roche invested $100 million in a production plant to commercially manufacture synthetic astaxanthin, the structure of which is nature identical (Springate, pers comm.). The astaxanthin product manufactured by Roche was named under the Carophyll® product range (Table 1.7) as Carophyll Pink™. Carophyll® products are designed to be free flowing spherical beadlets in which carotenoids are finely distributed in a starch-gelatine matrix, which provides stability and enables it to be added to animal diets as a raw material prior to processing (Latscha, 1990). The chemical pathway used to synthesise astaxanthin is a commercial secret. However, the manufacturing of Carophyll® products is based on the spray drying technique used to produce lipid soluble vitamins. Basically, synthetic lipid soluble vitamins or carotenoids in an oily solution are mixed with an aqueous solution of gelatine to form an emulsion. The emulsion is then spayed into the drying vessel with hot air and starch (Fitt, pers comm.). Spraying the emulsion causes it to disperse into droplets which are then coated in starch and dried by the hot air (Latscha, 1990).

Table 1.7 The carotenoid content and use of the Carophyll® product range (Latscha, 1990).

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Carotenoid Content</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carophyll Pink™</td>
<td>8% Astaxanthin</td>
<td>Fish &amp; Shrimp</td>
</tr>
<tr>
<td>Carophyll Red™</td>
<td>10% Canthaxanthin</td>
<td>Broilers, Eggs, Fish &amp; Shrimp</td>
</tr>
<tr>
<td>Carophyll Yellow™</td>
<td>5% Apocarotenoi acid ethyl ester</td>
<td>Broilers &amp; Eggs</td>
</tr>
<tr>
<td>Carophyll Orange™</td>
<td>5% Canthaxanthin &amp; 5% Apocarotenoi acid ethyl ester</td>
<td>Broilers &amp; Eggs</td>
</tr>
</tbody>
</table>

As shown in Table 1.7, Carophyll Pink™ is aimed, marketed and used by the aquaculture industry, primarily for use in the farming of salmonid species, namely rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). Yet, prior to the advent and manufacture of synthetic astaxanthin (Carophyll Pink™), the salmonid aquaculture industry used synthetic canthaxanthin (Carophyll Red™) as a dietary pigment. The
molecular structure of canthaxanthin (β-β-carotene-4,4'-dione: C₄₀H₅₂O₂) is very similar to that of astaxanthin (C₄₀H₅₄O₄), apart from astaxanthin, as its IUPAC name suggests (3,3’-dihydroxy-β-β-carotene-4,4'-dione) has a pair of hydroxyl groups, one at C3 and the other at C3’. However, the flesh colour of salmonids fed canthaxanthin had an orange hue, being markedly different to that of wild salmon whose flesh only contained astaxanthin and had a red hue. This obvious colour difference led researchers at Hoffmann-La Roche to elucidate a chemical pathway to synthesise astaxanthin that could be scaled up for commercial production.

1.3.3 Natural alternatives

Consumer pressure to restrict the use of synthetic food additives has led to the search for more economical natural alternatives to achieve pigmentation. A variety of natural materials have been evaluated as possible pigment sources for farmed salmonids, including paprika and dried flowers (Sommer et al., 1992). However, the degree of pigmentation was markedly dissimilar to that of wild salmonids (Torrissen et al., 1989). Suitable pigmentation has been gained with the use of krill, shrimp and crab waste and with the yeast Phaffia rhodozyma (Sommer et al., 1992), yet each source has been beset with problems. Waste products tend to be unstable, have a high lipid content and have a large variation in carotenoid content. Phaffia also has inherent problems including genetic instability, low carotenoid content (in many strains) and poor reproducibility during industrial fermentation (Johnson and Schroeder, 1996).

Industrial interest has now focused upon the merits of the microalga Haematococcus pluvialis, which has a high pigment content, up to 5% (dry weight) of which astaxanthin constitutes approximately 85% total carotenoid content (Benemann, 1992). Haematococcus pluvialis is relatively easy to cultivate in open ponds utilising a free carbon source (CO₂) and a free energy source (light), or in industrial fermentation vessels. The carotenoid content is stable and highly reproducible (Borowitzka, 1992), and initial
studies by Sommer et al. (1991) suggest that *Haematococcus pluvialis* could be the most economical natural alternative to pigment salmonids.

1.4 *Haematococcus pluvialis*

1.4.1 Structure and life stages of *Haematococcus pluvialis*

*Haematococcus pluvialis* is quite prevalent, being detected throughout the world in environments favourable for its growth, characteristically inhabiting ephemeral rain pools located on hard surfaces that are impermeable to water (Lorenz, 1997). Due to its prevalence, *Haematococcus pluvialis* may also be referred to as *Haematococcus lacustris* and *Saphaerella lacustris* (Lorenz, 1997). It should be noted, that from this point on the algae will be named either in full referring to its genus and species (*Haematococcus pluvialis*) or in brief, using its full species and partially referring to its genus (*H. pluvialis*).

*Haematococcus pluvialis* has the following taxonomy:

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Protista</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum</td>
<td>Chlorophyta</td>
</tr>
<tr>
<td>Class</td>
<td>Chlorophyceace</td>
</tr>
<tr>
<td>Order</td>
<td>Volvocales</td>
</tr>
<tr>
<td>Family</td>
<td>Haematococcaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Haematococcus</td>
</tr>
<tr>
<td>Species</td>
<td><em>pluvialis</em></td>
</tr>
</tbody>
</table>

In 1934 Elliot published a comprehensive study of the life cycle of *Haematococcus pluvialis* over several generations, detailing its cellular morphology at all phases relating them to its environment. Elliot’s observations are regarded as fundamental to the research of this species, and it is widely accepted as the definitive text on the life cycle of *Haematococcus pluvialis*, such that the vast majority of papers since 1934 cite Elliot’s work when referring to its life cycle (Young, pers comm.). Hence, the different life stages
of *H. pluvialis* described in sections 1.4.1.1 – 1.4.1.4 are based on Elliot (1934), unless referenced. Elliot described the life history of *Haematococcus pluvialis* as being comparatively simple in pure culture having four life stages (microzooid, macrozooid, palmella and haematocyst) that are dependent on environmental factors for their change. In favourable conditions the palmella stage alternates with the microzooid and macrozooid stages, where cells in the palmella stage undergo binary fission within the cytoplasmic membrane producing either small palmella cells or macrozooid cells.

1.4.1.1 Macrozooids

Macrozooids are spherical bi-flagellated motile cells that range in diameter from 8 to 50 μm. The flagella are located at the anterior of the cell at an acute angle (less than 90°). They contain a centrally located spherical nucleus, ranging in diameter from 2 to 10 μm. The cytoplasmic region surrounding the nucleus appears colourless and contains non-pigmented organelles such as mitochondria, ribosomes, Golgi apparatus and vacuoles (Green *et al.*, 1990b). Around the colourless cytoplasmic region lies a coloured cytoplasmic zone, which occupies the majority of the cell that Elliot described as the chromatophore. The chromatophore contains chloroplasts for photosynthesis and a red stigmata line that detects variations in photon density (light intensity) causing cellular movement in the water column. The cells usually move antagonistically to an attenuated irradiance suitable for optimal photosynthesis, this response is known as chemotaxis (Green *et al.*, 1990a; Hagen *et al.*, 1993). The cytoplasmic region (protoplast) is surrounded by a membrane, that is separated from the cell wall by a region that is described by Zlotnic *et al.* (1993) as watery jelly, that is traversed by cytoplasmic threads. Macrozooids may either grow to adulthood or they may loose their flagella reverting back to the palmella stage and then repeat the cycle.
1.4.1.2 Microzooids

Microzooids are also bi-flagellated motile cells that are cylindrical in shape and are generally smaller than macrozooids at less than 20 µm, but are able to swim with a greater vigour. The nucleus is located towards the anterior region of the cell, which is surrounded by a clear region of cytoplasm followed by the haematochrome region containing chloroplasts and the stigmata line. However, they differ from macrozooids as they do not have a protoplast membrane to separate the cytoplasm from the cell wall.

1.4.1.3 Palmella

Cells in the palmella stage grow to adulthood and are characterised by their lack of flagella, resulting in a lack of motility (Zlotnik et al., 1993). Apart from being non-flagellated, the palmella cells structure varies very little from that of the macrozooid cells as they also have a protoplast membrane and a haematochrome that may be centrally located or evenly dispersed through the cell.

1.4.1.4 Haematocysts

If the conditions become intolerable, regardless of life stage, the cells will endeavour to convert into haematocysts and it seems as if cells in the palmella stage are better adapted for conversion to this stage. This is probably due to cells in the microzooid and macrozooid stages having to undergo more physiological changes, namely the loss of their flagella, compared to those in the palmella stage. The extra physiological changes required, take longer to achieve and depending on how adverse the environmental conditions get, may result in death for most of the cells in the microzooid and macrozooid stages. Elliot described haematocysts as the resting stage in the life cycle of Haematococcus pluvialis cells that could survive conditions of desiccation. They were immotile due to the loss of their flagella and possessed a thick cell wall that was appreciably spaced from the protoplast, the most noticeable feature were their bright red colour. The haematochrome
changes colour from green to red. The intensity of the red pigment (astaxanthin) is dependent on the intensity of the light, in dim light the pigment is diffusely spread throughout the cytoplasm, whereas in bright light the pigment dominates the cytoplasm. When the environmental conditions become favourable haematocysts are converted to microzooids, which are rapidly transformed to young palmella cells, which may either mature to adult palmella or convert to macrozooids.

In more recent publications when referring to the life stages of *Haematococcus* workers tend not to use the four stages described by Elliot (1934) but for convenience only specify two stages, green vegetative cells and red aplanospores. Hence, from this point on *Haematococcus* cells will be referred to as green vegetative cells and aplanospores.

1.4.2 Caroteneogenesis

The term caroteneogenesis is derived from carotenoid (the collective name for terpenoid pigments) and genesis (from the Latin *gen* meaning to produce), being used to describe the *de novo* synthesis of secondary carotenoids by micro-organisms, algae, fungi and higher plants (Latscha, 1990). In a vast majority of organisms (including *Haematococcus pluvialis*) the production of terpenes, terpenoids and carotenoids occurs using the same anabolic pathway, the enzymes of which are either located in the cytoplasm or associated with a membrane (probably that of the chloroplast) (Armstrong, 1999). In the case of *Haematococcus pluvialis* cells, secondary carotenoid generally refers to astaxanthin, being the most predominant pigment constituting up to 90% of the total carotenoid content (Kobayashi *et al.*, 1997). Due to its prevalence, *H. pluvialis* inhabits areas that are favourable for growth but often encompass adverse climatic conditions. Thus, *H. pluvialis* has evolved a type of protection system to enable its cells to survive harsh environmental conditions. The two major physiological adaptations that allow the cells to endure adverse changes in their environment are the production of a thick cell wall and the caroteneogenesis of astaxanthin, forming aplanospores (haematocysts; section 1.4.1.4).
Many workers agree caroteneogenesis is triggered by several sub-optimal biotic and abiotic factors (Johnson and An, 1991; Grung et al., 1992; Kobayashi et al., 1993; Fan et al., 1994; Triki et al., 1997).

1.4.2.1 Factors affecting caroteneogenesis

Many factors have been suggested to initiate caroteneogenesis including mechanical stress (Chaumont and Thépenier, 1995). Those factors primarily regarded as inducing caroteneogenesis are temperature, light intensity, active oxygen species, salt stress, pH, cell division inhibitors and limited nutrients namely, nitrogen and phosphate (Boussiba et al., 1992; Chaumont and Thépenier, 1995; Lee and Ding, 1995). High temperatures not only affect the efficiency of enzyme-catalysed metabolic reactions but also have a direct affect on the aqueous environment in which the green vegetative cells reside. As the temperature increases the tertiary structure of their enzymes may be compromised which could result in their denaturation, also their aqueous environment will evaporate resulting in cellular desiccation (Elliott, 1934).

Low temperatures also affect the efficiency of enzyme-catalysed metabolic reactions, as the $Q_{10}$ (temperature coefficient) for the majority of enzyme-controlled reactions from 0 to 40°C is 2, *i.e.* for every 10°C increase the rate of reaction is doubled (Green et al., 1990b). When the temperature decreases so does the rate of reaction, but as the temperature reaches zero the enzyme is said to be inactivated, as its rate of reaction is negligible (Green et al., 1990b). Hence, the cells are no longer able to maintain normal activity, such as swimming, as this requires energy produced from the enzyme-controlled ‘dark reaction’ of photosynthesis (Carroll, 1989). As the temperature decreases to 4°C the water molecules become highly ordered due to their high affinity for each other and as the temperature reaches zero the water freezes producing a highly regular crystalline structure that is less dense than water, *i.e.* as water freezes it expands (Stryer, 1988). Thus, at low temperatures the cells become encysted which probably acts in two ways, the first as a thermal insulator
and the second to provide strength (Hagen et al., 1994a). A fortified cell wall will maintain its structural integrity during the formation of ice crystals (being difficult to breach), allowing the cell to retain its contents once the ice has thawed.

As stated (section 1.4.1.1) H. pluvialis contains a stigmata that allows the cells to undergo phototaxis, which in turn allows the cells to populate a water layer within the column with an attenuated irradiance that provides optimal photosynthesis (Hagen et al., 1993). However, excessive irradiation may damage the chloroplast by photobleaching the chlorophyll pigment, so to protect the cell against excessive irradiation they produce astaxanthin which acts as 'sun shade' for the chloroplasts (Hagen et al., 1994b). The concentration of astaxanthin is relative to the intensity of the light to which the cells are exposed (Fan et al., 1994). Photoprotection is also probably aided by the cell wall, as Young (pers comm.) described the thickness of the cell wall, as seeming to be correlated to the concentration of astaxanthin formed within the cells. The correlation seems to follow a hyperbolic trend which plateau at approximately 2 % total secondary carotenoid content (dry weight) (Young, pers comm.), even though H. pluvialis may produce up to 5 % total secondary carotenoid content (Triki et al., 1997).

Lee and Ding (1995) suggest excessive light may enhance rates of photosynthesis resulting in the production of excessive quantities of oxygen radicals inducing oxidative stress. Astaxanthin is a powerful antioxidant (section 1.6.3.4) that can quench the action of singlet oxygen species that may cause intra-cellular damage such as membrane lipid peroxidation and so limits oxidative stress. Kobayashi and Sakamoto (1999) suggest the presence of active oxygen species such as singlet oxygen (O₂) superoxide anion radicals (O₂⁻), hydroxy radicals (HO•) and hydrogen peroxide (H₂O₂) either in vivo or in the media cause oxidative stress and trigger the biosynthesis of astaxanthin.

An increase in salt concentration in the media in which Haematococcus pluvialis cells are residing, exerts stress as it relates to their normal aqueous environment decreasing in volume, resulting in a lower (more negative) solute potential. The decreasing solute
potential of the media (system 1) will result in a higher water potential for the water
compartment of the aqueous cytoplasm (system 2), that will eventually lead to water
diffusing out of the cells by osmosis to equilibrate the two systems (Green et al., 1990a).
However, if too large a volume of water is lost from system 2, the cells will no longer be
able to function in their normal manner, resulting in their death. Hence, salt stress causes
the cells to become encysted, as the cyst is made of a non-permeable polymer called
sporopollenin that stops water being lost from the cell (Mendes-Pinto et al., 2001).
Encystment also initiates the biosynthesis of secondary carotenoids, because if the media
volume decreases, it will inevitably lead to the cell being subjected to an increased light
intensity (Boussiba et al., 1992).
A change in pH initiates the biosynthesis of astaxanthin in a similar manner as salt stress,
but also has the added danger of denaturing the cell’s enzymes causing their metabolism to
cease operating and the cells to die. The lack of nutrients, particularly nitrogen and
phosphate, have been demonstrated to induce caroteneogenesis. This is due to nitrogen
being a mandatory component of protein synthesis and phosphate being obligatory for the
production of adenosine triphosphate (ATP), the universal currency of energy (Borowitzka
et al., 1991; Boussiba et al., 1992). So, if the media in which Haematococcus pluvialis
cells reside lack a suitable nitrogen source, the cells won’t be able to synthesise protein,
likewise, lack of phosphate could lead to limited metabolic functions (Borowitzka et al.,
1991). Hence, if Haematococcus pluvialis cells are unable to execute their metabolic
functions due to limited resources they will form into red encysted aplanospores, resting
until more favourable conditions are encountered.
The inhibition of cell division is linked to nutrient deficiencies, especially nitrogen and
phosphate, so inhibiting cell division has been suggested to initiate caroteneogenesis (Lee
and Ding, 1995). Boussiba et al. (1992) demonstrated that the addition of vinblastine (cell
division inhibitor) to vegetative green cells (grown under optimal conditions) triggered a
rapid accumulation of astaxanthin, showing that cell division and astaxanthin biosynthesis are directly linked and independent of nutrient status.

1.5 Application of carotenoids to aquaculture

1.5.1 Salmonid aquaculture

In the mid-1950s efforts began in earnest to raise salmon as a commercial food stuff, when Norwegian biologists started experimenting with Atlantic salmon smolts in a bid to achieve enhanced growth rates (Sedgwick, 1988). This research paved the way for success that has enabled Norway to become the world's top producer of Atlantic salmon, even though the industry is still regarded as being in its infancy. In 1972 the Norwegian salmon industry consisted of six farms with an annual production of approximately 50 tonnes, twenty years later the number of farms had increased to approximately 950, producing in excess of 200,000 tonnes annually (Monahan, 1993). This success has also been attributed to careful planning, this is due to the introduction of government regulations and research programmes, which has led estimations for Norwegian salmon production for the year 2001 to exceed 400,000 tonnes (Gulbrandsen, pers comm.).

The prosperity gained by Norwegian fish farmers has led to a commercial interest in the biochemistry of Salmonids, being fuelled by the industry's constant desire to maximise profit margins. As with any industry "time is money" and the very nature of salmonid aquaculture is based on rearing salmon and rainbow trout under controlled conditions from the egg to market (Monahan, 1993). Aquaculture is preferred to random capture fishing because not only the yield can be controlled but also the environment in which the fish are cultured, providing optimal conditions for growth by protecting them against predation and disease. Even though such practices, to a certain degree, creates an artificial environment to which the fish must adapt, it enables fish farmers to produce desirable, high quality products.
In first world countries such as the UK and Japan the demand for luxury fin-fish has increased. Their consumption has been advocated as being more healthy than consuming terrestrial animals, due to their high content of ω-3 and ω-6 fatty acids (Samples et al., 1997). It is thought that certain dietary lipids (namely ω-3 and ω-6 fatty acids) may have a preventative role in humans developing certain types of cancer, in particularly breast carcinoma. Early work carried out by Gonzalez et al. (1993) demonstrated a high fat diet (20 %) containing long chain ω-3 polyunsaturated fatty acids, namely eicosapentaenoic acid and docosahexaenoic acid, suppressed the growth of human breast carcinomas in female athymic nude mice infected with this cell line. In the European Union some of the most highly prized fin-fishes are the salmonids (rainbow trout and salmon) which have become the prime choice for intensive farming operations. Aquaculture has led to the development of various support industries, especially in the production of commercial feed formulations, which strive to meet the desire for optimum growth by formulating high energy feeds that concomitantly provide optimal nutrition to keep the fish healthy and sustain maximum feed utilisation.

In the wild, the Atlantic salmon and rainbow trout are opportunistic predators whose diets mainly consist of small fish and crustaceans, usually shrimp and prawns (Pethon, 1989). The latter contains the carotenoid astaxanthin, which salmonids absorb and deposit throughout their bodies. As humans first consumed wild salmon and rainbow trout that had pink flesh, producers of farmed salmon soon realised that habitual consumers of their product expected the same. As a direct response to consumer demand, the industry has adopted the practice of adding astaxanthin to the feed of farmed salmonids, as they are unable to synthesise astaxanthin de novo (Schiedt et al., 1986; March et al., 1990; Choubert and Storebakken, 1996). Such salmonids are entirely dependent on dietary supplements to achieve the desired flesh pigmentation (Storebakken and No, 1992). Hence, astaxanthin has become one of the key additives in aquafeeds destined for salmonids, which from a commercial view is purely aesthetic, only being incorporated to
enhance the pink colouration of their flesh. In fact, this consumer desire to have salmon with pink flesh has resulted in the market enforcing the notion that the more pigmented the salmon, the higher its value, even though astaxanthin does not possess any known human organoleptic properties (Christiansen et al., 1995a). This background has supplied salmonids with an elitist image, that in turn has provided their farmers with a distinct economic advantage, as they do not have to compete in the white fish market that has many more competitors in terms of the types of fish, and their producers.

1.5.2 The cost of astaxanthin

Astaxanthin has a dietary inclusion of 40-100 mg kg\(^{-1}\), yet constitutes approximately 20% of feed costs (Bjekeng et al., 1997) and 8% of the total production costs, making it an expensive commodity (Torrissen, 1995). This is mainly due to the fact that only two companies i.e. Hoffmann-La Roche (Basel, Switzerland) and BASF (Mannheim, Germany) manufacture a stable synthetic astaxanthin. In 1998 astaxanthin cost the global salmonid farming industry an estimated £ 175 million (Sterling) (McCoy, 1999). The metabolic pathways of astaxanthin (section 1.6.2) are not well understood in fish, but classic feeding experiments show salmon poorly utilise astaxanthin, with only 35% absorption in the gut, of which less than 50% is deposited in the white muscle tissue (Torrissen et al., 1989; Storebakken and Goswami, 1996). This equates to approximately £ 150 million (Sterling) wastage.

1.6 Salmonid utilisation of astaxanthin

1.6.1 Astaxanthin absorption

Absorption of any lipophilic compound, for example carotenoids, is defined as the movement of the ingested compound or their metabolites to the lymphatic system or portal circulation (Erdman et al., 1993). With respect to fish, particularly salmonids, the portal system is widely thought (even though controversial) as being the vehicle for lipid
transportation. However, no conclusive evidence has been proposed in defining teleostean fishes as having a discrete lymphatic system, such as those found in mammalian species (Vogel and Claviez, 1981). Astaxanthin absorption involves several stages, the first of which is the disruption of the food matrix. This is achieved mechanically by the fish chewing the feed pellets, followed by chemical disturbance due to the action of enzymes and gastric juices that release the carotenoid and oil into the gut lumen. The second step is emulsification, on release from the food matrix astaxanthin probably dissolves into lipid droplets, which become emulsified by the gastric juices forming a fine emulsion. Furr and Clark (1997) suggest the emulsion droplets have a triacylglycerol core (which may incorporate non-polar carotenoids) surrounded by a molecular mono-layer of protein, carbohydrates, phospholipids and polar carotenoids such as astaxanthin. The surface location of astaxanthin within the emulsion enables it and other polar carotenoids to spontaneously transfer to mixed micelles (the mechanism of which is unclear) (Furr and Clark, 1997). Nevertheless, the transfer of a carotenoid-containing emulsion to mixed micelles is only possible due to the presence of bile salts, without which the constitutional lipid droplets of the emulsion would not aggregate to form the mixed micelles. A difference between dietary lipids and carotenoids is that lipids do not require the micellar phase, which is obligatory for carotenoid uptake into the mucosal cells (which is the third step) (El-Gorab et al., 1975). It must be noted that the uptake of any compound just into the mucosal cells it not adequate. This is because mucosal cells only have a short half life, being readily sloughed off into the gastrointestinal tract, where they along with any absorbed carotenoids are ejected from the body as waste (Erdman et al., 1993). Lipids and carotenoids are thought to be absorbed into the mucosal cells of the pyloric region of the gut by passive diffusion. (Robinson and Mead, 1973; Sire et al., 1981; Parker, 1996). In the mucosa cells, the astaxanthin is integrated with protein moieties, forming lipoproteins of varying densities that are used to transport the carotenoid in the blood stream. As yet, the specific mechanism for the intra-cellular translocation of carotenoids in fish,
particularly salmonids, is not known (Parker, 1996). Transport of astaxanthin in the serum of salmonids is courtesy of non-covalent interactions with high density lipoproteins (80%) and very low density lipoproteins (20%) with the ratio varying dependant on sex and species (Ando et al., 1985; Torrissen et al., 1989). Deposition of astaxanthin occurs in most of the fishes tissue to varying degrees, but overall retention depends on many factors including diet composition, age, sexual maturation and feeding rates (Storebakken and No, 1992). Tyssandier et al. (1998) demonstrated rainbow trout fed diets containing different carotenoids (astaxanthin, zeaxanthin and lycopene) at an inclusion rate of 80 mg kg\(^{-1}\) were absorbed at different rates into the blood stream. The plasma concentration of astaxanthin (that has two hydroxyl and two ketone groups) was higher than that of zeaxanthin (that has two hydroxyl groups) \(i.e.\) astaxanthin is more readily absorbed than zeaxanthin, whereas hydrophobic lycopene that has no oxygenated groups was not detected in the plasma. Tyssandier et al. (1998) concluded that in rainbow trout the bioavailability of carotenoids is related to their polarity, where the more polar the carotenoid the higher its bioavailability.

1.6.2 Astaxanthin metabolism

As mentioned (section 1.6.1), astaxanthin is inadequately absorbed and its metabolism poorly understood. Experiments carried out to elucidate the metabolism of astaxanthin in rainbow trout generally targeted the skin and flesh, where the majority of astaxanthin accumulates. The results from such experiments led to the notion that the different metabolites of astaxanthin found in the different tissues had different reductive pathways. Schiedt et al. (1985) proposed that in the skin of rainbow trout, astaxanthin is reduced to \(\beta\)-adonixanthin by the loss of the C4' carbonyl group (Fig. 1.8), reduced again by the loss of the C4 carbonyl group to zeaxanthin (Fig. 1.8).
Fig. 1.8 The metabolism of astaxanthin in the skin of rainbow trout \((Oncorhynchus mykiss)\) (Goodwin, 1986).
Zeaxanthin is then oxidised to antheraxanthin by the addition of an epoxy group between C5 and C6, which becomes hydrogenated, forming a hydroxyl group at C5 forming deepoxineoxanthin (Fig. 1.8) (Goodwin, 1986). Schiedt et al. (1988a) proposed idoxanthin was also present in the skin of Atlantic salmon suggesting it was an intermediate metabolite being directly converted from astaxanthin. Schiedt et al. (1989) further suggested astaxanthin was metabolised into idoxanthin in the flesh of salmonids, even though Katsuyama et al. (1987) stated astaxanthin was not reductively metabolised in the white muscle of rainbow trout. Lagocki (1995) demonstrated astaxanthin constituted approximately 98% of the total carotenoid present in the plasma, similar to that of the red and white muscle, whereas the astaxanthin content of the liver only constituted 58% total carotenoid content. The majority of the other carotenoids present in the liver were not identified, but this led Lagocki (1995) to propose the liver to be a major organ for astaxanthin degradation. A possible explanation for the retention of idoxanthin in the flesh is due to its structure (Fig. 1.9), which like astaxanthin has four keto groups, with one hydroxyl group at C3 and one carbonyl group at C4 of the β-ionone ring and two hydroxyl groups on the β'-ionone ring at C'3 and C'4 (Schiedt et al., 1988b). Thus, idoxanthin potentially has eight stereoisomers (Schiedt et al., 1988b) all of which may bind to salmonid epaxial white muscle.

1.6.3 Functions and actions of astaxanthin in rainbow trout

Christensen (1996) determined that salmonids utilised astaxanthin for several biological activities, classifying them as either functions or actions (Table 1.8). In this sense, functions are characterised as any essential role that astaxanthin or its metabolites play in the well being of salmonids and actions as any physiological or pharmacological response to the administration of astaxanthin (Christensen, 1996).
Fig. 1.9 The chemical structure of idoxanthin (Schiedt, et al., 1998b)
Table 1.8 The biological functions and actions of astaxanthin by salmonid species (Christensen, 1996).

<table>
<thead>
<tr>
<th>Activity</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Function</td>
<td>Communication</td>
</tr>
<tr>
<td></td>
<td>Reproduction</td>
</tr>
<tr>
<td></td>
<td>Vitamin A production</td>
</tr>
<tr>
<td>Action</td>
<td>Eggs resistant to lower oxygen concentrations</td>
</tr>
<tr>
<td></td>
<td>Eggs resistant to elevated ammonia concentrations</td>
</tr>
<tr>
<td></td>
<td>Increased photoprotection</td>
</tr>
<tr>
<td></td>
<td>Reduced lipid peroxidation</td>
</tr>
</tbody>
</table>

1.6.3.1 Communication and reproduction

One of the most important uses of astaxanthin is communication, particularly with respect to reproduction, where visual recognition of the opposite sex is an important part of courtship (Torrissen and Christensen, 1995). Under experimental conditions Choubert et al. (1998) demonstrated astaxanthin played no significant role in the timing of ovulation, fertilisation rate, relative fecundity or fingerling growth in rainbow trout. Thus, in terms of reproduction astaxanthin only has two roles, the aforementioned communication and a protective role for eggs. Salmonids of both sexes, during their sexual maturation mobilise astaxanthin stored in their epaxial white muscle. Females transfer the astaxanthin to their gonads and eggs whereas males transport astaxanthin to their skin (Sivtseva and Dubrovin, 1982; Torrissen and Torrissen, 1985; Sigurgisladottir et al., 1994). Christensen (1996) suggests body colouration may also be a visual cue indicating the health status of the fish, enabling fish of the opposite sex to determine whether a particular fish would make a good mating partner. However, bright markings are a compromise between the requirement for communication and the risk of predation. The concentration of astaxanthin and the ratio of optical isomers in Atlantic salmon flesh and their eggs have been correlated to the concentration in their diet (Storebakken et al., 1985). Yet, the concentration of astaxanthin in salmonid eggs has also been linked to water quality, where eggs in poorly oxygenated water tend to have an elevated astaxanthin concentration as compared to those laid in well oxygenated water (Tacon, 1981). This led Christiansen (1996) to suggest that within
salmonid eggs, ketocarotenoids such as astaxanthin and canthaxanthin may have a respiratory function where they may act as intracellular oxygen reserves. Christiansen and Torrissen (1997) further suggest that eggs containing elevated concentrations of astaxanthin has no affect upon their size and enables them to tolerate other harsh environmental conditions such as elevated concentrations of ammonia and elevated water temperatures.

1.6.3.2 Vitamin A production
Salmonids like many animals are unable to synthesise vitamin A de novo, a deficiency of which may lead to hypovitaminosis, which can manifest itself in many different ways including keratinisation of mucosal tissue, visual disorders and poor growth (Latscha, pers comm.; Christensen et al., 1995a). Thus, vitamin A is a mandatory component of the salmonid diet, with rainbow trout requiring 2000-2500 IU kg\(^{-1}\) dry diet (Christiansen, 1996). However, both Al-Khalifa and Simpson (1988) and Storebakken et al. (1991) demonstrated that salmonids (rainbow trout and Atlantic salmon, respectively) fed vitamin A deficient diets supplemented with astaxanthin showed no disorders associated with vitamin A deficiency. On analysis of the gut, Al-Khalifa and Simpson (1988) concluded astaxanthin to be a vitamin A precursor, with astaxanthin being converted to vitamin A\(_1\) (retinol) and then into vitamin A\(_2\) (3-dehydroretinol) in the intestine. Guillou et al. (1989) determined the metabolic pathway for the conversion of astaxanthin to vitamin A\(_2\) whereby astaxanthin is first reduced to zeaxanthin and then centrally cleaved to yield two vitamin A\(_2\) molecules (Fig. 1.10). Guillou et al. (1989) further suggested that astaxanthin, converted to vitamin A\(_2\) in the gonads of both sexes, is done so without first being converted to vitamin A\(_1\). Thompson et al. (1995) demonstrated that vitamin A is required to maintain a specific function in the immune system of rainbow trout, namely leucocyte migration. Even though astaxanthin is a precursor of vitamin A, its presence in a vitamin A deficient diet had no effect on the specific or innate immune system of rainbow trout.
Fig. 1.10 The metabolic pathway for the conversion of astaxanthin to vitamin A₂ by rainbow trout (*Oncorhynchus mykiss*) (Guillou et al., 1989).
Thompson et al. (1995) concluded astaxanthin had very little potential as an immunostimulant in the culture of salmonid species.

1.6.3.3 Photoprotection

Ultraviolet (UV) light may penetrate to depths of 6 m or more in non-turbid water columns, so that when wild salmonid fry start feeding they do so in shallow water that leaves them exposed to harmful UV rays (Christiansen, 1996). This has led to the suggestion that carotenoid deposition in the skin of juvenile fish has a photoprotective role against UV radiation (Christiansen, 1996). Photoprotection is probably also applicable to salmonids that are intensively farmed, as the methods used, namely open top nets and high stocking densities, results in their exposure to UV radiation, so making the fish somewhat resistant to ‘sun burn’.

1.6.3.4 Antioxidant properties of astaxanthin

A normal characteristic of aerobic life is the structural damage to a wide variety of organic compounds including carbohydrates, lipids, proteins and DNA. This is primarily a consequence of reductive reactions that liberate singlet oxygen and reactive oxygen species in the form of the superoxide ion, the hydroxide ion, the hydroxyl radical and hydrogen peroxide (Gutteridge and Halliwell, 1994). Singlet oxygen ($^1$O$_2$) is a highly reactive electronically excited form of oxygen, whereas free radicals are molecules that contain one or more unpaired electrons. However, low level production of singlet oxygen and free radicals are mandatory to many normal biological processes, for example the superoxide ion is liberated by lymphocytes and fibroblasts as growth regulators, and hydrogen peroxide is produced by D-amino acid oxidase which uses oxygen to oxidise unwanted D-amino acids (Sies, 1986). Hence biological systems have developed powerful enzymatic (e.g. catalase and the superoxide dismutase) and non-enzymatic (e.g. $\alpha$-tocopherol and astaxanthin) antioxidant systems to counteract these potentially destructive reactive oxygen
species (Sies, 1986). Nevertheless, diverse biological processes such as ageing, carcinogenesis, inflammation and radiation lead to excessive free radical production, causing a shift in the prooxidant/antioxidant balance, in favour of the former. Allowing unactivated free radicals to roam free, results in the oxidation and damage of structural compounds in an unregulated manner, especially the peroxidation of lipid membranes. It is this damage inflicted by reactive oxygen species that has been termed oxidative stress (Sies, 1986). Carotenoids have the ability to quench both singlet oxygen and scavenge free radicals (Palozza and Krinsky, 1994). Free radicals either accept or donate electrons from other molecules in a bid to try and pair their electrons, which usually starts a chain reaction as during this process new free radicals are generated. The role of antioxidants is to interrupt the chain reaction, carotenoids are able to achieve this because of their unstable electron rich polyene structure that makes them susceptible to attack by electrophilic species to which they readily donate electrons (Britton et al., 1995). Carotenoids quench singlet oxygen by absorbing and dispersing their excited energy, which in doing so, converts the singlet oxygen to its ground state ($^1$O$_2$) and oxidises the carotenoid (Fig. 1.11) (Christiansen, 1996).

$^1$O$_2$ + Carotenoid $\rightarrow$ $^3$O$_2$ + $^3$Carotenoid

Fig. 1.11 The general mechanism by which carotenoids quench singlet oxygen (Christiansen, 1996).

Vitamin E is stored within both the plasma membrane and organelle membranes of cells, an ideal location for its primary function as a potent chain breaking antioxidant of bulk unsaturated lipids (Koga et al., 1994). The chromanol ring of $\alpha$-tocopherol is located on the hydrophobic surfaces of both membrane leaflets, while the phytol side chain interacts with the interior hydrophobic fatty acid chains (Buckley and Morrisey, 1992). In this orientation $\alpha$-tocopherol is able to protect the constituent phospholipids from peroxidation by free radicals. It seems anti-mortem that astaxanthin and vitamin E work synergistically
to scavenge free radicals and reduce lipid peroxidation, which if unchecked post-mortem in fillets and steaks manifests, itself as rancid off flavours (Bell et al., 1998). Both Bjerkeng and Johnsen, (1995) and Bell et al. (1998) demonstrated that the synergy between vitamin E and astaxanthin continues post-mortem. Hence, the addition of vitamin E and astaxanthin into salmonid diets does not just increase the aesthetic appeal of the fillets and steaks to the customer, but also helps to maintain product quality during storage. Under the correct conditions the presence vitamin E and astaxanthin can elongate the storage period (Sigurgisladottir et al., 1994; Bjerkeng and Johnsen, 1995).

1.6.4 Salmonid muscle structure

In salmonids, lipids are chiefly stored in white muscle tissue which is composed of parallel muscle fibres separated by three layers of connective tissue (endomysium, preimysium and epimesium) (Bell et al., 1998). At the point where the muscle fibre terminates the layers of connective tissue fuse together forming a strong sheet of connective tissue called myosepta, between which a large numbers of adipocytes are located that readily accumulate excessive lipids (Bell et al., 1998). Both red and white muscle act as a storage depot for astaxanthin (Sivtseva and Dubrovin, 1982) with each muscle type having a similar potential to retain astaxanthin (Lagocki, 1995). A study using sockeye salmon white muscle led Henmi et al. (1989) to propose the β-ionone rings of astaxanthin and canthaxanthin bind to a hydrophobic pocket on the surface of the actomyosin (Fig. 1.12). Using resonance Raman spectroscopy on pigmented sockeye salmon and coho salmon muscle, Henmi et al. (1990a) showed astaxanthin and canthaxanthin bound to actomyosin only caused a small shift in wave number when compared to their respective standards. The results suggested astaxanthin and canthaxanthin were mildly distorted in the plane of the carbon backbone, implying that they were only weakly bound to the actomyosin (Henmi et al., 1990a).
Fig. 1.12 The model proposed by Henmi et al. (1989) displaying the hydrophobic pocket on the surface of salmon actomyosin to which astaxanthin binds.
The ability to bind astaxanthin and canthaxanthin to white muscle is not a specific trait of salmonid species, as Henmi et al. (1990b) demonstrated in vitro, the muscle of white fish such as jack mackerel and stone flounder also had the ability to bind carotenoids. During this study, Henmi et al. (1990b) established a correlation between the surface hydrophobicity of actomyosin and the binding of ketocarotenoids, where by the more hydrophobic the actomyosin, the greater number of astaxanthin molecules can bind via hydrophobic bonds. These results led Henmi et al. (1990b) to suggest that in vivo the lipoproteins transporting carotenoids to the muscle are first bound to a receptor on the cell surface. They are then taken into the cell by endocytosis and transported by an unknown system that selects astaxanthin and/or canthaxanthin that conveys them to the myofibrillar protein that has the ability to bind these ketocarotenoids. All the evidence suggests that ketocarotenoids such as astaxanthin and canthaxanthin form hydrogen bonds with the actomyosin protein. Yet, Bjerkeng et al. (1990) suggests rainbow trout utilise astaxanthin up to 1.5 times more efficiently than canthaxanthin, in agreement with Storebakken et al. (1986) result. This may be due to the fact that each β-ionone ring of astaxanthin contains a carbonyl group and a hydroxyl group, both of which can form a hydrogen bond with the actomyosin. Whereas each β-ionone ring of canthaxanthin only contains a carbonyl group, so can only form one hydrogen bond with actomyosin, which is possibly less stable and perhaps more readily lost from the muscle.

1.6.4.1 Factors affecting muscle retention of astaxanthin

The concentration of astaxanthin within white muscle not only varies between species (Table 1.9) but also within a species, being dependant on age, weight, diet, sex, stage of maturation, and genetic predisposition (Storebakken and No, 1992; March and MacMillian, 1996)
Table 1. The concentration of astaxanthin measured in the muscle of wild salmonid species (Torrissen et al., 1989).

<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th>[Ax] $^a$ (mg kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Oncorhynchus nerka</em></td>
<td>Sockeye salmon</td>
<td>26 - 37</td>
</tr>
<tr>
<td><em>Oncorhynchus kisutch</em></td>
<td>Coho salmon</td>
<td>9 - 21</td>
</tr>
<tr>
<td><em>Oncorhynchus tschawytscha</em></td>
<td>Chinook salmon</td>
<td>8 - 9</td>
</tr>
<tr>
<td><em>Oncorhynchus gorbuscha</em></td>
<td>Pink salmon</td>
<td>4 - 6</td>
</tr>
<tr>
<td><em>Oncorhynchus keta</em></td>
<td>Chum salmon</td>
<td>3 - 8</td>
</tr>
<tr>
<td><em>Salmo salar</em></td>
<td>Atlantic salmon</td>
<td>3 - 11</td>
</tr>
<tr>
<td><em>Oncorhynchus mykiss</em></td>
<td>Rainbow trout</td>
<td>1 - 3</td>
</tr>
</tbody>
</table>

$^a$ Astaxanthin concentration; it must be noted that the concentrations shown are not definitive as it does not take into account the weight, sex, stage of maturation or the carotenoid content of the fish’s diet.

The age of the fish is important especially in salmonid aquaculture, where a European Union directive states that salmonids can not be fed synthetic carotenoids particularly, astaxanthin and canthaxanthin, before the age of six months (Gulbrandsen, pers comm.).

In a model proposed by Torrissen et al. (1989) the concentration of astaxanthin in the muscle of salmonids is linearly correlated to the weight of the fish. However, this model makes the assumption that the rate of growth decreases as their weight increases, which implies that the retention efficiency increases with weight. The weight of the fish and the concentration of astaxanthin in the muscle are both related to diet; if the fish find a diet unpalatable they will not eat it, resulting in poor or even retarded growth and poor pigmentation of the flesh (Refstie, pers comm.). If a diet is nutritionally unbalanced this will also result in poor pigmentation and growth. The sex and stage of maturation of a fish can be dealt with together, as described (section 1.6.3.1) during maturation females mobilise astaxanthin to their gonads and eggs and males to the skin (Table 1.10) (Bjerkeng et al., 1992).

Pre-maturation, the gonads of immature females constitute less than 1 % body weight (wet weight), but post maturation they can constitute up to 25 % body mass (Torrissen and Torrissen, 1985). This is because during the maturation process, that takes seven to eight months, the fish (both males and females) do not eat, instead relying on their reserves of body fat and muscle for sustenance (Torrissen and Torrissen, 1985). As yet it seems there is no genetic explanation for the innate ability of rainbow trout to utilise astaxanthin in terms of metabolism and uptake (Storebakken and No, 1992).
Table 1.10 The percentage astaxanthin content of several rainbow trout tissues measured pre and post maturation (Torrissen and Torrissen, 1985).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Immature(^a) (% Ax)(^b)</th>
<th>Mature male (% Ax)(^b)</th>
<th>Mature Female (% Ax)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flesh</td>
<td>55.6</td>
<td>3.5</td>
<td>24.2</td>
</tr>
<tr>
<td>Skin</td>
<td>40.3</td>
<td>88.0</td>
<td>33.9</td>
</tr>
<tr>
<td>Liver</td>
<td>4.1</td>
<td>4.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Gonad</td>
<td>0</td>
<td>4.1</td>
<td>38.4</td>
</tr>
<tr>
<td>Weight(^c) (kg)</td>
<td>3.6</td>
<td>2.2</td>
<td>3.5</td>
</tr>
</tbody>
</table>

\(^a\) Measurement carried out on both males and females; \(^b\) Percentage astaxanthin content; \(^c\) Mean weight of the fish analysed.

However, work carried out using immature diploid and triploid rainbow trout fed diets containing canthaxanthin showed no significant difference in flesh colour or muscle retention of this carotenoid (Torrissen et al., 1989).
1.7 Project aims

The overall aim of the following experiments was to test the viability of using *Haematococcus pluvialis* as a source of astaxanthin to pigment the flesh of salmonid species. The initial experiments (seven in total, Fig 1.13) were designed to study the commercial viability of *Haematococcus pluvialis* as a product in terms of its stability and application to commercial diets. These experiments also encompassed the development of two new methodologies required to test the use of *Haematococcus pluvialis* in the field (feed trials). The first to be developed was an efficient method to extract either synthetic or *Haematococcus pluvialis*-derived astaxanthin from cold pressed and commercially produced expanded diets. The second method developed was a novel use of image analysis to quantify the colour of fillets from rainbow trout that had been fed either synthetic or *Haematococcus pluvialis*-derived astaxanthin. The final experiments (five in total, Fig 1.14) were feed trials, all of which used rainbow trout (*Oncorhynchus mykiss*) as a model for farmed salmonid species. The majority of these feed trials (4.1, 4.2, 5.1 and 5.2) were designed to test the efficacy of *Haematococcus pluvialis* as a dietary pigment, either in cellular form (trials 4.1 and 4.2) or as various extracts (trials 5.1 and 5.2). The final experiment (trial 6.1) was designed to test *in vivo*, the efficacy of a novel additive (Corbinol) purported to increase the bioavailability of synthetic astaxanthin *in vitro*. It was envisaged that the sequence of experimental studies would serve to evaluate the potential of *Haematococcus pluvialis*-derived astaxanthin as an effective natural alternative to its synthetic counterpart. The central objective was to test the efficacy of the algal derived carotenoid under conditions applicable to commercial feed production and salmonid aquaculture practice.
Determine the carotenoid content of the basal diet ingredients (Experiment 3.1).

Develop an efficient method to extract the experimental astaxanthin (synthetic & natural) from test diets either cold pressed or extruded (Experiment 3.2).

Assess the most appropriate method of applying synthetic astaxanthin and Haematococcus pluvialis cells (containing astaxanthin) mixed with cod liver oil as top-coat to commercially made (expanded) diets (Experiment 3.3).

Analyse the effect of water erosion on experimental sources of astaxanthin applied to a commercial diet as a top-coat (Experiment 3.4). Quantify the partition coefficient of astaxanthin in a two phase system containing water and cod liver oil (Experiment 3.5).

Analyse the effect of emulated solar radiation on experimental sources of astaxanthin dissolved in organic solvent (as standards) and mixed with cod liver oil to be applied to a commercial diet as a top-coat (Experiment 3.6).

Develop a quantitative method of measuring the distribution of astaxanthin retained in the flesh of rainbow trout after consuming diets containing the experimental sources of astaxanthin (Experiment 3.7).

Fig. 1.13 Summary flow chart of the work (initial experiments) undertaken in the Chapter 3.
Assess the efficacy of processed (NatuRose™) and non processed (experimental) *Haematococcus pluvialis* cells against a synthetic commercial source of astaxanthin (Carophyll Pink™) presented to rainbow trout (*Oncorhynchus mykiss*) in cold pressed diets. Also identify a dose response (retention of astaxanthin within epaxial muscle) to astaxanthin dietary inclusion for NatuRose™ and Carophyll Pink™ (Trial 4.1).

Assess the efficacy of algal derived astaxanthin (NatuRose™ *H. pluvialis* cells) against synthetic (Carophyll Pink™) astaxanthin, presented to rainbow trout in commercially produced expanded diets. Also assess the stability of NatuRose™ and Carophyll Pink™ exposed to simulated commercial extruder conditions (Trial 4.2).

Assess the efficacy of an immobilised total carotenoid extract derived from NatuRose™ (*H. pluvialis*) against NatuRose™ algal cells and synthetic Carophyll Pink™, presented to rainbow trout top-coated on to a commercially produced expanded basal diet. (Trial 5.1).

Assess the efficacy of a mono-esterified astaxanthin extract derived from NatuRose™ (*H. pluvialis*), a di-esterified astaxanthin extract derived from NatuRose™ and total carotenoid extract derived from NatuRose™ against synthetic Carophyll Pink™, presented to rainbow trout top-coated on to a commercially produced expanded basal diet. (Trial 5.2).

Assess the effect of Corbinol (2-palmitoylaminopropionic acid) on the bioavailability of synthetic astaxanthin (Carophyll Pink™) and the residual carotenoids present in a commercially produced expanded diet when fed to rainbow trout (Trial 6.1).

Fig. 1.14 Summary flow chart of the work (final experiments / feed trials) undertaken in the Chapters 4, 5 and 6.
CHAPTER 2

General materials and methods
2.1 Introduction

The methodologies described in the following sections were used in all five feed trials (Chapters 4, 5 and 6), some were also used for the experiments carried out in chapter 3, where appropriate. However, those methods specific to a particular experiment or feed trial are described within the appropriate chapter.

2.2 Sampling

The rainbow trout (*Oncorhynchus mykiss*) used in all five feeding trials (4.1, 4.2, 5.1, 5.2 and 6.1) were all triploid females obtained from Hatchlands Trout Farm (Rattery, Devon, UK). The number of rainbow trout sampled in each experiment was dependent upon experimental design and the expected growth rate of the fish. This is due to the growth rate of rainbow trout being correlated to the energy content (lipid content) of their feed, with high oil content diets being regarded as providing increased rates of growth compared to low oil content diets. The diets used in the five feeding trials varied in oil content. Expected growth rates are dependent on stocking density and the initial weight of the fish at the start of the experiment. Stocking density (being relative to tank size and water volume) is critical because salmonids are social animals that have a structured social hierarchy within a group. If the number of fish within a group is too low (low stocking density) then the fish become territorial, fighting with others to defend their territory (Mork *et al.*, 1999). Hence, a decrease in social interaction is observed which results in a decrease in food intake, which in turn causes their rate of growth to decrease. If this situation continues the fish will become emaciated and eventually die. Likewise, if the stocking density is too high the fish will also fight as the competition for food increases, which again results in poor rates of growth. Initial weight is an important consideration, as the rate at which salmonids grow is not constant throughout their lives, as smaller fish (< 50 g) can attain higher growth rates than larger fish (> 150 g). Hence, initial weight (week 0) is used to predict the final weight of the fish on termination of the experiment. All these factors
have a direct effect on the number of time intervals at which fish were sampled and the length of time between each sampling. Thus, sampling for each trial is described in the appropriate chapter under the sectional heading 'experimental design'.

2.3 Experimental systems

Fish were held in twelve tanks (each measuring 1 m x 0.5 m x 1 m) with a water volume of 400 litres. Fish were acclimated to the culture conditions four weeks prior to the start of each experimental period (feed trial). Throughout the experimental period, the photoperiod was maintained at the spring equinox 12 h light : 12 h dark (by electronically controlled time switches) as described by Choubert et al. (1991). Water temperature was maintained at 15 ±0.5°C (JCW Refrigeration, Plymouth, UK), the optimal environmental temperature for rainbow trout growth and pigmentation (No and Storebakken, 1991). Water was re-circulated at a flow rate of 1 L³ hr⁻¹ per tank. This with the aid of compressed air being pumped into each tank via an air diffuser maintained the dissolved oxygen content at a level greater than 70% (> 7.0 mg L⁻¹) of saturation. The solubility of oxygen in fresh water is dependent upon temperature and partial pressure, which in this context refers to elevation (height above sea level). Combining the effects of water temperature (15°C) and the elevation of the aquarium where the feeding trials were executed, which is negligible (regarded as being at sea level: 0 elevation), relates to a maximum dissolved oxygen concentration of 10.07 mg L⁻¹ (Lawson, 1995). Water pH was maintained at pH 6.7 ±0.5 (the optimal environmental pH for rainbow trout growth and pigmentation) by buffering the system with sodium bicarbonate and calcium carbonate. Each parameter (water temperature, pH and dissolved oxygen concentration) was monitored daily and any deviation from the optimum was addressed accordingly. Maintaining the aforementioned water quality parameters at their optimum levels for all the feeding trials limited the number of variables between them and concomitantly eliminated several significant factors that could effect the growth rate of the fish used in the feeding trials (Aas, pers comm.).
2.4 Diets, feeding and rearing.

2.4.1 Diet preparation

Diets for each feed trial were prepared depending on the source and physical nature of the pigments being used. Thus, diet preparation for each trial is described in the appropriate chapter under the same section heading.

Three sources of pigment were used (in different combinations) in the five feeding trials. The first was a synthetic astaxanthin product called Carophyll Pink™ (Hoffmann-La Roche, Basel, Switzerland) that was used in all five feeding trials (4.1, 4.2, 5.1, 5.2 and 6.1) as a positive control. The Carophyll Pink™ product is composed of a gelatine-starch matrix, guaranteed to contain 8% (dry weight) free astaxanthin with a 1:3, cis:trans isomeric ratio, where the trans isomer contains a racemic mixture of the three stereo isomers (3S,3'S; 3R,3'S meso and 3R,3'R) in a ratio of 1:2:1 (Schiedt, 1988). The Carophyll Pink™ product is designed to form beadlets with a mean diameter of 0.3 mm having a violet-red hue with a bulk density of 0.56 - 0.65 kg L⁻¹, providing a particle number of 0.1 million g⁻¹ (Springate and Nickell, 2000). As stated in section 1.3.2 the chemical pathway to synthesise astaxanthin is a commercial secret, but the formation of its beadlets is the same as that of vitamins, spay drying under vacuum. The second source of astaxanthin was natural, contained within the cells of the microalga Haematococcus pluvialis, produced by Liverpool John Moores University. This was an experimental batch of Haematococcus pluvialis the cells of which had intact cell walls and a total carotenoid content of 1.00%. The media, culture conditions (used to produce this single batch of experimental H. pluvialis) and its carotenoid profile were all unknown. This product was presented as freeze dried cells that had a red hue and was only used in trial 4.1. The third source of astaxanthin was a natural product called NatuRose™ (Cyanotech Corporation, Kona, Hawaii, USA). The NatuRose™ product comprised of freeze dried Haematococcus pluvialis cells, the walls of which had been disrupted. The media, culture conditions (used
to produce the NatuRose™ product) and the method of cell wall disruption were all confidential (commercial secrets). *Haematococcus* meal contains approximately 70% astaxanthin monoesters, 10% astaxanthin diesters, 5% free astaxanthin and the remaining 15% consists of a mixture of β-carotene, lutein, canthaxanthin, zeaxanthin and other carotenoids (Lorenz, 1999). The carotenoid profile of the NatuRose™ product was stable the total carotenoid content varied from batch to batch, with a target of 1.5 % dry weight, which resulted in the cells having a red hue and a mean diameter of 45 μm. The NatuRose™ product or its extracts were used in all five feeding trials (4.1, 4.2, 5.1, 5.2 and 6.1).

2.4.2 Diet Storage

Diets for all trials were packed in airtight plastic containers and stored at -20°C (domestic freezer) until used. The use of a domestic freezer was deemed adequate, as the astaxanthin content of diets stored at -20°C has been demonstrated to remain stable for up to 9 months, only resulting in 24% degradation (Storebakken *et al.*, 1987).

2.4.3 Feeding Regime

To meet the daily feeding rate, fish were fed (by hand) twice daily and weighed bi-weekly in order to adjust the feed ration in accordance with the change in biomass. During the acclimation period, the fish were fed to satiation using a non-pigmented expanded feed (Standard Expanded 40, Trouw Aquaculture, Wincham, Cheshire, UK). During the experimental period (weeks 0-12 for trials 4.1, 4.2 and 6.1, weeks 0-10 for trial 5.1 and weeks 0-6 for trial 5.2) feed ration was calculated to chart levels as determined by Biomar Limited, to ensure good growth and minimal feed wastage. The mass of each diet fed to each group of fish, in each trial, was used to calculate the feed conversion ratio (FCR) and the specific growth rate (SGR) of the fish. FCR and SGR were calculated using equations 2.i (Whyte, *pers comm.*) and 2.ii (Bjerkeng *et al.*, 1997), respectively.
2.i \[ FCR = \frac{\text{Feed Intake (g)}}{\text{Live weight gain (g)}} \]

2.ii \[ SGR = \frac{(\ln \text{Final biomass} - \ln \text{Initial biomass}) \times 100}{\text{Number of days of feeding}} \]

2.5 Analytical Procedures

2.5.1 Soxtherm method for extraction of astaxanthin in feeds

Five grams of diet was added to a cellulose (porous) thimble (three replicates per diet were analysed). The thimble was inserted into a Soxtherm beaker containing 120 ml dichloromethane (DCM) (a volume which completely immersed the feed sample). DCM was chosen as it has a low boiling point (42°C) and it is the organic solvent in which astaxanthin has the greatest solubility. The beaker was placed onto a Soxtherm hot plate (150°C), and the DCM was allowed to recirculate for 25 min, via a condenser situated above the beaker. After this time approximately 50% of the DCM was evaporated (being collected in a storage reservoir), so the thimble and feed were no longer immersed in DCM. The remaining DCM was recirculated a second time for 20 min, after which the DCM was allowed to evaporate. The process was terminated when approximately 20 ml of DCM remained in the beaker. The remaining DCM containing the sample was made up to volume using a 100 ml volumetric flask. This was subsequently transferred to an amber bottle and stored at 4°C until analysed. Prior to analysis the presence of any moisture in the samples was extracted by adding 5 g anhydrous sodium sulphate (Na₂SO₄) and filtered using a 0.45 µm PTFE syringe tip filter (Phenomenex, UK). The development of the Soxtherm method is described and discussed in Chapter 3 (experiment 3.2). This method was used to extract the astaxanthin from those diets containing Carophyll Pink™ (free astaxanthin) and the total carotenoid content from those diets containing NatuRose™ (H. pluvialis) in Chapters 3 (experiment 3.3), 4 (trials 4.1 and 4.2) and 6 (trial 6.1).
2.5.2 Enzyme method for extraction of astaxanthin in feeds

Approximately 50 g of pelleted diet was weighed into a coffee grinder (Braun, Germany) and homogenised for 30 s. A 4 g (+ 0.1 g) aliquot of the ground feed was placed in a 50 ml polypropylene conical bottom centrifuge tube to which 100 mg Maxatase (Genencor International, Lieden, Holland) and 19 ml distilled water was added. Each sample was vortexed (Whirlimixer, Fisons, UK) for 15 s and placed in a heated sonicating water bath (Sonimatic, Langford Ultrasonics, West Midlands, UK) where they were sonicated and incubated at 60°C for 35 min. After incubation each sample was placed in the dark for 60 min, allowing them to cool to ambient temperature. Then 15 ml of methanol containing 500 mg L\(^{-1}\) 2,6-di-t-butyl-p-cresol (BHT, as an antioxidant) and 12 ml of dichloromethane was added to each sample and vortexed for 15 s, then incubated in the dark for 10 min at room temperature (20 °C). After incubation each sample was vortexed for a further 15 s and then centrifuged (Mistral 3000, Fisons, UK) for 10 min (3000 rpm), at 16 °C. After centrifugation the hypophase of each sample was removed and a 3 ml aliquot pipetted into an amber vial and the solvent evaporated (blown down) under a gentle stream of nitrogen. Samples were re-suspended in 2 ml hexane, carefully decanted into sealable eppendorf tubes, and then centrifuged (Micro-Centaur MSB010.CX2.5, Sanyo Gallenkamp PLC, Uxbridge, UK) at 13,000 rpm for 5 min to precipitate any insoluble lipid prior to HPLC quantification. This method was used to extract the astaxanthin from those diets containing Carophyll Pink™ (free astaxanthin) and the total carotenoid content from those diets containing NatuRose™ (H. pluvialis) in Chapter 5 (trials 5.1 and 5.2).

2.5.3 Fillet colour analysis

The perceived colour of the fillets was observed on fresh unskinned (right hand) fillets using the naked eye. Fillet colour analysis was carried out using the Roche SalmoFan™; a method adapted from the Roche Colour Card system described by Skrede et al. (1990).
The fillets were laid on a light grey coloured bench (that reduced light interference) and observed under constant light conditions. An artificial light source (T8 standard Halophosphate fluorescent tube, GE Lighting, Lancashire, UK) that produced a colour temperature of 6300 K and had a colour rendering index (Ra) > 90 was used in accordance with Roche operating conditions (Roche, 2000). The intensity of pigmentation was scored on a scale from 20 to 34 (arbitrary units). Each fillet was scored by each of the three members of a sensory panel, of which each member was deemed to have normal colour vision after successfully completing an Ishihara test (colour blindness test) (Ishihara, 1971).

2.5.4 Tissue preparation
At each sampling time (section 2.2) all the sampled fish were anaesthetised (individually) with phenoxy-ethanol 200 mg L\(^{-1}\) (Sigma Chemical Co., Poole, Dorset, UK) and weighed. The fish were then slaughtered by a blow to the head, cut open and their viscera excised and discarded. The fish were filleted, one of which was used as the source of white muscle to determine carotenoid content and the other was used for the Roche colour score (SalmoFan\textsuperscript{TM}) assessment.

2.5.5 Analysis of astaxanthin in white muscle
2.5.5.1 Sample preparation
Two grams of epaxial white muscle was excised from under the dorsal fin (Fig. 2.1) of the left hand fillet of each fish, carefully avoiding red muscle, which has been demonstrated to contain less astaxanthin than white muscle (Lagocki, 1995). The area under the dorsal fin is the widest part of the fillet to which the consumer’s eye is naturally drawn, making it the most desirable area of the fillet and has been designated by the Norwegian quality council as the Norwegian quality cut (NQC) (Nickell and Bromage, 1998a).
Fig. 2.1 The shaded area represents the region of the left hand fillet from which the sample of epaxial white muscle was excised and consequently analysed to determine the astaxanthin concentration of the fillet.
The NQC is regarded as the prime cut for steaks and provides a good representation of the astaxanthin concentration in the whole fillet (Lagocki, Unpublished data). The muscle sample (2 g from each fish sampled) was accurately weighed into a 25 ml centrifuge tube. Distilled water and methanol containing 500 mg L\(^{-1}\) BHT was added to each sample in a ratio of 1 g tissue : 1 ml distilled water : 1 ml methanol. The samples were homogenised (Silverson, Chesham, Buckinghamshire, UK) for 30 s, and then chloroform was added in a ratio of 3 ml chloroform : 1 g tissue and homogenised for a further 30 s. All samples were incubated in the dark for 10 min at room temperature (20°C), homogenised for a further 30 s and then centrifuged (Mistral 3000, Fisons, UK) for 10 min at 3000 rpm, at 16°C. After centrifugation a 3 ml aliquot of hypophase was filtered using PTFE syringe tip filters (0.45 μm; Phenomenex, UK), directly into amber sample vials and immediately sealed. The samples were stored at -20°C until subsequent analysis. Prior to analysis the presence of any moisture in the samples was extracted by adding 0.5 g anhydrous sodium sulphate (Na\(_2\)SO\(_4\)) and filtered using a 0.45 μm PTFE syringe tip filter (Phenomenex, UK).

2.5.5.2 Chromatographic conditions
The samples were analysed isocratically on a Spherisorb S5CN nitrile column (PhaseSep, Queensferry, Clywyd, UK) (length 250 mm; internal diameter 4.6 mm; particle size 5 μm), using 20% acetone in hexane as the mobile phase. All solvents were HPLC grade and degassed. The flow was 1.5 ml min\(^{-1}\). These conditions provided adequate separation of the \(cis\) and \(trans\) isomers of astaxanthin extracted from flesh and diet samples containing free astaxanthin (Carophyll Pink™) (Fig. 2.2a). It also provided adequate separation of astaxanthin mono and di-esters in diets containing \(Haematococcus pluvialis\) (Fig. 2.2b).
Fig. 2.2 Typical HPLC chromatograms for a. the carotenoid profile for rainbow trout (*Oncorhynchus mykiss*) epaxial white muscle (Lagocki, 1995); b. the carotenoid profile for a diet containing *Haematococcus pluvialis* (Bowen, unpublished data).
The astaxanthin content of diets containing NatuRose™ and Liverpool John Moores experimental *Haematococcus pluvialis* is expressed as total carotenoid content, as mono and di-esterified astaxanthin and the other carotenoids present in *Haematococcus* may have an effect on pigmenting the white muscle tissue of rainbow trout. The HPLC used was a Kontron Instruments HPLC system, 332 detector, 325 Pump, degasser deg-103 (Kontron Instruments, Watford, Hertfordshire, UK). The detection wavelength was set at 470 nm and peak areas were integrated using a Kontron PC Integration Pack (Kontron Instruments, Watford, Hertfordshire, UK).

2.6 Standards

In order to quantify the samples, an external standard method was employed. Standards of known concentration (approximately 3 mg L\(^{-1}\)) were made from crystalline all-E-astaxanthin (Sigma Chemicals Co., Poole, Dorset, UK) dissolved in 4.5 % chloroform, in hexane. The concentration of the standard solution was measured each time samples were analysed by spectrophotometry.

2.6.1 Quantification of standards and samples by spectrophotometry

The concentration of astaxanthin standards and diets and samples containing *Haematococcus pluvialis* were quantified using a spectrophotometer (PU8720, Philips, UK) and either equations 2.iii (Lagocki, unpublished data) and/or 2.iv (Bjerkeng and Liaaen-Jensen, 1990). Combining the fact that the carotenoid profile of *Haematococcus pluvialis* is relatively complex (Fig. 2.2b) with the lack of appropriate standards, dictated that the carotenoid concentration of samples containing *H. pluvialis* (cells or extracts) were determined spectrophotometrically and expressed as total carotenoid concentration. Each sample was scanned by the spectrophotometer from 350-600 nm and its spectrum printed (Fig. 2.3). As Fig. 2.3 shows the residual absorption is relatively high, but did not have to be taken into consideration when calculating the concentration of the standard. However,
the residual absorption of a solution containing either individual carotenoid extracts or the total carotenoid content extracted from *H. pluvialis* cells was taken into consideration when calculating the concentration, by correcting the total absorbance. Equation 2.iii was used to calculate the corrected absorbance (represented by the double ended arrow in Fig. 2.3) from the total absorbance (measured at $\lambda_{\text{max}}$) and the residual absorbance.

2.iii Corrected Absorbance = Total Absorbance − Residual Absorbance

The corrected absorbance calculated in equation 2.iii was then used in equation 2.iv to calculate the carotenoid concentration of the solution.

2.iv Carotenoid concentration (mg L$^{-1}$) = \[
\frac{10 \times \text{Abs} \times \text{Vol} \times \text{Dil}}{E \ 1\%, \ 1\ \text{cm}}
\]

Where:

10 = Constant used to correct the formation of

Abs = Corrected absorbance

Vol = Volume of the extract

Dil = Dilution

$E \ 1\%, \ 1\ \text{cm}$ = Extinction coefficient of a 1% solution in a 1 cm cuvette of a specified solvent (diluent).

The extinction coefficients and the relative absorbance maxima for crystalline astaxanthin (standard), *Haematococcus pluvialis* and zeaxanthin are listed in table 2.1.

Table 2.1 The extinction coefficients ($E \ 1\%, \ 1\ \text{cm}$) and absorbance maxima ($\lambda_{\text{max}}$) for several carotenoids used in the experimental Chapters (3-6).

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>$E \ 1%, \ 1\ \text{cm}$</th>
<th>$\lambda_{\text{max}}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astaxanthin$^a$</td>
<td>2100</td>
<td>470</td>
<td>Bjerkeng <em>et al.</em>, 1997</td>
</tr>
<tr>
<td>Zeaxanthin$^b$</td>
<td>2340</td>
<td>478</td>
<td>Manz, 1983</td>
</tr>
<tr>
<td><em>H. pluvialis</em> extract$^c$</td>
<td>2100</td>
<td>470</td>
<td>Young, <em>pers comm.</em></td>
</tr>
</tbody>
</table>

$^a$ All trans-crystalline (Hoffmann-La Roche, Basel, Switzerland); $^b$ Required for experiment 3.1; $^c$ Total carotenoid content (NatuRose™, Cyanotech, Kona, Hawaii, USA).
Fig. 2.3 The uv/vis spectrum of all-trans-crystalline astaxanthin dissolved in 4.5% chloroform in hexane (Bjerkeng and Liaane-Jensen, 1990).
2.7 Carotenoid Retention

The percentage retention of astaxanthin (in epaxial muscle) throughout the experimental period was calculated (equation 2.v) as net apparent retention (NAR). NAR is expressed as a function of feed intake and dietary astaxanthin concentration (being measured at strategic points in order to account for stability related losses), biomass gain and epaxial muscle astaxanthin concentration. The constant of 0.6 is used to compensate for the muscle mass, where the remaining 40% biomass accounts for the fishes head, skeleton, viscera and skin (Bjerkeng et al., 1997). The calculation was applied to the growth that occurred in the experimental period for each feed trial.

\[
NAR(\%) = \frac{(0.6 \times Wt_F \times [Ax]_F) - (0.6 \times Wt_i \times [Ax]_i)}{FI \times [Ax]_D} \times 100
\]

Where:

- \( Wt_F \) = Mean final live weight
- \([Ax]_F\) = Mean final muscle astaxanthin concentration
- \( Wt_i \) = Mean initial live weight
- \([Ax]_i\) = Mean initial muscle astaxanthin concentration
- \( FI \) = Total feed intake per fish in an individual tank
- \([Ax]_D\) = Mean diet astaxanthin concentration

2.8. Statistical analysis

Statistical analyses were carried out on the data for all the feed trials using Minitab 12. The data for each trial was subjected to a two-way analysis of variance, using a two factor nested design with a significance level of \( P<0.05 \). The means for dietary treatments were ranked by Duncans Multiple Range Test.
Two factor nested design (Model I):

\[ y_{ijk} = \mu + D_i + T_{(ij)} + E_{(ij)k} \]

Where:

- \( i = 1, \ldots, I \)
- \( j = 1, \ldots, J \)
- \( k = 1, \ldots, n_{(ij)} \)

- \( y_{ijk} \): the \( k \)th response of the \( j \)th level within the \( i \)th main group.
- \( \mu \): overall mean.
- \( D_i \): mean effect due to the \( i \)th diet.
- \( T_{(ij)} \): mean effect due to the \( j \)th tank within the \( i \)th diet.
- \( E_{(ij)k} \): error associated with the \( j \)th tank within the \( i \)th diet.

Restriction  \( \sum N_i R_i = 0 \)

Where:

- \( N_i = \sum n_{(ij)} \) and  \( \sum \sum n_{(ij)} T_{(ij)} = 0 \)

Assumption  \( E_{(ij)k} \sim N(0, \sigma^2) \) and independent.

Results from the experiments showed no significant difference between tanks receiving the same diet; i.e. none of the variance of astaxanthin concentration in the fish epaxial muscle could be explained by the factor “Tanks”. Thus from this point on all fish receiving the same diet were assumed to come from the same block (Tank) enabling the model to be simplified to Model II
One way analysis of variance to see the effect of diet on astaxanthin concentration retained within flesh and perceived colour (Roche Colour Score) of the flesh (Model II):

\[ y_{ij} = \mu + D_i + E_{ij} \]

Where:

\( i = 1, \ldots, I \)
\( j = 1, \ldots, J \)

\( y_{ij} \) = the \( j \)th response of the \( i \)th diet.
\( \mu \) = overall mean.
\( D_i \) = mean effect due to the \( i \)th diet.
\( E_{ij} \) = error associated with the \( j \)th response of the \( i \)th diet.

Using Model II tested the null hypothesis

\( H_0 = \) No mean effect due to diet
\( H_1 = \) There is a mean effect due to diet

Restriction \( \sum D_i = 0 \)

Where:

\( N_i = \sum n(i)j \) and \( \sum \sum n(i)j \epsilon(i)j = 0 \)

Assumption \( E_{ij} \sim N(0, \sigma^2) \sigma^2 \) is constant.

A test size of 0.05 indicated that there were differences between the diets. Therefore the \( H_0 \) was rejected in favour of \( H_1 \). Follow up analysis was carried out to find the least significant difference (LSD) using Duncans Multiple Range Test.

The assumptions about the error structure of the model that was made were justified by carrying out a scatter plot (normal probability plot) of the residuals and a histogram of the residuals, both of which proved to be random around \( \emptyset \) (Fig 2.4).
Fig 2.4 The efficacy of the statistical model, tested by plotting a histogram of the residuals and a normal probability plot of residuals, where the response was the concentration of astaxanthin (Ax) in the white muscle of those fish sampled at the final sampling session.
A linear regression test was then carried out for weight (independent variable) against muscle astaxanthin concentration (dependent variable) and repeated for weight against Roche Colour Score, to identify possible outliers. This was followed by the Kruskal Wallis Test that uses the median (not the mean) to eradicate outliers. The Kruskal Wallis Test provided very small p values showing Model II was applicable for weight. Finally the model was refitted without the outliers indicated by the first linear regressions. The results from these analyses were effectively the same as those including the outliers.
CHAPTER 3

Method development and product stability
3.1 Introduction

The seven experiments that constitute the present chapter were designed to study the viability of using cellular *Haematococcus pluvialis* as a commercial product (a natural source of astaxanthin) for application to commercially-produced expanded diets. Each of the following experiments is linked to one or more of the other experiments. However, in terms of continuity and ease of understanding, each of the seven experiments is treated (introduced, the data analysed and discussed) individually.

3.2 Aims

The main aims of the following experiments were:

1. Analyse the carotenoid content of the ingredients used to formulate the basal diet and to evaluate any potential effect the ingredients may have on the overall pigmentation of the rainbow trout flesh (Experiment 3.1).

2. Develop a simple rapid accurate method to extract astaxanthin (from either a natural [NatuRose™, *Haematococcus pluvialis*] or a synthetic source [Carophyll Pink™]) from cold pressed and commercially produced diets with the pigment being added either pre or post extrusion. Also to determine the stability of Carophyll Pink™ and NatuRose™ astaxanthin during the production of cold pressed feeds (Experiment 3.2).

3. Assess the most appropriate method of adding NatuRose™ and Carophyll Pink™ (astaxanthin) to commercially produced diets. Using rheology to evaluate the affect of applied shear on the flow behaviour of cod liver oil containing natural and synthetic forms of astaxanthin and the retention of astaxanthin on to extruded pellets (Experiment 3.3).
4. Analyse the effect of water erosion on the stability of NatuRose™ and Carophyll Pink™ astaxanthin added to commercially produced diets as a top-coat, under simulated farm conditions (Experiment 3.4).

5. Quantify the partition coefficient of synthetic astaxanthin (Carophyll Pink™) in a two phase system containing distilled water and cod liver oil (Experiment 3.5).

6. Analyse the stability of astaxanthin (from both NatuRose™ and Carophyll Pink™) when exposed to emulated solar radiation (sunlight); as standards (dissolved in organic solvents) and the form (mixed with cod liver oil) used to top-coat commercially produced diets (Experiment 3.6).

7. Develop a method to quantitatively measure the colour and optically map its distribution in rainbow trout fillets (following the feeding of pigmented diets) using image analysis (Experiment 3.7)
Experiment 3.1 Determination of the carotenoid profile of the basal diet ingredients.

3.1.1 Introduction

As described in section 1.5.1, salmonids are unable to synthesise astaxanthin de novo, so this is added to their feed as a supplement in a bid to enhance the aesthetic appeal (colour) of their flesh to the consumer. Astaxanthin is used instead of other carotenoids, as it provides the characteristic pink/red hue associated with the flesh of wild salmonids. Astaxanthin also has a higher flesh retention quotient (section 2.7, net apparent retention) than other carotenoids (Gulbrandsen, pers comm.).

Latscha (1990) proposes carotenoids to be some of the most abundant compounds found in nature, some of which have the ability to pigment salmonid flesh. This suggests that some of the ingredients used to make the basal diet, for the subsequent feeding trials (Chapters 4-6) may contain one or more carotenoids that could potentially pigment the flesh of the experimental fish [rainbow trout (Oncorhynchus mykiss)] used in these investigations. Hence, analysis of the basal diet ingredients to elucidate their carotenoid profiles seems prudent in order to ascertain any potential effect the ingredients may have on the overall pigmentation of the rainbow trout flesh. Also, it should be ascertained whether the inclusion rate of the experimental astaxanthin sources has to be modified to compensate for the presence of any such flesh-pigmenting carotenoids, so enabling the target inclusion level to be achieved.

3.1.2 Materials and methods

3.1.2.1 Experimental designs

The carotenoid content of extruded wheat (Pascoe’s Ltd., Bridgewater, Somerset, UK) fishmeal (LT94, Norsildmel, Bergen, Norway), cod liver oil (Seven Seas, Marfleet, Hull, UK), vitamin premix (Biomar Ltd., Grangemouth, UK) and mineral premix (Biomar Ltd.,
Grangemouth, UK) was determined by HPLC (section 2.5.4.2) against external standards (section 2.6) and quantified spectrophotometrically (section 2.6.1). The analysis of each ingredient was carried out in triplicate.

3.1.2.2 Carotenoid extraction method

The astaxanthin concentration of extruded wheat, fishmeal, vitamin and mineral premix were determined by weighing 2 g samples of each ingredient into a 20 ml glass vial. This was adapted for cod liver oil, where a 2 ml sample was pipetted into a 20 ml glass vial. 2 ml distilled water and 2 ml methanol containing an antioxidant 500 ppm BHT (2,6-tert-butyl-4-methy1phenol; Sigma Chemical Co., Dorset, UK) were added to each sample. All samples were homogenised (Silverson, Chesham, Buckinghamshire, UK) for 15 s. 6 ml dichloromethane was added to the samples and homogenised for a further 15 s. The samples were incubated in the dark for 10 min at room temperature (20°C), homogenised again for 15 s and then centrifuged (Mistral 3000, Fisons, UK) for 10 min at 3000 rpm, at 16°C. After centrifugation a 3 ml aliquot of hypophase was filtered using PTFE syringe tip filters (0.45 μm; Phenomenex, UK), directly into amber sample vials and immediately sealed. The samples were stored at -20°C until analysed. Prior to analysis the presence of any moisture was extracted by adding 0.5 g of anhydrous sodium sulphate (Na₂SO₄) and filtered using PTFE syringe tip filters (0.45 μm; Phenomenex, UK).

3.1.2.3 Statistical analysis

The statistical analyses were carried out using the Statgraphics™ statistics computer software. The data was subjected to a (n=3) one way analysis of variance, using a factor nested design with a significance level of P<0.05. The means for different treatments were ranked by Duncan's Multiple Range Test.
3.1.3 Results

Only two of the five ingredients of the basal diet contained carotenoids (Table 3.1)

Table 3.1 The total carotenoid (TC) content of the basal diet ingredients; n=3, ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>[TC] (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fishmeal (LT94) a</td>
<td>ND</td>
</tr>
<tr>
<td>Cod liver oil b</td>
<td>ND</td>
</tr>
<tr>
<td>Mineral premix c</td>
<td>ND</td>
</tr>
<tr>
<td>Vitamin premix c</td>
<td>0.29 ±0.03</td>
</tr>
<tr>
<td>Wheat (Extruded) d</td>
<td>1.08 ±0.09</td>
</tr>
</tbody>
</table>

a Norsildmel, Bergen, Norway; b Seven Seas, Marfleet, Hull, UK; c Biomar Ltd., Grangemouth, UK; d Pascoe's Ltd., Bridgewater, Somerset, UK; e Not Detected.

The results showed that wheat contained a significantly (P<0.01) higher carotenoid concentration compared to the vitamin premix (1.08 and 0.29 mg kg⁻¹ respectively). In both ingredients the only carotenoid identified was zeaxanthin (Whyte, pers comm.). No residual carotenoids were detected in the fishmeal, cod liver oil or the mineral premix.

3.1.4 Discussion

An evaluation of the ingredients of the basal diet showed that extruded wheat and vitamin premix contained the carotenoid zeaxanthin. However, zeaxanthin has a diminutive effect on white muscle or skin pigmentation of rainbow trout as it is a metabolite of astaxanthin and is poorly absorbed in the gut (Schiedt, 1988; Bjerkeng, 1992). The carotenoid profile of a commercial non-pigmented feed containing the same extruded wheat and vitamin premix was also analysed. This feed contained 0.17 mg kg⁻¹ zeaxanthin. Further tests included the flesh of five rainbow trout that had received this feed for several months prior to being analysed. A visual observation of the fillets, showed a white appearance. Analysis of flesh samples by HPLC, resulted in no carotenoids being detected. So, the target dietary
Inclusion levels for astaxanthin did not have to be adjusted to compensate for the carotenoid content of the basal diet.
3.2.1 Introduction

The addition of astaxanthin to experimental diets is relatively easy regardless of source (natural or synthetic), and can be achieved by using one of two methods. The first method involves mixing the pigment into the raw ingredients prior to extrusion. The second method entails adding the pigment as a top-coat to the feed pellets post extrusion. Each method has its advantages and disadvantages, which will become apparent, and will be discussed later. Nevertheless, which ever method is adopted, it is important to accurately measure the astaxanthin content of the diet before presentation to the fish, so the stability of the different astaxanthin sources can be monitored throughout the experimental period and the net apparent retention can be calculated. The accepted method for extracting astaxanthin from commercial and experimental diets was developed by the technical division at Hoffmann-La Roche (Basel, Switzerland), the world's leading manufacturer of stable synthetic astaxanthin (Carophyll Pink™). However, this method (termed the Roche method) is costly in terms of time, as it entails approximately twenty steps and uses relatively large volumes of several toxic organic solvents including diethyl ether and methanol. The Roche method also utilises several pieces of specialised equipment not readily found in biochemistry laboratories, such as a temperature control sonicating water bath and a rotary evaporator. The method initially states 10 g of pelleted diet is weighed into a 250 ml conical flask to which 100 ml distilled water and 100 mg Maxatase™ (Genecor International, Lieden, Holland) are added and then homogenised. After homogenisation, the flasks are transferred to the sonicating water bath (incubated for 30 min at 50°C), being employed to aid the task of the immobilised enzyme (Maxatase™) the action of which releases the astaxanthin into solution. After incubation the majority of the lipid-soluble astaxanthin floats on the surface of the aqueous solution. The method then
states, that the flask should be shaken vigorously and a $10$ g aliquot of the emulsion be quickly poured into another beaker. This stage raises concern, as the definition of vigorously is not elaborated upon. One has to make the assumption that a homogenous emulsion will be formed after agitation and the $10$ g aliquot is representative of the mixture. This step seems obvious and easy to achieve, but in practice obtaining a homogenous emulsion by shaking is difficult. As Lagocki (unpublished data) demonstrated, the distribution of astaxanthin in the emulsion is highly variable using the aforementioned method. Hence, the problems associated with the Roche method led to the development of a new method of extracting astaxanthin from pelleted diets. This new extraction method was developed from one of the fundamental chemical properties of astaxanthin i.e. it is lipid soluble. The traditional method for quantification of total lipid content of solid samples (the Soxhlet method) was adapted to extract the carotenoids from the experimental feed pellets. The method should work efficiently for extracting both synthetic and natural sources of astaxanthin, and from this point on shall be referred to as the "Soxtherm method".

3.2.2 Materials and Methods

3.2.2.1 Experimental design

Eleven experimental diets containing varying quantities of synthetic (Carophyll Pink™) and natural (NatuRose™, *Haematococcus pluvialis*) astaxanthin were made in $0.5$ kg batches. Diets 1 - 6 contained $0$, $20$, $40$, $60$, $80$, $100$ mg kg$^{-1}$ Carophyll Pink™ (Hoffmann-La Roche, Basel, Switzerland), respectively, whereas diets 7 - 11 contained $20$, $40$, $60$, $80$, $100$ mg kg$^{-1}$ NatuRose™ (Cyanotech Corporation, Hawaii, USA) *Haematococcus*, respectively. The astaxanthin content of each diet was analysed from triplicate samples taken at three stages during production: 1. after the mixing of the raw ingredients; 2. After cold pelleting; 3. after drying in an oven at $45^\circ$C for 72 hours. The remaining portion of
the two cold pressed diets (containing Carophyll Pink™ and NatuRose™) targeting 60 mg kg⁻¹ astaxanthin were then used to optimise the time required for both evaporation steps required for the Soxtherm protocol. Up to 70 min was allowed for the first evaporation step, increasing in 5 min increments, and up to 40 min for the second evaporation step, again increasing in 5 min increments. Optimisation of this method was repeated using an extruded (commercially produced) diet, top coated with either Carophyll Pink™ or NatuRose™. The efficacy of this method was also analysed for extracting astaxanthin from the pigmented flesh of rainbow trout. Six rainbow trout (mean weight 300 g) were purchased from a fish farm (Hatchlands Trout Farm, Devon, UK) that had received a commercial pigmented (50 mg kg⁻¹ Carophyll Pink™) diet (Trouw Expanded 40, Cheshire, UK) for 12 weeks prior to analysis. The astaxanthin content of the flesh samples and those diets (2-6) containing Carophyll Pink™ were quantified by HPLC (section 2.5.5) against external standards (section 2.6). The total carotenoid content of the unpigmented diet (0) and those diets (7-11) containing NatuRose™ were quantified spectrophotometrically (section 2.6.1). Each diet and fish was analysed in triplicate, the results of which were subject to statistical analysis (section 3.1.2.3).

3.2.2.2 Diet preparation

3.2.2.2.1 Cold pressed diets

The composition of the basal diet (Table 3.2) was designed to promote optimal growth. Each diet was produced in 0.5 kg batches (University of Plymouth) mixed using a commercial (3 kg capacity) blender (Hobart A-120, London, UK) and cold pressed using 3 mm die (Hobart A-120, London, UK). The synthetic astaxanthin (Carophyll Pink™) contained 8% (dry weight) astaxanthin immobilised in a gelatine-starch matrix that was pre-treated, by adding 5 ml distilled water to varying quantities (Table 3.3) of the Carophyll Pink™ product for diets 2 – 6. The Carophyll Pink™ solution was heated to
50°C using a temperature controlled water bath (Sonimatic, Langford Ultrasonics, West Midlands, UK) in order to dissolve its gelatine-starch matrix.

Table 3.2 Cold pressed basal diet formulation.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Inclusion (g kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT-94 Fish-meal a</td>
<td>540.0</td>
</tr>
<tr>
<td>Wheat-meal b</td>
<td>185.3</td>
</tr>
<tr>
<td>Soybean (Solvent Extracted) c</td>
<td>150.0</td>
</tr>
<tr>
<td>Fish Oil d</td>
<td>110.0</td>
</tr>
<tr>
<td>Vitamin Premix d</td>
<td>5.0</td>
</tr>
<tr>
<td>Mineral Premix d</td>
<td>4.0</td>
</tr>
<tr>
<td>Chromium Oxide e</td>
<td>2.5</td>
</tr>
<tr>
<td>Choline Chloride e</td>
<td>2.0</td>
</tr>
<tr>
<td>Yttrium Oxide e</td>
<td>1.0</td>
</tr>
<tr>
<td>Inositol e</td>
<td>0.2</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
</tr>
</tbody>
</table>

a Norsildmel, Bergen, Norway; b Pascoe’s Ltd., Bridgewater, Somerset, UK (Astaxanthin was added at the expense of extruded wheat); c Cherwell Valley Silos Ltd. Twyford, Oxon, UK; d Biomar Ltd., Grangemouth, UK; e Sigma Chemical Co., Poole, Dorset, UK; f Inert marker.

The Carophyll Pink™ solution was then added to the feed mix prior to being cold pressed.

Diets 7 - 11 containing *Haematococcus pluvialis* derived astaxanthin was mixed into the appropriate diets in its native form prior to cold pressing. The commercial source of *H. pluvialis* (NatuRose™) added to diets 7 - 11 had a total carotenoid content of 1.5% (dry weight) and the varying quantities added to each diet is summarised in Table 3.3.

Table 3.3 Quantities of the two astaxanthin (Ax) products Carophyll Pink™ and NatuRose™ required to pigment 0.5 kg cold pressed diets 1-11.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Ax Source</th>
<th>Target [Ax] (mg kg⁻¹)</th>
<th>Required weight of Ax (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N/A a</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>CP b</td>
<td>20</td>
<td>0.125</td>
</tr>
<tr>
<td>3</td>
<td>CP</td>
<td>40</td>
<td>0.250</td>
</tr>
<tr>
<td>4</td>
<td>CP</td>
<td>60</td>
<td>0.375</td>
</tr>
<tr>
<td>5</td>
<td>CP</td>
<td>80</td>
<td>0.500</td>
</tr>
<tr>
<td>6</td>
<td>CP</td>
<td>100</td>
<td>0.625</td>
</tr>
<tr>
<td>7</td>
<td>NR c</td>
<td>20</td>
<td>0.667</td>
</tr>
<tr>
<td>8</td>
<td>NR</td>
<td>40</td>
<td>1.333</td>
</tr>
<tr>
<td>9</td>
<td>NR</td>
<td>60</td>
<td>2.000</td>
</tr>
<tr>
<td>10</td>
<td>NR</td>
<td>80</td>
<td>2.667</td>
</tr>
<tr>
<td>11</td>
<td>NR</td>
<td>100</td>
<td>3.333</td>
</tr>
</tbody>
</table>

a Not applicable; b Carophyll Pink™ (Hoffmann-La Roche, Basel, Switzerland); c NatuRose™ (Cyanotech, Kona, Hawaii, USA).
3.2.2.2 Extruded diets

The composition of the unpigmented basal diet (Table 3.4) was designed to promote optimal growth, as determined by Biomar Ltd. The basal diet was produced in one 150 kg batch (Biomar Ltd., Brande, Denmark) and top coated with the appropriate pigment in 0.5 kg batches (University of Plymouth, Devon, UK). Diet 12 containing synthetic astaxanthin (60 mg kg\(^{-1}\) Carophyll Pink™) was pre-treated by heating in order to dissolve its gelatine matrix. 0.375 g Carophyll Pink™ (8 % astaxanthin content) was added to 5 ml distilled water and heated to 50°C to form an astaxanthin solution. The astaxanthin solution was then mixed with 60 g cod liver oil (Seven Seas, Hull, UK) to form a suitable astaxanthin emulsion. Diet 13 containing *H. pluvialis* (60 mg kg\(^{-1}\) NatuRose™) derived astaxanthin had a total carotenoid content of 1.5% (dry weight). 2g NatuRose™ (in its native form) was mixed into the 60 g cod liver oil, producing an astaxanthin suspension. The astaxanthin solution (diet 12) and astaxanthin suspension (diet 13) were slowly (10 minute period) poured over the basal diet (440 g for both diets) concomitantly being agitated by a mixing paddle (Hobart A-120, London, UK); after addition, the pigment and the basal diet were left mixing for 15 min to ensure homogeneity.

Table 3.4 Extruded basal diet formulation.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Inclusion (g kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT-94 Fish-meal(^a)</td>
<td>380.0</td>
</tr>
<tr>
<td>Norsemink Fish-meal(^a)</td>
<td>170.0</td>
</tr>
<tr>
<td>Wheat Gluten(^b)</td>
<td>50.0</td>
</tr>
<tr>
<td>Wheat-meal(^b)</td>
<td>196.5</td>
</tr>
<tr>
<td>Fish Oil(^b)</td>
<td>190.0</td>
</tr>
<tr>
<td>Vitamin Premix(^b)</td>
<td>7.5</td>
</tr>
<tr>
<td>Mineral Premix(^b)</td>
<td>6.0</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
</tr>
</tbody>
</table>

\(^a\) Norsildmel, Bergen, Norway; \(^b\) Biomar, Ltd., Grangemouth, UK.

3.2.2.3 Soxtherm method for extraction of astaxanthin in feeds

Five grams of diet (or rainbow trout flesh) was added to a cellulose (porous) thimble (three replicates per diet and flesh were analysed). The thimble was inserted into a Soxtherm
beaker containing 120 ml dichloromethane (DCM) (a volume which completely immersed the feed sample). DCM was chosen as it has a low boiling point (42°C) and it is the organic solvent in which astaxanthin has the greatest solubility (30 g L⁻¹) (Johnson and An, 1991). The beaker was placed onto a Soxtherm hot plate (150°C), and the DCM was allowed to recirculate for up to 70 min, via a condenser situated above the beaker. After this time approximately 50 % of the DCM was evaporated (being collected in a storage reservoir), so the thimble and feed were no longer immersed in DCM. The remaining DCM was recirculated a second time for up to 40 min, after which the DCM was allowed to evaporate. The process was terminated when approximately 20 ml of DCM remained in the beaker. The remaining DCM containing the sample was made up to volume using a 100 ml volumetric flask. This was subsequently transferred to an amber bottle and stored at 4°C until analysed. Prior to analysis the presence of any moisture in the samples was extracted by adding 5 g anhydrous sodium sulphate (Na₂SO₄) and filtered using a 0.45 μm PTFE syringe tip filter (Phenomenex, UK).

3.2.3 Results

No losses or degradation of either free astaxanthin in the synthetic Carophyll Pink™ or total carotenoids in natural astaxanthin source (NatuRose™) occurred at any production stage of processing the cold pressed feeds (Table 3.5). These results show there is no appreciable dose response effect in the degradation of astaxanthin during the production of cold pressed diets. They also show that the use of the Soxtherm system to extract carotenoids from experimental diets is an efficient method for application. Figures 3.1a and 3.1b display the optimum evaporation times required for the total extraction of carotenoids (from cold pressed and extruded diets) for Carophyll Pink™ and NatuRose™, respectively.
Table 3.5. Stability of synthetic (Carophyll Pink™) and natural *Haematococcus pluvialis* (NatuRose™) astaxanthin (Ax) measured at 3 stages during the production of cold pressed diets, n=3, ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Ax Source</th>
<th>Target [Ax] (mg kg⁻¹)</th>
<th>Stage 1 [Ax] (mg kg⁻¹)d</th>
<th>Stage 2 [Ax] (mg kg⁻¹)e</th>
<th>Stage 3 [Ax] (g kg⁻¹)f</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N/Aa</td>
<td>0</td>
<td>0.00 ±0.00</td>
<td>0.00 ±0.00</td>
<td>0.00 ±0.00</td>
</tr>
<tr>
<td>2</td>
<td>CPb</td>
<td>20</td>
<td>19.92 ±0.27</td>
<td>19.87 ±0.66</td>
<td>20.32 ±0.57</td>
</tr>
<tr>
<td>3</td>
<td>CP</td>
<td>40</td>
<td>39.51 ±0.64</td>
<td>39.21 ±0.47</td>
<td>39.69 ±0.23</td>
</tr>
<tr>
<td>4</td>
<td>CP</td>
<td>60</td>
<td>58.96 ±1.13</td>
<td>60.21 ±1.89</td>
<td>59.83 ±0.89</td>
</tr>
<tr>
<td>5</td>
<td>CP</td>
<td>80</td>
<td>80.09 ±0.59</td>
<td>79.02 ±1.42</td>
<td>81.21 ±0.98</td>
</tr>
<tr>
<td>6</td>
<td>CP</td>
<td>100</td>
<td>98.82 ±1.34</td>
<td>97.32 ±2.17</td>
<td>99.21 ±0.63</td>
</tr>
<tr>
<td>7</td>
<td>NRc</td>
<td>20</td>
<td>21.31 ±0.42</td>
<td>19.13 ±0.39</td>
<td>18.69 ±0.90</td>
</tr>
<tr>
<td>8</td>
<td>NR</td>
<td>40</td>
<td>39.03 ±0.84</td>
<td>38.49 ±0.53</td>
<td>38.87 ±0.32</td>
</tr>
<tr>
<td>9</td>
<td>NR</td>
<td>60</td>
<td>58.72 ±0.77</td>
<td>59.17 ±1.89</td>
<td>60.83 ±2.09</td>
</tr>
<tr>
<td>10</td>
<td>NR</td>
<td>80</td>
<td>81.47 ±0.72</td>
<td>81.00 ±1.44</td>
<td>77.92 ±1.20</td>
</tr>
<tr>
<td>11</td>
<td>NR</td>
<td>100</td>
<td>100.63 ±0.85</td>
<td>99.06 ±1.48</td>
<td>99.74 ±2.98</td>
</tr>
</tbody>
</table>

a Not applicable (unpigmented basal diet); b Carophyll Pink™ (Hoffmann-La Roche, Basel, Switzerland) measured its free astaxanthin content; c NatuRose™ (Cyanotech, Hawaii, USA) measured its total carotenoid content; d After the mixing of the raw ingredients; e After cold pelleting; f After drying in an oven at 45°C for 72 hours.
Fig. 3.1a Extraction of free astaxanthin (Ax) Carophyll Pink™ from extruded and cold pressed diets using the Soxtherm method.

Fig. 3.1b Extraction of the total carotenoid content of NatuRose™ (Haematococcus pluvialis) from extruded and cold pressed diets using the Soxtherm method. Step 1 refers to the initial evaporation stage where the pelleted diet is immersed in dichloromethane (DCM); step 2 refers to the recovery (final evaporation step) of Ax from the pellets when there no longer immersed in the (DCM) n=3, ± standard deviation of the mean (error bars to small to be identified).
The results show the optimum evaporation time is 25 and 20 min and for steps 1 (initial evaporation step whilst the pellets are immersed in DCM) and 2 (final recovery step where the pellets are no longer immersed in DCM) respectively. These optimum times were applicable for both cold pressed and extruded diets, and for extracting free astaxanthin from Carophyll Pink™ and the total carotenoid content of Haematococcus pluvialis (NatuRose™). The extruded diet containing Carophyll Pink™ liberated higher concentrations of astaxanthin than the cold pressed diet (containing Carophyll Pink™) at 5, and 10 min for step one. At 20 and 25 min in step one the liberation of astaxanthin from the extruded diet became significantly (P<0.01) lower than the cold pressed diet. The significant differences identified at 25 min in step one were also noted at time zero for step 2. The same significant differences were observed for the diets containing NatuRose™ at 5 and 10 min in step one. However, no significant differences (P>0.05) were identified between the two diets containing NatuRose™ at any of the other sampling times in step one or in any of the sampling times in step two. However, the concentration of carotenoid extracted from both cold pressed and extruded diets was significantly (P<0.05) higher for Carophyll Pink™ (free astaxanthin) than NatuRose™ (total carotenoid) at 5 and 10 min, but significantly (P<0.05) lower at 25 min in step 1. In step two the extruded diet containing NatuRose™ liberated significantly (P<0.05) higher concentrations of astaxanthin than Carophyll Pink™ at time 0 and 5 min.

3.2.4 Discussion

Astaxanthin proved to be extremely stable during the production of cold pressed diets, as all the pigmented diets containing either Carophyll Pink™ or NatuRose™ demonstrated almost 100% recovery at all stages of processing. So, cold pressed diets do not require extra carotenoids to attain the desired astaxanthin concentration in the diets, due to
processing losses. These results also indicate that the fractures and cavities induced into the cell covering of *Haematococcus* by down stream processing to increase bioavailability of the astaxanthin, does not affect its stability (Lorenz, 1999). The desired dietary inclusion rates for both forms of astaxanthin calculated prior to extrusion were equivalent to those levels measured post extrusion. These results also show that the Soxtherm method is efficient for carotenoid extraction, attaining 100% recovery from fish feed. The significant differences noted between the cold pressed and extruded diets containing NatuRose™ or Carophyll Pink™ were probably due to the fact that the pigments were top-coated onto the extruded diet, whereas the pigments were part of the pellet matrix in the cold pressed diets. The significant differences observed when comparing the two pigment sources occurs due to the fact that Carophyll Pink™ was dissolved in water prior to being added to the diets, whereas the NatuRose™ products comprises of cells in which the carotenoid pigments reside, so probably take longer to be extracted. Nevertheless, the Soxtherm method is not suitable for muscle tissue. During its development, the application of this method to extract astaxanthin from epaxial white muscle tissue was also tested. The extract of white muscle when analysed by HPLC, resulted in no astaxanthin being detected in any of the fish, even though the samples visibly contained pigment. This may be due to DCM being unable to disrupt the electrostatic bond that forms when either the 3 or 3' hydroxyl group on astaxanthin binds to a hydrophobic pocket on the surface of epaxial muscle actomyosin (Henmi *et al.*, 1989). This also showed that muscular fat deposits extracted from the muscle samples by the Soxtherm method do not retain astaxanthin.
Experiment 3.3 Rheological analysis of the experimental astaxanthin sources when mixed with cod liver oil.

3.3.1 Introduction

Epaxial white muscle deposition of astaxanthin is dependent on growth rate, which in turn, is dependent upon diet composition and nutrient balance (Torrissen, 1985). Hence, the application and retention of test pigments to experimental diets is of great importance. The efficacy of the different pigment sources must be tested in vivo, by presentation to the fish in experimental diets that can be produced either by cold pressing or extrusion. Yet, in the context of pigmentation, both methods are beset with problems. Cold pressed diets have a low oil content, approximately 11%. The major energy component of such diets is protein, which is metabolically more expensive than oil, resulting in poor growth. Alternatively, extruded diets provide optimal growth rates as they contain a high lipid content of up to 40%. To attain such high lipid levels, extrusion technology operates under high temperatures and pressures, which consequently leads to astaxanthin losses. These losses may be up to 40% when producing small batches (50 kg) of experimental expanded diet (as described in section 4.5). This problem does not arise in the production of cold pressed diets as mild production conditions (low temperature and pressure) are employed minimizing astaxanthin degradation (section 4.5). Marrying the benefits of the two production methods may lead to the creation of reproducible high oil content extruded diets with the desired astaxanthin content, where the astaxanthin is mixed with oil and applied to the feed pellets as a top-coat.

Pigmenting small batches of extruded pellets by top-coating may limit their astaxanthin content. The proposed method will be achieved by pouring an astaxanthin oil solution over the pellets, which are concomitantly agitated by a mixing paddle. During application to the pellets, the oil is exposed to shear forces that may vary the resistance to flow and hence its viscosity. If the oil demonstrates pseudoplasticity (shear thinning) then its “retention” to the surface of the pellets will be restricted. As a consequence, diets of lower than expected
carotenoid content will be produced. The latter could potentially reduce the concentration of astaxanthin retained in the epaxial white muscle of the test fish.

3.3.2 Materials and Methods

3.3.2.1 Experimental design

Five samples of cod liver oil (500 ml) containing either *Haematococcus pluvialis* (50 mg kg⁻¹ dietary inclusion rate) or astaxanthin extracted from a synthetic source (50 mg kg⁻¹ dietary inclusion rate) in different solvents (Table 3.6) were analysed using control stress rheometry to determine the effect of increasing shear on viscosity at 20°C. Then 60 ml of each oil sample containing astaxanthin were applied as a top-coat to 440 g of unpigmented expanded diet and the astaxanthin content of the diet determined. The astaxanthin content of the oil samples and diets containing Carophyll Pink™ were quantified by HPLC (section 2.5.5.2) against external standards (section 2.6). The total carotenoid content of the oil samples, unpigmented diet and diets containing NatuRose™ were quantified spectrophotometrically (section 2.6.1). The carotenoid contained within each diet was extracted using the Soxtherm method (section 2.5.1). Each diet and oil was analysed in triplicate, the results of which were subject to statistical analysis (section 3.1.2.3).

3.3.2.2 Sample preparation

462 g (w/v, specific gravity 0.924 at 20°C) of cod liver oil (Seven Seas Ltd., Hull, UK) was used for each sample. The profile of the basal diet (Table 3.7) allowed for 12% (120 g kg⁻¹) oil to be added as a top-coat, where 462 g cod liver oil equates to the top-coat for 3.85 kg of basal diet. Sample 1 was an unpigmented control containing only cod liver oil. The quantity of each astaxanthin source required to pigment 3.85 kg of basal diet is presented in Table 3.6.
Table 3.6. The quantity of astaxanthin (Ax) at an inclusion level of 50 mg kg\(^{-1}\) required for 3.85 kg diet, and the solvents and volumes required to extract astaxanthin from Carophyll Pink\(\text{TM}\).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ax Source</th>
<th>Ax Content (%)</th>
<th>Ax Source Weight (g)</th>
<th>Solvent</th>
<th>Solvent Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (^{a})</td>
<td>N/A (^{b})</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>NR (^{c})</td>
<td>1.25</td>
<td>15.40</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>3</td>
<td>CP (^{d})</td>
<td>8</td>
<td>2.41</td>
<td>Distilled water</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>CP</td>
<td>8</td>
<td>2.41</td>
<td>DCM (^{e})</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>CP</td>
<td>8</td>
<td>2.41</td>
<td>Chloroform (^{f})</td>
<td>75</td>
</tr>
</tbody>
</table>

\(^{a}\) Control; \(^{b}\) Not applicable; \(^{c}\) NatuRose\(\text{TM}\) (Cyanotech, Kona, Hawaii, USA); \(^{d}\) Carophyll Pink\(\text{TM}\) (Hoffman La-Roche, Basel, Switzerland); \(^{e}\) Dichloromethane (BDH, Poole, UK); \(^{f}\) BDH, Poole, UK.
15.4 g NatuRose™ (*H. pluvialis*) derived astaxanthin (dried cells) was mixed into the cod liver oil (500 ml) in its native form (sample 2). In order to dissolve the gelatine-starch matrix coating, the synthetic astaxanthin (Carophyll Pink™) was pre-treated prior to its addition to the oil in the following ways. Sample 3, 50 ml distilled water was added to 2.41 g Carophyll Pink™ and incubated at 50°C for 30 min. The aqueous Carophyll Pink™ solution was added to 500 ml cod liver oil and homogenised (Silverson, Chesham, Buckinghamshire, UK) for 30 s at 3000 rpm to form an emulsion.

Samples 4 and 5, 3.0 g Carophyll Pink™ was added to 10 ml distilled water and 0.5 g Maxatase (Genecor International, Lieden, Holland) in a 100 ml polypropylene conical bottom centrifuge tube, and incubated at 50°C in a sonicating water bath (Sonimatic, Langford Ultrasonics, West Midlands, UK) for 30 min. After incubation, 10 ml ethanol containing 500 mg dm⁻³ 2,6-di-t-butyl-p-cresol (BHT), as an antioxidant, was added to each sample. This was homogenised (Silverson, Chesham, Buckinghamshire, UK) for 30 s, then 30 ml dichloromethane (DCM) or chloroform were added to samples 4 and 5 respectively. Samples 4 and 5 was homogenised for a further 30 s, incubated in the dark for 10 min at room temperature (20 °C), homogenised again for 30 s and then centrifuged (Mistral 3000, Fisons, UK) for 10 min (3000 rpm), at 16 °C. After centrifugation the hypophase was removed and the samples filtered (0.20 μm nylon syringe tip; Whatman, UK) to form an astaxanthin DCM stock solution and an astaxanthin chloroform stock solution. The concentration of both astaxanthin stock solutions were determined by HPLC. An aliquot (sufficient to pigment 3.85 kg of basal diet at a dietary inclusion level of 50 mg kg⁻¹) was taken from each of the astaxanthin DCM and astaxanthin chloroform stock solutions and made up to 75 ml in volumetric flask with DCM and chloroform, respectively. The 75 ml astaxanthin DCM solution and 75 ml astaxanthin chloroform solutions were then added to 500 ml cod liver oil to form samples 4 and 5, respectively.
3.3.2.3 Controlled stress rheometer

The viscosity of the oil samples was measured using a double concentric cylinder geometry (Fig. 3.2) on a controlled stress rheometer (Carri-Med Rheometer CSL2 500, TA Instruments, Surrey, UK). The dimensions of the cylinders were R1 = 2.000 mm, R2 = 2.038 mm, R3 = 2.195 mm and R4 = 2.238 mm (Fig. 3.2) The temperature was set at 20°C, the shear stress varied from 0.1 – 3.5 Pa, over a ramp duration of 3 min. A range of separate experiments were performed to determine the optimum ramp time and results suggest that this ramp time (3 min) produced equilibrium data.

3.3.3.4 Diet preparation

The composition of the unpigmented basal diet (Table 3.7) was designed to promote optimal growth, as determined by Biomar UK Ltd. Each diet was produced in 0.5 kg batches, mixed using a commercial (3 kg capacity) blender (Hobart A-120, London, UK). 440 g of unpigmented expanded diet (Biomar Ltd, Grangemouth, UK) was added to the bowl and top coated with 60 g of oil containing either the natural or synthetic source of astaxanthin. The oil samples were slowly poured over the feed pellets by hand, and mixed for 15 min. The total carotenoid content of diets 1 (control) and 2 (50 mg kg⁻¹ H. pluvialis) were quantified spectrophotometrically. The astaxanthin content of diets 3, 4 and 5 containing Carophyll Pink™ (50 mg kg⁻¹) was quantified by HPLC, each diet being analysed in triplicate.

Table 3.7 Extruded basal diet formulation.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Inclusion (g kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT-94 Fish-meal</td>
<td>380.0</td>
</tr>
<tr>
<td>Norsemink Fish-meal</td>
<td>170.0</td>
</tr>
<tr>
<td>Wheat Gluten</td>
<td>50.0</td>
</tr>
<tr>
<td>Wheat-meal</td>
<td>196.5</td>
</tr>
<tr>
<td>Fish Oil</td>
<td>190.0</td>
</tr>
<tr>
<td>Vitamin Premix</td>
<td>7.5</td>
</tr>
<tr>
<td>Mineral Premix</td>
<td>6.0</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
</tr>
</tbody>
</table>

a Norsildmel, Bergen, Norway; b Biomar Ltd., Grangemouth, UK.
Fig. 3.2 Double concentric cylinder system. In this configuration the ratio $R_1 : R_2 = R_3 : R_4$ and shear stress is calculated using either $R_1$ and $R_2$ or $R_3$ and $R_4$ in the equation

Shear stress factor ($F_r$) = \frac{(R_1)^2 + (R_3)^2}{4\pi (R_2)^2 H ((R_1)^2 + (R_2)^2)}
3.3.3 Results

All the flow curves for the oil samples (Fig. 3.3) were linear, where the sample (3) containing Carophyll Pink™ dissolved in water had the highest gradient followed by the control (sample 1) and the sample (2) containing Haematococcus cells which were very similar. The sample (4) containing synthetic astaxanthin dissolved in dichloromethane had the lowest gradient and was similar to that containing synthetic astaxanthin dissolved in chloroform (sample 5). The viscosities of the samples (Table 3.8) were directly correlated to the gradient of the flow curves. The astaxanthin concentration of the experimental diets (Table 3.8) varied depending upon the type of solvent and the source of astaxanthin used.

Table 3.8 The relative poise (viscosity) of the experimental oil solutions and astaxanthin (Ax) content of the experimental diets; n=3, ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ax Source</th>
<th>Solvent</th>
<th>Viscosity (mPa.s)</th>
<th>[Ax] (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 †</td>
<td>N/A b</td>
<td>N/A</td>
<td>65.30</td>
<td>ND g</td>
</tr>
<tr>
<td>2</td>
<td>NR c</td>
<td>N/A</td>
<td>67.70</td>
<td>53.97 ±0.12</td>
</tr>
<tr>
<td>3</td>
<td>CP d</td>
<td>Distilled water</td>
<td>82.00</td>
<td>40.07 ±1.15</td>
</tr>
<tr>
<td>4</td>
<td>CP</td>
<td>DCM e</td>
<td>23.20</td>
<td>44.29 ±1.23</td>
</tr>
<tr>
<td>5</td>
<td>CP</td>
<td>Chloroform f</td>
<td>32.00</td>
<td>47.44 ±1.86</td>
</tr>
</tbody>
</table>

† Control; b Not applicable, c NatuRose™ (Cyanotech, Kona, Hawaii, USA); d Carophyll Pink™ (Hoffman La-Roche, Basel, Switzerland); e Dichloromethane (BDH, Poole, UK); f BDH, Poole, UK; g Non-detected.
Fig. 3.3 Comparison of the shear stress profiles (flow behaviour) for synthetic (Carophyll Pink™) and natural (NatuRose™) astaxanthin over the range of shear rates applied to the samples. Where:

- **Cells** = *Heamotococcus pluvialis* (NatuRose™) cells
- **DCM** = Free astaxanthin (Carophyll Pink™ extract) dissolved in dichloromethane
- **Control** = Cod liver oil
- **Water** = Free astaxanthin (Carophyll Pink™ extract) dissolved in water
- **CHCL₃** = Free astaxanthin (Carophyll Pink™ extract) dissolved in chloroform
- **Newtonian** = Newtonian behaviour
3.3.4 Discussion

The rheological flow measurements used in this experiment were obtained using a controlled stress rheometer. Prior to analysis a range of geometries were considered. Experiments with cone and plate, and parallel plates were rejected in favour of the double concentric geometry as the former were not sensitive enough to detect the small differences in viscosity. In the double concentric geometry a greater amount of test liquid was in contact with the upper measuring cylinder, leading to more accurate viscosity predictions. All the flow curves demonstrated a linear variation through the origin of shear stress, with shear rate confirmed by the high regression coefficients ($R^2$ values, Fig. 3.3), indicating that the flow behaviour of all the fluids tested can be classified as Newtonian, over the range of the shear stresses considered. Although the cod liver oil sample containing water and Carophyll Pink™ (CP) suggested some evidence of shear thinning at low shear stress.

The control sample containing pure cod liver oil had a viscosity of 65.300 mPa.s. The samples containing extracts from the synthetic astaxanthin source (Carophyll Pink™) varied depending on the type of extraction method employed and the solvent used. Addition of organic solvents (chloroform and dichloromethane used to extract astaxanthin from Carophyll Pink™) to cod liver oil, caused its Newtonian viscosity to decrease. This is due to the mobile (low viscosity) organic solvents being miscible with the more viscous cod liver oil, where the organic solvents act as additives that dilute the cod liver oil on mixing, causing its viscosity to decrease. Resulting in a viscosity of 23.200 and 32.000 mPa.s for dichloromethane (DCM) and chloroform, respectively.

The lower viscosity of the cod liver oil DCM solution compared to that of the cod liver oil chloroform solution may be due to the relative viscosities of the diluent solvents. The viscosity of DCM is 0.413 mPa.s, being markedly lower than 0.537 mPa.s for chloroform at 25°C (Lide, 1996). The difference in the relative viscosities of the solvents is reflected in the corresponding viscosities of the solvent cod liver oil solutions. In addition, this
difference may also be due to the number and type of inter-molecular interactions that could form between cod liver oil when mixed with either DCM or chloroform. As DCM, chloroform and cod liver oil are all miscible organic solvents, the only interactions that will probably form are non-bonding hydrophobic interactions (Braven, *pers comm*.).

The addition of synthetic astaxanthin dissolved in distilled water, caused the viscosity of cod liver oil to increase. Even though the viscosity of water is 1.002 mPas at 20°C (Lide, 1996). The increased viscosity is probably due to the immobilisation of synthetic astaxanthin (Carophyll Pink™) in a starch-gelatine matrix. This is rationalised by the fact that the solid starch gelatine-matrix is soluble in water at ambient temperature (20°C). Starch and gelatine may form weak inter-molecular interactions (hydrogen bonds and van der Waals forces) with water and the component fatty acids and lipid soluble vitamins of cod liver oil.

The sample containing *H. pluvialis* had a similar viscosity (67.700 mPas) as the control, pure cod liver oil (65.300 mPas). This 10.4% increase in viscosity is rationalised by the *Haematococcus* cells only constituting a phase volume of 1.13% and being relatively large having a mean diameter of 45 microns (Lorenz, 1999). Newtonian liquids containing particulates which constitute a phase volume of less than 10% have little affect on viscosity, only causing an optimal increase of 40% (Barnes, 1989). This is due to the spatial arrangement of the Haematococcus cells forming a homogeneous suspension, where the cells probably have very little or no interaction.

The dietary carotenoid content of the five diets is presented in Table 3.8, being expressed as total carotenoid content in the case of *H. pluvialis* and free astaxanthin content for the synthetic astaxanthin. Total carotenoid content is quantified in the case of *H. pluvialis* because NatuRose™ *Haematococcus* meal contains approximately 70% astaxanthin monoesters, 10% astaxanthin di-esters, 5% free astaxanthin and the remaining 15% consists of an admixture of β-carotene, lutein, canthaxanthin, zeaxanthin, violaxanthin, neoaxanthin and echinenone (Lorenz, 1999; Grung *et al.*, 1992). Esterified (mono- and di-) astaxanthin,
Lutein and canthaxanthin have all demonstrated an ability to pigment salmonid species (Latscha, 1990). This compares with the synthetic source (Carophyll Pink™, Hoffmann-La Roche, Basel, Switzerland) being guaranteed to contain 8% free astaxanthin with a 1:3, cis:trans isomeric ratio, where the trans isomer contains a racemic mixture of the three stereo isomers (3S,3'S; 3R,3'S meso and 3R,3'R) in a ratio of 1:2:1 (Schiedt, 1988).

The astaxanthin concentration of diets 4 and 5 containing synthetic astaxanthin extracted in DCM and chloroform respectively, were not significantly different (P>0.05). This resulted in an astaxanthin content of 44.29 and 47.44 mg kg⁻¹ for diets 4 and 5 respectively. The retention of astaxanthin in diets 4 and 5 is probably due to astaxanthin having a similar solubility in DCM (30 mg ml⁻¹) and chloroform (10 mg ml⁻¹) (Johnson and An, 1991). All the pigmented diets (3, 4 and 5) containing CP, targeted 50 mg kg⁻¹ free astaxanthin. The retention of astaxanthin for diets 4 (88.6% of the target level) and 5 (94.9% of the target level) is due to production losses, as oil containing astaxanthin was noticeably retained in the mixing bowl and on the mixing paddle. Both diets 4 and 5 retained a significantly (P<0.01) higher astaxanthin concentration than diet 3 (40.07 mg kg⁻¹) that had been coated in a solution of CP dissolved in water and mixed with cod liver oil. Low retention of astaxanthin in diet 3 (80.1% of the target level) can also be attributed to the same type of production losses noted for diets 4 and 5. However, results from a preliminary experiment showed production losses of oil when added as a top coat to extruded diets remained constant regardless of the solvent used to dissolve the astaxanthin. Hence, the lower astaxanthin content of diet 3 compared to diets 4 and 5 may be rationalised by observations that organic solvents such as DCM, chloroform and acetone were more readily absorbed by extruded feed pellets than inorganic solvents such as water. This greater absorption can be attributed to the composition of the basal diet (Table 3.7), where more than 80% of its content are organic compounds, mainly proteins, carbohydrates and lipids.

Diet 2, though containing NatuRose™ targeted 50 mg kg⁻¹ total carotenoid, resulted in total carotenoid content of 53.97 mg kg⁻¹ (7.9% higher than the target level) being significantly
(P<0.01) higher than the other diets. The manufactures of the NatuRose™ product reported the batch used in this experiment to contain 1.25% total carotenoid by dry weight. As NatuRose™ is a natural product each batch contains differing quantities of carotenoid. To maintain product continuity, different batches are blended. However, no accredited method has yet been developed to quantitatively assess the carotenoid content of *H. pluvialis*, on which the NatuRose™ product is based. This factor in conjunction with the residual production losses, suggests that the actual carotenoid content of NatuRose™ is higher than that quoted by the manufacturer.

Diet 1 (control) had no detectable astaxanthin, yet had a total carotenoid content of 0.17 mg kg⁻¹. This is rationalised from the results of experiment 3.1 that evaluated the ingredients of the basal diet and showed extruded wheat and vitamin premix to contain the carotenoid zeaxanthin. As discussed in experiment 3.1, zeaxanthin has a diminutive effect on white muscle or skin pigmentation of rainbow trout as it is a metabolite of astaxanthin and is poorly absorbed in the gut (Schiedt, 1988; Bjerkeng, 1992). So, the target dietary inclusion levels for astaxanthin do not have to be adjusted to compensate for the carotenoid content of the basal diet.
Experiment 3.4 Determining the stability of astaxanthin (applied as a top-coat to a commercial diet) when immersed in a turbulent water column.

3.4.1 Introduction

During degradation studies of pigmented diets, a number of factors have to be taken in to consideration, namely the effect of solar radiation and water erosion.

In experimental conditions, the feeding of fish is carefully monitored to ensure an equal distribution of feed within the group to achieve good growth rates and minimal wastage. Similarly, the experimental diets can be stored under optimal conditions. However, on a fish farm the conditions, scale of the operation and time makes the storage of diet under its optimal conditions very difficult in practice, so for convenience diets are usually stored on location. Such constraints also make careful monitoring of feed intake very difficult to quantify in practice. In this respect, the fish are generally fed in excess of their dietary intake, resulting in some feed remaining in the water. The voracious feeding nature of salmonids usually results in a rapid fulfilment of the satiation response, with a relatively rapid return of appetite (as compared to other cold fresh water species) (Mork, pers comm.). Consequently, the uneaten feed pellets residing in the water will most likely be consumed in a reasonable time. However, the integrity of feed pellets (both dietary and structurally) stored in an aqueous environment is questionable. As water is absorbed (causing the pellets to soften and swell), water soluble nutrients (namely vitamins) will leach out and as the pellets start to structurally degrade, the oil containing astaxanthin will slowly be released. Due to the expense of astaxanthin and its sensitive chemical nature, a growing trend amongst feed manufacturers is to add the astaxanthin to feeds as a top-coat post extrusion in a bid to reduce processing losses and cost (whereby the astaxanthin is mixed with oil and applied to the pelleted diets under vacuum). Top-coated diets are probably more likely to lose astaxanthin when placed in water as the surface oil containing astaxanthin will be initially washed off the pellets rather than being released after the
structural integrity of the pellet has been breached. Thus, erosion of feed pellets when added to water may be an important factor in loss of astaxanthin.

3.4.2 Materials and methods

3.4.2.1 Experimental design

Triplicate 5 g samples of two experimental diets top coated with either a natural (NatuRose™, *H. pluvialis* 50 mg kg⁻¹) or synthetic (Carophyll Pink™ 50 mg kg⁻¹) source of astaxanthin were placed into sealable polyethylene bags (5.5 x 7.5 cm) to which 10 ml distilled water was added. The bags were placed in an agitating water bath and incubated at 15°C for up to 10 min in darkness. Samples were taken at 0, 15, 30, 60, 120, 210, 300, 450 and 600 s. Gentle agitation was employed to emulate the moving water column caused by wave action, the swimming action of fish and water and air being pumped into tanks/ponds. The astaxanthin content of the water samples containing Carophyll Pink™ were quantified by HPLC (section 2.5.5.2) against external standards (section 2.6). The total carotenoid content of the water samples containing NatuRose™ were quantified spectrophotometrically (section 2.6.1). Triplicate samples were analysed at each time interval, the results of which were subject to statistical analysis (section 3.1.2.3).

3.4.2.2 Extraction of astaxanthin from the water column

After incubation the water was removed from each bag by carefully cutting off one corner and allowing the water to drain into a 20 ml glass vial. A 2 ml aliquot was taken, to which 2 ml methanol containing 500 ppm 2,6-di-t-butyl-p-cresol (BHT, added as an antioxidant) was added and mixed (Whirlimixer, Fisons, UK) for 15 s. 6 ml dichloromethane was added to the sample and mixed for a further 15 s. The samples were incubated in the dark for 10 min at room temperature (20°C), mixed for a further 15 s and then centrifuged (Mistral 3000, Fisons, UK) for 10 min at 3000 rpm, at 16°C. After centrifugation a 3 ml aliquot of
hypophase was filtered using PTFE syringe tip filters (0.45 μm; Phenomenex, UK), directly into amber sample vials and immediately sealed. The samples were stored at -20°C until analysed. Prior to analysis the presence of any moisture in the samples was extracted by adding 0.5 g anhydrous sodium sulphate (Na₂SO₄) and filtered using a 0.45 μm PTFE syringe tip filter (Phenomenex, UK).

3.4.2 Results

The percentage loss of carotenoids from pigmented feed pellets immersed in water is presented in Fig. 3.4, being assessed as free astaxanthin content for diet 1 (Carophyll Pink™) and as total carotenoid content for diet 2 (NatuRose™, _H. pluvialis_). The water in which the diets were immersed was analysed at zero seconds (the start of the experimental period) then at strategic intervals and finally at 600 s (10 min; on termination of the experiment). Both diets showed a continuous loss of pigment throughout the experimental period. At the initial sampling (0 s) both diets contained similar carotenoid contents (diet 1, 50.32 ±0.51 mg kg⁻¹ free astaxanthin and diet 2, 50.96 ±1.21 mg kg⁻¹ total carotenoid content) and the distilled water used to immerse the diets contained an undetectable quantity of carotenoids. Thereafter, diet 1 demonstrated a significantly (P<0.01) higher loss of pigment than diet 2. Diet 2 containing NatuRose™, (_H. pluvialis_) showed a total carotenoid loss of 4.80 % after 60 s, which increased to 7.57 % loss after 300 s, and finally 10.45 % at 600 s. Diet 1 containing synthetic Carophyll Pink™ proved to be the least stable of the two astaxanthin sources tested, demonstrating a 14.36 % loss of free astaxanthin after 60 s which increased to 45.28 % after 300 s and finally 65.82 % at 600 s.
Fig. 3.4 Carotenoid loss from extruded feed pellets top-coated with Carophyll Pink™ (CP, Diet 1) or NatuRose™ (NR, Diet 2) *Haematococcus pluvialis* mixed with cod liver oil then immersed in a turbulent water column; n=3, ± standard deviation of the mean (error bars too small to be identified).
3.4.4 Discussion

The results for the percentage loss of carotenoids from pigmented feed pellets immersed in water were based on a feeding regime that is employed on the majority of fish farms, i.e. feeding to excess. The decision to terminate the experiment after 10 minutes was based on observations made whilst feeding stock rainbow trout to excess. A combination of the flow rate of water (1000 L hr$^{-1}$ tank$^{-1}$) and design of the recirculation systems employed, resulted in excess feed pellets being expelled from the tanks within 10 minutes of being released into the water. It should be noted that the carotenoid content of both dietary treatments attained the target inclusion level of 50 mg kg$^{-1}$. Hence, the loss of the test carotenoids applied to diets 1 (Carophyll Pink™) and 2 (NatuRose™, H. pluvialis) would be of a similar potential given the fact that both sources of astaxanthin were mixed with cod liver oil and applied as a top coat to the feed pellets. The rate of carotenoid loss due to water erosion for both diets followed an exponential trend, as confirmed by their high regression coefficients (R$^2$ values; Fig. 3.4). Exponential loss of astaxanthin into the aqueous media demonstrated first order kinetics. At all the subsequent sampling times after the initial sampling (0 s) diet 1 demonstrated a significantly (P<0.01) higher loss of pigment into the aqueous environment than diet 2. This may be due to the H. pluvialis cells being weakly attracted to the surface of the feed pellets, due to the formation of electrostatic bonds formed during mixing. Electrostatic charges may have been formed not only when the cells were added to the oil but also when the oil/cell suspension was mixed with basal feed pellets. The surface area of the pellets is increased due to its rough superficies, which is also porous and could enable some of the cells to partially enter the body of the pellets. Such factors would probably afford some resistance to the cells being sloughed off by the action of the moving water column. Obviously, a portion of the cells would have remained in the oil as an homogenous suspension constituting a phase volume of less than 1 %. The cells are also relatively large having a mean diameter of 45 μm (Lorenz, 1999). Conjoining these facts will probably result in the cells having very little or
no interaction. As demonstrated in experiment 3.3, *H. pluvialis* cells suspended in cod liver oil caused the viscosity of the oil to increase by approximately 10 %, concomitantly maintaining the oils Newtonian properties. Thus, the surface oil is readily sloughed off the pellets as the samples were agitated. As the oil is washed off the surface of the pellets, they start to absorb water causing them to swell and eventually compromising their structural integrity. The loss of surface oil would also allow certain lipid and water soluble vitamins A, D3, K3, B1 and C to leach out of the pellets (Gadient, 1986 and Hilton *et al.*, 1981). Once compromised, the outer surface of the pellets starts to degrade causing the water to become turbid, simultaneously releasing more cells into the water. Combining all these truisms rationalises the exponential trend observed during this study. The increased loss of Carophyll Pink™ as compared to NatuRose™ may be due to Carophyll Pink™ essentially being water soluble, as demonstrated in the preparation of the Carophyll Pink™/cod liver oil suspension (where Carophyll Pink™ was pre-dissolved in 5 ml distilled water prior to being mixed with 195 g cod liver oil). Perhaps the ratio of water to oil is an important factor, when in favour of the latter (for example during preparation of the pigmented diet) Carophyll Pink™ is maintained in an emulsion. However, when the ratio is in favour of the former (for example when the pellets are immersed in water) perhaps the emulsion readily separates, with the astaxanthin being maintained in aqueous solution. This is probable as the water soluble starch and gelatine that constitute 92 % dry weight of the Carophyll Pink™ product forms weak inter-molecular interactions (hydrogen bond and van der Waals forces) with astaxanthin when in aqueous solution. Hence, after being immersed in distilled water for 10 min (600 s) diet 2 containing the natural astaxanthin source NatuRose™ only showed approximately 10 % loss of its total carotenoid content, whereas diet 1 containing (Carophyll Pink™) demonstrated approximately 66 % loss of free synthetic astaxanthin.
Experiment 3.5 Determination of the partition coefficient for astaxanthin in a two phase system (containing water and cod liver oil).

3.5.1 Introduction

As described in experiment 3.4 (section 3.4.1) the expense of astaxanthin and its sensitive chemical nature has led feed manufacturers to add astaxanthin to its feeds as a top-coat post extrusion in a bid to reduce processing losses and costs. The results of experiment 3.4 demonstrated that astaxanthin is readily lost from diets top coated with either Carophyll Pink™ (synthetic astaxanthin) or NatuRose™ (a natural source of astaxanthin) when placed in a turbulent water column. As previously stated, astaxanthin is lipid soluble and readily dissolves in cod liver oil, but oil and water are immiscible liquids which form discrete layers post mixing. However, due to the law of partition, where a solute (astaxanthin) dissolved in a solvent (cod liver oil) is mixed with an immiscible solvent (water) heterogeneously, an interface is formed between the two solvents and the solute distributes itself between the two solvents (Good and Denny, 1985). Hence, a quantity of astaxanthin will probably be lost from the surface of feed pellets when immersed in water (regardless of the effects of water erosion) due to the natural partitioning that occurs between the cod liver oil and water.

3.5.2 Materials and methods

3.5.2.1 Experimental design

The partition coefficient for synthetic astaxanthin (Carophyll Pink™) between cod liver oil and distilled water was determined. The astaxanthin content of both phases (water and cod liver oil) quantified by HPLC (section 2.5.5.2) against external standards (section 2.6).
3.5.2.2 Sample preparation

Carophyll Pink™ was pre-treated by adding 62.5 mg (8% astaxanthin content) to 1.0 ml distilled water and heated to 50°C in order to dissolve its starch-gelatine matrix, forming an astaxanthin solution. The astaxanthin solution was then mixed with 99.0 g cod liver oil (Seven Seas, Hull, UK) to form an astaxanthin emulsion. The emulsion was poured into a 500 ml separating funnel to which 200 ml distilled water was added. The funnel was capped and inverted several times allowing the two phases to mix. After mixing, 1g sodium chloride was added to the funnel to disrupt the Carophyll Pink™ emulsion (Jervis, pers comm.). The funnel was recapped and then incubated in the dark at 15°C for 24 hours to ensure the two phases had separated. After, incubation the astaxanthin content of both phases was determined, and the partition coefficient calculated using equation 3.i (Goodman and Denny, 1985)

3.i. \[ K = \frac{\text{Water [Ax]}}{\text{Oil [Ax]}} \]

Where:
- \( K \) = Partition coefficient
- Water [Ax] = Astaxanthin concentration of the aqueous phase
- Oil [Ax] = Astaxanthin concentration of the oil phase

3.5.2.3 Astaxanthin extraction methods

3.5.2.3.1 Hypophase (water)

The hypophase was drained from the separating funnel into a 250 ml beaker and mixed for 15 min using a magnetic stirrer. After mixing, triplicate 2 ml samples of water were pipetted into 20 ml glass vials to which 2 ml methanol containing 500 ppm BHT was added to the sample and mixed (Whirlimixer, Fisons, UK) for 15 s. 6 ml dichloromethane was added to the sample and mixed for a further 15 s. All samples were incubated in the
dark for 10 min at room temperature (20°C), mixed for a further 15 s and then centrifuged (Mistral 3000, Fisons, UK) for 10 min at 3000 rpm, at 16°C. After centrifugation a 3 ml aliquot of hypophase was filtered using PTFE syringe tip filters (0.45 μm; Phenomenex, UK), directly into amber sample vials and immediately sealed. The samples were stored at -20°C until analysed. All samples were analysed (quantified) by HPLC. Prior to analysis the presence of any moisture was extracted by adding 0.5 g of anhydrous sodium sulphate (Na₂SO₄) and filtered using PTFE syringe tip filters (0.45 μm; Phenomenex, UK).

3.5.2.3.2 Hyperphase (cod liver oil)

The hypophase was drained from the separating funnel into a 250 ml beaker and mixed for 15 minutes using a magnetic stirrer. After mixing, triplicate 2 ml samples of water were pipetted into 20 ml glass vials to which 2 ml methanol containing 500 ppm BHT and 2 ml distilled water was added and mixed for 15 s (Whirlimixer, Fisons, UK). 6 ml dichloromethane was added to the sample and mixed for a further 15 s. Then as described in the second paragraph of section 3.3.5.2.1 (hypophase).

3.5.3 Results

The partition coefficient of astaxanthin between distilled water and fresh cod liver oil is presented in Table 3.9. The majority of the astaxanthin remained in the oil phase.

Table 3.9. Mean concentration of astaxanthin in each phase (oil and water), of the two phase system used to determine the partition coefficient for astaxanthin; n=3, ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Phase</th>
<th>[Astaxanthin] b (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>1.47 ±0.04</td>
</tr>
<tr>
<td>Cod liver oil a</td>
<td>48.53 ±0.05</td>
</tr>
<tr>
<td>Partition Coefficient</td>
<td>0.03</td>
</tr>
</tbody>
</table>

a Seven Seas, Hull, UK; b Carophyll Pink™ (Hoffmann-La Roche, Basel, Switzerland)
3.5.4 Discussion

The partition coefficient (K) for synthetic astaxanthin (Carophyll Pink™) in a two phase system (an astaxanthin cod liver oil emulsion mixed with distilled water) was determined to be 0.03 (K=0.03), which resulted in only 3% of the free astaxanthin being transferred to the aqueous phase. This result confirms the observation made in experiment 3.4 (section 3.4.4) that the loss of Carophyll Pink™ from the feed pellets to the water in the main part is due to the oil being washed off the pellets. Nevertheless, after initially mixing the two phases, no external agitation was applied to the liquid column, which essentially remained static with the only movement observed being that of the two phases separating. However, the 3% astaxanthin residing in the aqueous phase would have probably been higher in experiment 3.4, which used feed pellets, because the water column was externally agitated causing turbulent flow. The turbulent flow of the aqueous phase would have a direct affect on the size of the laminar region that exists around each pellet. A reduction in the laminar region would probably have a direct affect on the mass transfer of the astaxanthin from the oil phase to the aqueous phase resulting in an elevated diffusion coefficient. Diffusion coefficients in certain two phase systems (such as the aforementioned oil/water system) have been demonstrated to increase, when altering the flow from laminar to turbulent (James, pers comm.).
Experiment 3.6 Determining the stability of astaxanthin when dissolved in either organic solvents or cod liver oil and exposed to simulated solar radiation.

3.6.1 Introduction

Hydrocarbons released into the environment are susceptible to modification by several biotic and abiotic factor processes, including bio-degradation and photo-degradation. Latscha (1990) propounds astaxanthin to be photosensitive, readily undergoing degradation when exposed to light. However, the source of light and its spectrum are also of importance, such that photo-degradation experiments should be performed using natural sun-light, as solar radiation contains a broad range of wavelengths that encompasses ultra violet (UV), visible and infra red (IR) radiation (Goodman and Denny, 1985). Solar radiation is quite restrictive to use as its intensity varies both on a daily and seasonal basis, with variations in its wavelength distribution being dependent on latitude and general weather conditions including cloud cover (Kirk, 1986). Even when food pellets are immersed in water the astaxanthin content is still susceptible to photo-degradation. Moderately high solar altitudes and mixed layer depth water bodies can result in approximately 80-90% incident sunlight being absorbed by the water, as described by Roof (1982) in the Black sea model (Fig. 3.5). Combining these factors makes using sunlight inconvenient for photo-degradation experiments. The use of emulated (artificial) sunlight has been widely advocated in photo-degradation studies of organic compounds in preference to natural sunlight, as artificial radiation offers greater stability and reproducibility (Klein and Pilpel 1974; Thominette and Verdu, 1984; Fukuda et al., 1988).
Fig. 3.5 The "Black sea model", the numerical values represent those typical of moderately high solar altitude and mixed layer depths (tens of meters); the various shaped symbols in the mixed depth layer depict different chromophores such as astaxanthin (Roof, 1982).
Several artificial light sources have been used in photo-degradation studies, including day light lamps, black light fluorescent lamps, mercury lamps (high, medium and low pressure derivatives) and xenon arc lamps (filtered and unfiltered) (Gould, 1989). The choice of artificial lamp is critical as they all provide different wavelength distributions, some of which differ quite markedly from that of solar radiation (Fig. 3.6). Light emitted from the sun with wavelengths lower than 290 nm are the most energetic, but are predominantly absorbed by ozone in the upper atmosphere (Giese, 1976). Lamps which emit high energy UV radiation between 185 - 295 nm will result in enhanced rates of photo-degradation and product yield compared to ambient solar radiation (Hansen, 1975). Hence, in this type of study, wavelengths greater than 295 nm are considered to be of greatest importance. Consequently, the use of artificial light sources generally require filters such as borosilicate glass to attain the desired wave-length band (Draper, 1985). According to Roof (1982), the lamp of choice is a filtered xenon arc lamp, as it most closely resembles the spectral distribution and light intensity of solar radiation (Fig. 3.6).

Furthermore, artificial lamps have the advantage of shortening the irradiation time, due to increased light intensities, as demonstrated by Zepp (1982). Zepp studied the rate constants for the degradation of three aromatic hydrocarbon compounds (napthalene, anthracene, napthacene) when irradiated with several artificial light sources (normalised by filtering to remove wave lengths < 300 nm) and sun light. The results (Table 3.10) of this study showed values < 1 indicated photo-degradation due to solar irradiation was more rapid than exposure to artificial light. Conversely, values > 1 indicated artificial light caused more rapid degradation; where sunlight is designated a value of 1.
Fig. 3.6 The spectra of the major light sources used in photolysis experiments. It can be clearly seen that the spectrum of an ozone free xenon arc lamp filtered at 290 nm closely follows the solar spectrum (Zepp, 1982).
Table 3.10 Relative rate constants calculated for photodegradation of Napthalene, Anthracene and Napthacene with various light sources (Zepp, 1982).

<table>
<thead>
<tr>
<th>Light source</th>
<th>Napthalene</th>
<th>Anthracene</th>
<th>Napthacene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summer sunlight</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Winter sunlight</td>
<td>0.55</td>
<td>1.02</td>
<td>1.25</td>
</tr>
<tr>
<td>Sunlamp</td>
<td>11.60</td>
<td>0.55</td>
<td>0.12</td>
</tr>
<tr>
<td>Blacklight</td>
<td>0.17</td>
<td>1.13</td>
<td>0.04</td>
</tr>
<tr>
<td>Medium pressure mercury lamp</td>
<td>12.70</td>
<td>0.57</td>
<td>0.31</td>
</tr>
<tr>
<td>Xenon lamp</td>
<td>2.6</td>
<td>0.94</td>
<td>0.65</td>
</tr>
</tbody>
</table>

3.6.2 Materials and method

3.6.2.1 Experimental design

Two standards (200 ml) containing either all-\(E\)-trans crystalline astaxanthin or a \(H.\) pluvialis total carotenoid extract were exposed to ultra violet (UV) and visible radiation using a Suntest CPS xenon arc lamp (Heraeus Instruments GmbH, Germany) (Fig. 3.7). UV and visible radiation was emitted from a 1.8 kW to 1 x strength emulated sunlight for up to 8 hours. Samples were taken at 0, 5, 10, 15, 30 min and then every 30 min thereafter up to 480 min (8 h). During irradiation the temperature of the samples was maintained at 15°C. The experiment was repeated by emulating the environment in which the pigments reside whilst on the surface of the feed pellets. This was achieved by forming Carophyll Pink\(^\text{TM}\) / cod liver oil emulsion and \(H.\) pluvialis (NatuRose\(^\text{TM}\)) cell / cod liver oil suspension. The astaxanthin content of the oil samples containing Carophyll Pink\(^\text{TM}\) were quantified by HPLC (section 2.5.5.2) against external standards (section 2.6). The total carotenoid content of the NatuRose\(^\text{TM}\) standard and oil samples containing NatuRose\(^\text{TM}\) were quantified spectrophotometrically (section 2.6.1). Triplicate samples were analysed at each time interval, the results of which were subject to statistical analysis (section 3.1.2.3).
Fig 3.7 Light stability (photolysis) experimental set up set. Where

1 = Suntest CPS unit
2 = Xenon lamp
3 = Lamp potentiometer
4 = Lamp potentiometer setting regulator
5 = Lamp switch
6 = Lamp compartment
7 = Wooden block
8 = Stainless steel tank containing ethylene glycol
9 = Jack to support tank
10 = Thermocouple and thermometer
11 = Photolysis quartz tube holder
12 = Chiller unit
13 = Photolysis quartz tube
3.6.2.2 Sample preparation

3.6.2.2.1 Standards subjected to emulated solar radiation

The method used to prepare the crystalline all-E-astaxanthin standard for the light degradation study (treatment 1) was the same as that used to prepare the external standard (section 2.6) used for HPLC analysis. The H. pluvialis (NatuRose™) standard was prepared by adding 120 mg NatuRose™ cells to 500 ml DCM in a volumetric flask. The cells were left for 12 h, at ambient temperature constantly being agitated using a magnetic stirrer, to allow all the carotenoids to be extracted (treatment 2). The solution was filtered using 0.20 μm filter paper (Whatman, UK) placed in a Buchner funnel.

3.6.2.2.2 Oil samples

The synthetic astaxanthin (Carophyll Pink™) was pre-treated by adding 125 mg Carophyll Pink™ (8 % astaxanthin content) to 5 ml distilled water and heated to 50°C in order to dissolve its starch-gelatine matrix, forming an astaxanthin solution. The astaxanthin solution was then mixed with 195 g cod liver oil (Seven Seas, Hull, UK) to form an astaxanthin emulsion. 800 mg NatuRose™ Haematococcus (1.25 % total carotenoid content) was mixed into 200 g cod liver oil in its native form producing a cellular astaxanthin suspension. The cellular astaxanthin suspension (treatment 4) astaxanthin emulsion (treatment 3) and the two astaxanthin standards (treatments 1 and 2) were then carefully poured into four 50 ml quartz tubes (25 cm long; 2 cm external diameter; 1.8 cm internal diameter; 19/26 socket ground silica joint) and tightly capped with hollow quartz stoppers which project 2 cm (H. Baumbach & Co. Ltd., Ipswich, Suffolk, UK).

3.6.2.3 Suntest CPS system

The xenon arc lamp was situated horizontally between two parabolic reflectors (Fig. 3.8). A quartz glass dish fitted with an infra red radiation (IR) filter (reflective surface) that transmitted UV and visible radiation was situated below the lamp.
Fig. 3.8 The xenon arc lamp (cross sectional view) as installed in the Suntest CPS unit. Where

1 = Xenon burner
2 = UV mirror
3 = Visible light mirror
4 = Quartz-glass dish with reflective coating
5 = Additional filter (UV glass or special glass)
6 = Parabolic reflector
7 = Sample level
UV and visible components of the xenon radiation emitted vertically were reflected back onto the sample platform (tank) by a mirror situated above the lamp. This mirror transmitted the IR radiation on to the samples. All samples were held in a stainless steel tank (Zeneca Agrochemicals, Bracknell, Berkshire, UK) containing glycerol (Parker, pers comm.). The quartz tubes containing the samples were placed into metal holders immersed in the glycerol. Glycerol was used in preference to water to avoid loss of coolant due to evaporation.

The temperature of the glycerol was maintained by being recirculated through an industrial chiller (Thermo circulator unit 05/CTCHG, Conair Churchill Ltd., Uxbridge Middlesex, UK) then back to the sample tank via insulated polyethylene tubing. The temperature of the glycerol was monitored using four thermocouples fixed at different locations within the tank (location 1-next to the glycerol inlet; location 2-next to the glycerol outlet, locations 3 and 4 were spaced equi-distant between the glycerol inlet and outlet) connected to a data logger (Squirrel meter, Grant Instruments Ltd., Cambridge, UK) that recorded the temperature every 30 s. The xenon arc lamp and subsequently the air within the Suntest unit was cooled by an integral fan.

Prior to sample irradiation, the lamp and chiller unit were switched on for one hour to allow the lamp to reach its maximum radiation intensity and to allow the glycerol to equilibrate to the desired temperature. The tank holding the tubes was positioned directly under the lamp (at a distance of approximately 20 cm) using a laboratory jack. The radiation intensity of the xenon lamp was measured prior to the present experiment by Smith (unpublished data). The light intensity was measured using a UDT 81 optometer fitted with a broad band filter that integrated total energy from 200 to 800 nm at an incident with the quartz tubes at seven locations across the tank (Fig. 3.9) and at all eight xenon lamp potentiometer settings, as described by Ali (1994). The results showed a linear increase between lamp potentiometer setting and radiation intensity for each location (Fig. 3.10).
Fig. 3.9 A front view of the xenon arc lamp compartment of the Suntest CPS unit showing the seven locations at which the radiation intensity measurements were taken under the lamp (Smith, *pers comm.*).
Fig. 3.10 Light intensity measurements taken at seven locations under the xenon arc lamp, over the range of potentiometer settings (Smith, unpublished data).
The intensity varied depending on the location, yet this variation was less than 5% for any combination of locations. Hence, the mean value was accepted as the radiation intensity incident on the sample surface.

3.6.2.3.1 Extrapolation of the photodegradation period to Florida summer sunlight

In order to relate the rate of astaxanthin degradation caused by its exposure to the filtered xenon arc lamp, the lamps light intensity was compared with the intensity of Florida (latitude 30° North) summer sunlight (Smith, pers comm.). Assuming a 12 hour light day equation 3.ii was used to calculate the intensity of the xenon arc lamp in terms of Florida sunlight (Smith, pers comm.).

3.ii.  

\[
1 \text{ Florida day} = \frac{(0.75 \times 12 \times 20.13)}{0.335 \times Z}
\]

Where: \(Z\) = The intensity of the xenon arc lamp incident on the sample surface

The mean (200-800) nm light intensity at lamp potentiometer setting 1 (used in the present experiment) was 720 W m\(^{-2}\) (Smith, unpublished data). Selected equivalent Florida days and hours sunlight to which the astaxanthin samples were exposed, are shown in Table 3.11, determined using equation 3.ii.

Table 3.11 Xenon arc lamp exposure times and their equivalent Florida (latitude 30° North) summer sunlight times.

<table>
<thead>
<tr>
<th>Exposure time (hours)</th>
<th>Florida days</th>
<th>Florida hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75</td>
<td>1.00</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>2.67</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>5.33</td>
<td>64</td>
</tr>
<tr>
<td>6</td>
<td>8.00</td>
<td>96</td>
</tr>
<tr>
<td>8</td>
<td>10.67</td>
<td>128</td>
</tr>
</tbody>
</table>
3.6.2.4 Astaxanthin extraction methods

3.6.2.4.1 Astaxanthin standards

At each sampling, 3 ml of each standard was pipetted into a 20 ml glass vial to which 1 ml methanol containing 500 ppm BHT was added and mixed (Whirlimixer, Fisons, UK) for 15 s. 1 ml distilled water was added to the sample and mixed for a further 15 s. All samples were incubated in the dark for 10 min at room temperature (20°C), mixed for a further 15 s and then centrifuged (Mistral 3000, Fisons, UK) for 10 min at 3000 rpm, at 16°C. After centrifugation a 3 ml aliquot of hypophase was filtered using PTFE syringe tip filters (0.45 μm; Phenomenex, UK) directly into amber sample vials and immediately sealed. The samples were stored at -20°C until analysed. Prior to analysis the presence of any moisture was extracted by adding 0.5 g of anhydrous sodium sulphate (Na₂SO₄) and filtered using PTFE syringe tip filters (0.45 μm; Phenomenex, UK). The samples containing free astaxanthin (all-£-trans crystalline astaxanthin and Carophyll Pink™) were quantified by HPLC. Those samples containing H. pluvialis total carotenoid extract were quantified spectrophotometrically (section 2.6.1).

3.6.2.4.2 Oil samples

At each sampling, 1 ml of oil was pipetted into a 20 ml glass vial to which 1 ml methanol containing 500 ppm BHT and 1 ml distilled water was added and mixed (Whirlimixer, Fisons, UK) for 15 s. 3 ml dichloromethane was added to the sample and mixed for a further 15 s. Then as described in the second paragraph of section 3.6.2.4.2 (astaxanthin standards).

3.6.3 Results

Figure 3.11 displays the percentage loss of carotenoids from an all-£-trans crystalline astaxanthin standard (treatment 1: Ax-Std), a H. pluvialis (NatuRose™) total carotenoid...
extract standard (treatment 2: NR-Std), Carophyll Pink™ dissolved in cod liver oil (treatment 3: CP in oil) and H. pluvialis (NatuRose™) cells suspended in cod liver oil (treatment 4: NR in oil) when exposed to artificial sun light. Samples containing either the all-E-trans crystalline astaxanthin standard or Carophyll Pink™ were assessed for their free astaxanthin content, whereas those containing either H. pluvialis (NatuRose™) cells or its extract were assessed for their total carotenoid content. Samples were taken at zero hours (the start of the experimental period) then at various intervals and finally at 8 hours (on termination of the experiment). All four treatments showed continuous degradation throughout the experimental period. After 2 hours exposure (equivalent to 32 hours Florida summer sunlight), treatment 1 (Ax-Std), exhibited significantly (P<0.01) more degradation than treatment 2 (NR-Std), and treatment 3 (CP in oil) showed significantly (P<0.01) more degradation than all the other treatments. However, there was no significant difference (P>0.05) in the amount of degradation between treatments 2 and 4 (NR in oil), and between treatments 1 and 4. This represented a carotenoid loss of 9.57, 10.81, 12.20 and 23.67 % for treatments 2, 4, 1 and 3, respectively. After 4 hours exposure (equivalent to 64 hours Florida summer sunlight), treatments 1 and 2 exhibited significantly (P<0.01) more degradation than treatment 4, and again treatment 3 showed significantly (P<0.01) more degradation than all the other treatments. However, there was no significant difference (P>0.05) in the amount of degradation between treatments 1 and 2. This represented a carotenoid loss of 15.01, 18.52, 19.32 and 40.40 % for treatments 4, 1, 2 and 3, respectively. After 6 hours (equivalent to 96 hours Florida summer sunlight) exposure all the treatments showed significantly different (P<0.001) rates of degradation from each other.
Fig. 3.11 Carotenoid degradation of astaxanthin during exposure to solar radiation emulated by a filtered xenon arc lamp (Suntest CPS); n=3, ± standard deviation of the mean (error bars to small to be identified). Where:

Ax Std = Crystalline all-\(E\)-trans astaxanthin standard dissolved in 4.5% dichloromethane (DCM) in hexane

NR Std = All carotenoids extracted from NatuRose™ using 4.5% DCM in hexane and used as a standard for *Haematococcus pluvialis*

CP in Oil = Carophyll Pink™ solution mixed with cod liver oil

NR in Oil = NatuRose™ cells suspended in cod liver oil
Where treatment 1 (Ax-Std) exhibited significantly (P<0.001) more degradation than treatment 4 (NR in oil), treatment 2 (NR-Std) showed significantly (P<0.001) more degradation than treatments 1 and 4, and treatment 3 (CP in oil) once more showed significantly (P<0.001) more degradation than all the other treatments. This characterised a carotenoid loss of 14.17, 26.57, 31.01 and 43.51 % for treatments 4, 1, 2 and 3, respectively. The final samples taken from all treatments after 8 hours exposure (equivalent to 128 h Florida summer sunlight) demonstrated further degradation, and followed the same trends and significant differences as seen for those samples taken after 6 h exposure. This represented a carotenoid loss of 19.25, 35.33, 41.90 and 45.43 % for treatments 4, 1, 2 and 3, respectively.

3.6.4 Discussion

It is evident from the results of the percentage loss of carotenoids from synthetic (Carophyll Pink™) and natural (H. pluvialis, NatuRose™) sources of astaxanthin when exposed to simulated sun light that carotenoid degradation occurred regardless of the media (organic solvents or cod liver oil) in which they were “dissolved”. As in experiment 3.4 (dietary carotenoid loss due to water erosion), all four treatments displayed an exponential trend for carotenoid degradation due to light exposure. This was again confirmed by the high regression coefficients (R² values; Fig. 3.11) gained for all the treatments. This was further confirmed by plotting log concentration against time, which demonstrated the degradation reaction of all the treatments followed first order kinetics as demonstrated by Mortensen and Skibsted (1997) using pulsed laser light to degrade astaxanthin dissolved in chloroform. The overall rate of carotenoid degradation was significantly different (P<0.01) for all the treatments tested, where treatment 3 (Carophyll Pink™ in oil) > treatment 2 (NatuRose™ standard) > treatment 1 (Astaxanthin standard) > treatment 4 (NatuRose™ in oil). Thus, treatment 3 demonstrated the greatest degradation
rate and treatment 4 the lowest. Treatment 4 demonstrating the lowest rate of carotenoid
degradation is rationalised by the fact that it contained NatuRose™ (suspended in cod liver
oil) which section 4.4 illustrates (using electron microscopy) to be composed of intact H.
pluvialis cells whose walls contained small vacuoles. Therefore, the presence of the cell
wall provides protection for the extrachloroplastic secondary carotenoid (astaxanthin) lipid
vacuoles which reside in the protoplast (Zlotnik et al., 1993). Many workers suggest the
transformation of green H. pluvialis cells in a vegetative state into immotile aplanospores
that commence production of secondary carotenoids and the development of a cell wall is
initiated by environmental stress (Borowitzka et al., 1991; Chaumont and Thépenier, 1995;
Harker et al., 1995; Lee and Ding, 1995 and Millard et al., 1993). Excessive irradiation is
proposed as one of the major initiating factors in the production of secondary carotenoids
(Hagen et al., 1993; Hagen et al., 1994 and Fan et al., 1994). Thus, the protection afforded
by the aplanospore cell wall against intense irradiation is ironically, only possible due to
the vegetative cells only producing a cell wall after being excessively irradiated. The
degradation of astaxanthin may be due to the production of highly reactive oxygen species
such as the hydroxyl radical (HO•) formed by an iron catalysed Fenton reaction (Fe²⁺ +
H₂O₂ → OH⁻ + HO• + Fe³⁺), as described by Kobayashi et al., (1993) who found an Fe²⁺
enriched media enhanced carotenoid formation, determining iron to be an integral
component of the media. Iron is a mandatory element required by all living cells as it is an
active participant in numerous metabolic pathways, especially catalysis (Lagocki, 1995).
The main catalytic advantage of iron resides in its redox properties which may be altered
by its interaction with co-ordinating ligands, enabling a variation in the ease in which it
accepts electrons (Lagocki, 1995). However the catalytic advantages of iron also make it
potentially toxic, as reactions engaging oxygen prefer a univalent reductive pathway (as in
the Fenton reaction) which results in free radical formation, manifesting as oxidative stress.
As the addition of a pro-oxidant (Fe²⁺) is advocated to increase the carotenoid content of H.
pluvialis aplanospores, a potential irony could exist for astaxanthin. Astaxanthin (the most
powerful antioxidant known to date) is possibly degraded to relieve oxidative stress initiated by light operating in synergy with pro-oxidants to form several free radicals species such as the super oxide radical ($O_2^-$) and the hydroxyl radical (HO•). This may have caused the 19 % reduction in the total carotenoid concentration after 8 hours irradiance (equivalent to 128 hours Florida summer sunlight).

Treatment 1 containing all-E-trans crystalline astaxanthin prepared as a standard dissolved in 4.5 % dichloromethane in hexane displayed an increased carotenoid degradation rate as compared with treatment 4 (NatuRose™ in oil). This is rationalised by the fact that all the astaxanthin present was in the free form and afforded no protection from cod liver oil or the presence of a cell wall. Thus, the 36 % degradation observed after 8 hours irradiance was probably only due to photo-oxidation, and not chemical oxidation. The chemical oxidative products of astaxanthin are semi-astacene and astacene, neither of which was detected in any of the samples taken from any of the treatments. However, it is possible that semi-astacene and astacene were subject to further photo-oxidation, which resulted in the number of double carbon bond in their chromophore being less than six, rendering them colourless. The photo-oxidative products of astaxanthin were not quantified by either HPLC or spectral analysis because the products were not identified. Under spectral analysis, with increased exposure to light the samples revealed shouldering in the UV region. Nevertheless, these could not be quantified as the extinction coefficients of these compounds were unknown. Likewise, for quantification by HPLC, external standards of known concentration are required. The HPLC analysis did not reveal any degradation products, because they demonstrated absorption in the UV region, (as a result are colourless) and the HPLC detector was set at 470 nm (visible region). Lack of detection may also be due to some of the degradation products being volatiles, which would have been released into the atmosphere at each sampling, when the stoppers were removed from the quartz test tubes.
Treatment 2 containing the *H. pluvialis* standard obtained the second highest rate of carotenoid degradation. This standard was employed to demonstrate whether astaxanthin esters are also susceptible to photo-oxidation. In this context, the results were as predicted with the *H. pluvialis* standard showing a 217% higher loss of total carotenoids than the *H. pluvialis* oil suspension (treatment). This is rationalised by the carotenoids in the *H. pluvialis* standard not having the protection of a cell wall. This increased rate of carotenoid degradation as compared with treatment 1, could be linked to the profile of the non-carotenoid moiety (R groups) of the astaxanthin esters, which constitute 85% of the total carotenoid content. Certain R groups to which the astaxanthin is ester bonded may act as sensitisers to the organic solvents (4.5% dichloromethane and 95.5% hexane) in which the carotenoids were extracted (from the *H. pluvialis* cells). If any of the R groups did act as sensitisers then it is conceivable for them to initiate oxidation of all forms of astaxanthin (free, mono-ester and di-ester) present, in addition to the photo-oxidation caused during irradiance. All these factors probably work synergistically including the aforementioned role of Fe²⁺ to increase the overall rate of carotenoid degradation that resulted in 42% of the total carotenoid being degraded.

Treatment 3 contained Carophyll Pink™ (50 mg kg⁻¹, free astaxanthin) dissolved in cod liver oil, as used to top-coat the reference diet (positive control) in three of the five feeding trials. This treatment displayed the greatest rate of carotenoid degradation resulting in 45% loss of free astaxanthin after 8 hours irradiance. This elevated loss, as compared to the all-\(E\)-trans astaxanthin standard (treatment 1), is possibly due to the 1:3, \(cis : trans\) isomeric ratio of astaxanthin in the Carophyll Pink™ product, where the cis-isomers more readily undergo photolysis. More likely, the elevated rate of pigment degradation is due to component a of the cod liver oil acting as sensitisers, as discussed previously.
Experiment 3.7 Image analysis of the SalmoFan™ as a standard for rainbow trout fillets.

3.7.1 Introduction

The rate of deposition of astaxanthin in salmonid epaxial white muscle depends upon many factors including age, weight, feeding rate and genetic disposition (Choubert et al., 1997). Nevertheless, for salmonid producers faced with increased global demand for salmon and rainbow trout, product quality is paramount, with salmonid flesh colour being an important criterion used for marketing (Torrissen and Christiansen, 1995). This has given rise to the necessity to distinguish between variance in flesh tones, as different export markets have distinct preferences for flesh pigmentation (Christiansen et al., 1995a).

Traditionally, the measurement of perceived colour of fillets and steaks has either been carried out by trained sensory panels using colour cards (standardised colour surfaces, introduced in 1988 by Hoffmann-La Roche) or instrumentally by a chromameter (Minolta Limited, Milton Keynes, UK) to measure reflectance (Christiansen et al., 1995b; Johnsen and Whatne, 1990). However, each method is beset with problems. The Roche Colour Card™ was developed for the salmonid industry to enable farmers to be able to determine the colour of salmon flesh quickly and inexpensively (Christiansen et al., 1995b). The results from the colour card are influenced by the presentation and illumination of the fillets and differences in people’s visual perception of colour, making this system very subjective (Foss et al., 1987). Roche colour score values are quoted on the basis of whole numbers, with the Roche Colour Card™ incorporating two scoring systems. The first scoring system numbered 1-8 was used to score steaks and the other numbered 11-18 was used to score fillets (Smith et al., 1992). The Colour Card™ was superseded in 1997 by the introduction of the SalmoFan™ system, consisting of a fan with 15 different coloured blades numbered 20 – 34 (Plate 3.1), being applicable for both steaks and fillets. The numerical values on the SalmoFan™ are arbitrary, where a score of 20 colour score units...
(CSU) represents very pale pink flesh and a score of 34 CSU a very intense pink/red flesh colour, making its use qualitative. Whereas, the aforementioned chromameter is a quantitative method that can accurately measure the colour of the fillets, taking into account all the different hues available. It also provides reproducible, accurate data logging that can be statistically analysed. The chromameter relies on measuring the reflectance of light shone on to the surface being measured. In order to collect enough available reflected light the iris requires a minimum diameter of 3mm (an area of 7.07 mm²). The maximum aperture for the chromameter is 15 mm (an area of 176.71 mm²), which limits its use, as several readings are required at different points along the fillet, traditionally being taken at the head, below the dorsal fin and the tail (Nickell and Bromage, 1998b). When being used on fillets, the iris covers an area that may encompass white coloured intramuscular fat that is deposited between muscle myotomes. Measuring the colour of the intramuscular fat with epaxial muscle obviously produces biased results.

An alternative way to measure the colour and deposition of astaxanthin in salmonid fillets and steaks is by image analysis. In this method a still image is acquired by a colour camera linked to a computer controlled analysing system. As the camera has focus and zoom functions it may easily be manipulated to acquire an image of the whole fillet. The camera is used to acquire colour information (chromaticity) as a single complex number, Z for each pixel (Frey, pers comm.). In this method the fillet is acquired as separate red (R) green (G) and blue (B) images. The intensity of each colour component (RGB) is measured separately, represented as grey values, which range from 0 (black) to 256 (white saturation), giving three sets of data for each fillet (Russell, pers comm.). The whole image is divided into a number of transects allowing any spatial distribution of colour to be observed. Once the image of a fillet has been digitised and stored, further analysis may be carried out to separate (independently measure) the white appearance of the lipid deposits between the myotomes, enabling a more representative colour of the fillet to be determined.
Plate 3.1. The colour variation observed between the 15 blades (colour scores 20-34) that constitute the SalmoFan™ (Hoffmann-La Roche, Basel, Switzerland) gradation.
3.7.2 Materials and methods

3.7.2.1 Experimental design

The individual blades of the SalmoFan™ were separated and the red, green and blue colour components of each blade were analysed from an image acquired using a camera linked to a computer. The data obtained identified which blades would be used as calibrants when applying this technique to rainbow trout fillets. This permitted a programme (macro) to be written allowing the colour of rainbow trout fillets and its distribution throughout the fillet to be quantitatively analysed.

3.7.2.2 Image analysis

Images of the blades were acquired via a Hitachi HV-C20 3CCD camera and Canon TV zoom lens positioned 1 m above the SalmoFan™ leaflets. Illumination was provided by a 100 w tungsten bulb set at 45° at a distance of 500 mm. The camera was linked to the image analyser (Quantimet Q570, Cambridge instruments, Cambridge, UK) via three leads (one for each colour component) (Fig. 3.12). The macro (a program written to drive the Quantimet image analyser program to perform specific tasks) was then used to set both gain (contrast, c) and offset (brightness, b) for each of the three colour components of the camera, red (R), green (G) and blue (B). This calibration of the three contrasts were then used to set the same chromaticity for every run. The six settings ($R_c, R_b, G_c, G_b, B_c$ and $B_b$) used in this first macro were then incorporated into a second macro, to be used when analysing fish fillets.
Fig. 3.12 The general layout of the Quintamet C 570 image analyser. Where

1 = Light source, angle poise lamp set at 45° containing a 100 W tungsten bulb
2 = Hitachi colour camera with 3 leads (red, green and blue) connected to the image processor
3 = Colour television to observed the image acquired by the camera
4 = Computer containing the image processor
3.7.3 Results

The results gained from analysing the SalmoFan™ using image analysis are shown in Fig. 3.13. Directly below the analysis is a grey wedge of a linear grey change for all 256 grey levels available. The three colour components [red (R), green (G) and blue (B)] of the image are further analysed graphically (Fig. 3.13). It is obvious that the change in both green and blue components of the individual SalmoFan™ leaflets is relatively constant (linear) for all 15 Roche colour scores, whereas the red component is far more irregular, even reversing the apparent difference at two leaflets (RCS 24 and 26).
Fig. 3.13 The SalmoFan™ as imaged by the 3 CCD colour camera for each colour component. The three colours (RGB) are displayed below as grey scale images. The lower greyscale is a ‘wedge’ generated by the Q570 to illustrate a linear grey scale change from 0 to 256. The graph displays the grey level results from the SalmoFan™ using the individual colour components of the 3 CCD camera. The results are the average grey value for each fan blade as observed in the top image. In addition to the three colour components under tungsten illumination (CT3200/3600) there is also the red result under daylight illumination (CT5100/5300) Repeated sampling on the same image varied by less than 1%.
3.7.4 Discussion

Under image analysis, the results from the SalmoFan™ showed a surprising variation in the red component of the chromaticity of each blade. The rate of grey level change between blades varies greatly for red whilst the blue and green components are evenly spaced and very similar to each other. Using different colour temperatures makes no noticeable difference in the reflected grey level changes. Thus the red component of the SalmoFan™ is in irregular steps for chromaticity whereas both the blue and green are evenly spaced in grey change. When combined, the three images of red, green and blue provided a resulting colour image that was almost a perfect imitation of the SalmoFan™. The results enabled a second macro to be designed that enabled the acquisition of each colour component separately (at the correct gain and offset) for analysing the fillets used from trials 4.1 and 5.2. The analysis was performed by taking six transects down each fillet at evenly spaced intervals from anterior to posterior. The resulting grey levels for each of the three images were then saved to disk. Each file represents one of the three colour (RGB) components and having six transects of 185 pixels provides 1110 rows of data. These three files for each fillet were then merged into a spreadsheet for statistical analysis.
CHAPTER 4

The efficacy of *Haematococcus pluvialis* astaxanthin fed to rainbow trout in cold pressed laboratory diets (trial 4.1) and extruded commercial diets (trial 4.2)
4.1 Introduction

As mentioned in chapter 1 (section 1.3.3), the high cost of adding synthetic astaxanthin to nutritionally balanced formulated salmonid feeds, combined with consumer pressure to restrict the use of synthetic food additives, has led scientists to explore the use of more economical natural alternatives to pigment the flesh of farmed salmonid species. A wide range of species covering all three major taxonomical kingdoms (animal, plant and protista) contain astaxanthin in various chemical guises i.e. in its free form or either bound to fatty acids (esterified form) or proteins (carotenoproteins). The quantity and combination of astaxanthin forms varies greatly, being species dependent, some of which could potentially be used to pigment salmonid flesh. After a species has been identified and its carotenoid profile and astaxanthin concentration established, then the next logical step is to test the efficacy of the pigment source in vivo. As stated in section 1.3.3, a variety of natural materials have been evaluated as possible pigment sources for farmed salmonids, including paprika, dried flowers, krill, shrimp and crab waste (Sommer et al., 1992). Yet, due to a myriad of problems, all of the aforementioned natural sources have been dismissed as not being commercially viable. However, great interest is being shown in the microalga Haematococcus pluvialis, as it has many desirable assets for commercial production, such as a high pigment content of which astaxanthin is the primary constituent (Benemann, 1992). Most importantly, Haematococcus pluvialis is relatively easy and inexpensive to cultivate (Borowitzka, 1992). The in vivo testing of animal food additives (such as astaxanthin) entails designing and executing feeding trials whereby the experimental astaxanthin source is incorporated into test diets, which are then fed to replicate groups of salmonids, such as rainbow trout (Oncorhynchus mykiss). However, the molecular structure of astaxanthin dictates its chemical characteristics especially in terms of its stability. Unfortunately for astaxanthin, the system of conjugated double bonds within the carbon back bone that constitutes the chromophore (providing astaxanthin with its characteristic red hue) also renders it open to physical and chemical attack, being
readily oxidised and sensitive to temperature, acids and bases (Latscha, 1990). Therefore, it is prudent to test the stability of this additive (as with any other additive), not only after being incorporated within the matrix of a pelleted diet but also to test its stability whilst being processed. The results of experiment 3.2 demonstrated that astaxanthin from both sources (synthetic Carophyll Pink™ and natural _H. pluvialis_), when added as a raw micro ingredient prior to production of cold pressed diets, remained stable throughout the production process.

Cold pressed diets are produced in small batches (of up to 5 kg) at room temperature and under relatively low pressure (of up to 2 atmosphere) (Vassvik, _pers comm._). Cold pressed diets are produced to a specific recipe to obtain optimal growth within the constraints of the pellet matrix, in terms of the maximum inclusion of oil, which is determined by their method of production. Small batch production allows the conditions of production to be controlled within narrow limits to ensure minimal variation between diets containing different sources of astaxanthin. The scale and gentle production methods employed to manufacture cold pressed diets allows the ingredients to be thoroughly mixed, particularly with respect to the homogeneity of all the micro ingredients added to a diet. Another consideration is whether the experimental natural astaxanthin source will demonstrate a dose response similar to that of synthetic astaxanthin. Work carried out by Torrissen _et al._ (1989) established that the level of synthetic astaxanthin (Carophyll Pink™) presented to Atlantic salmon (_Salmo salar_) in their diet has a direct correlation to the concentration retained in their flesh. Trial 4.1, which used a commercial source of _H. pluvialis_ was designed to identify if different inclusion levels of _H. pluvialis_ in the diet would invoke a similar dose response as Carophyll Pink™.

One of the major problems associated with the use of _H. pluvialis_ is attaining the desired target level of carotenoids. This is because _H. pluvialis_ is batch cultured with each batch being slightly different with respect to its carotenoid content. Due to this fact, the producers of the commercial source of _H. pluvialis_ used in the present feed trials (4.1 and
4.2) rely on batch blending to attain the desired content (Lorenz, 1999). The primary aim of trial 4.1 is to test the efficacy of *Haematococcus pluvialis* in cold pressed diets as compared to a synthetic source of astaxanthin (Carophyll Pink™). However, commercial diets are not normally produced by cold pressing the ingredients, but employ extrusion technology that operates under high temperatures and pressures, allowing the formation of expanded diets whose pellets are more stable. The extrusion technology employed also allows increased production rates, enabling large batches (of up to several hundred tonnes) of a specific diet to be manufactured. Combining the fact that production conditions, pellet matrix and diet formulation of expanded diets greatly differ from those of cold pressed diets with the fact that fish farmers only feed expanded diets to their fish, dictates that the bioavailability of *Haematococcus pluvialis*-derived astaxanthin must also be assessed within an expanded diet matrix. Hence, the primary aim of trial 4.2 is to test the efficacy of *H. pluvialis* in a commercially extruded diet as compared to Carophyll Pink™.
4.2 Aims

The main aims of the present experiments were:

1. Assess the efficacy of *Haematococcus pluvialis* presented in cold pressed diets (Trial 4.1).

2. Identify a dose response (retention of astaxanthin in epaxial muscle) to astaxanthin dietary inclusion levels for a natural source *Haematococcus pluvialis* (NatuRose™) and a synthetic source Carophyll Pink™ (Trial 4.1).

3. Assess the efficacy of an experimental source of *Haematococcus pluvialis* against a synthetic commercial source Carophyll Pink™ (Trial 4.1).

4. Assess the stability and efficacy of synthetic Carophyll Pink™ and algal (NatuRose™) sources of astaxanthin presented in commercially produced diets (Trial 4.2).

5. Assess the stability of synthetic Carophyll Pink™ and algal NatuRose™ astaxanthin exposed to simulated commercial extruder conditions (Trial 4.2).
4.3 Materials and Methods

4.3.1 Experimental designs

4.3.1.1 Trial 4.1

Five hundred and forty rainbow trout were divided into 12 tanks (45 fish tank\(^{-1}\), utilising 2 experimental systems: section 2.3). The fish were fed one of six diets containing different levels and sources of astaxanthin (Table 4.1) with two replicates (tanks) per diet (Fig. 4.1).

Table 4.1 Desired (target) astaxanthin content of the experimental diets.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Astaxanthin source</th>
<th>Target [Astaxanthin] (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Synthetic(^a)</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>Synthetic(^a)</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td><em>Haematococcus</em>(^b)</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td><em>Haematococcus</em>(^b)</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td><em>Haematococcus</em>(^c)</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>Control (non-pigmented)</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) Carophyll Pink\(^TM\) (Hoffmann-La Roche, Basel, Switzerland). \(^b\) NatuRose\(^TM\) (Cyanotech Corporation, Hawaii, USA). \(^c\) Experimental *Haematococcus pluvialis* (John Moores University, Liverpool, UK).

Fish were sampled at four intervals during the experimental period, 20 fish were used in sample one (week 0), 36 in sample two (at week 4), 36 in sample three (week 8) and 120 used in sample four (week 12) on termination of the trial. At each sampling time the perceived colour of the fillets of each of the randomly selected fish was recorded using the SalmoFan\(^TM\) (section 2.5.3) and the concentration of astaxanthin in the epaxial white muscle was quantified by HPLC (section 2.5.5) against external standards (section 2.6). This data was used to calculate the feed conversion ratio (section 2.4.3), specific growth rate (section 2.4.3) and net apparent retention of astaxanthin (section 2.7). In addition, the fillets were subject to image analysis (section 4.3.3), and the rate of muscle related drip loss was also assessed (section 4.3.4). Also, the three sources of astaxanthin were observed using scanning electron microscopy (section 4.3.5).
Fig. 4.1 A schematic plan of the two recirculation systems used in trial 4.1, in relationship to the distribution of experimental diets amongst the tanks. Where:

- Diet 1 = 50 mg kg\(^{-1}\) Carophyll Pink\(^{TM}\) (synthetic astaxanthin)
- Diet 2 = 10 mg kg\(^{-1}\) Carophyll Pink\(^{TM}\)
- Diet 3 = 50 mg kg\(^{-1}\) NatuRose\(^{TM}\) (Haematococcus pluvialis)
- Diet 4 = 10 mg kg\(^{-1}\) NatuRose\(^{TM}\)
- Diet 5 = 50 mg kg\(^{-1}\) Liverpool John Moores experimental Haematococcus pluvialis
- Diet 6 = Unpigmented control
4.3.1.2 Trial 4.2

Three hundred and sixty rainbow trout were divided into 12 tanks (30 fish tank\(^{-1}\), utilising 2 experimental systems: section 2.3). The fish were fed one of three diets containing different levels and sources of astaxanthin (Table 4.2) with four replicates (tanks) per diet (Fig. 4.2).

Table 4.2 Desired (target) astaxanthin content of the experimental diets.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Astaxanthin source</th>
<th>Target [Astaxanthin] (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Synthetic(^{a})</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td><em>Haematococcus</em>(^{b})</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td><em>Haematococcus</em>(^{b})</td>
<td>70</td>
</tr>
</tbody>
</table>

\(^{a}\) Carophyll Pink™ (Hoffmann-La Roche, Basel, Switzerland) control diet. \(^{b}\) *Haematococcus pluvialis* NatuRose™ (Cyanotech, Hawaii, USA).

Fish were sampled at four intervals during the experimental period, 20 fish were used in sample one (week 0), 36 in sample two (at week 4), 36 in sample three (week 8) and 120 used in sample four (week 12) on termination of the trial. At each sampling time the perceived colour of the fillets of each of the randomly selected fish was recorded using the SalmoFan™ (section 2.5.3) and the concentration of astaxanthin in the epaxial white muscle was quantified by HPLC (section 2.5.5) against external standards (section 2.6). This data was used to calculate the feed conversion ratio (section 2.4.3), specific growth rate (section 2.4.3) and net apparent retention of astaxanthin (section 2.7). No unpigmented control diet was required for trial 4.2, as the fish in trial 4.1 after 12 weeks of receiving diet 6 (unpigmented control) retained no detectable carotenoids in their flesh.
Fig. 4.2 A schematic plan of the two recirculation systems used in trial 4.2, in relationship to the distribution of experimental diets amongst the tanks. Where:

Diet 1 = 50 mg kg\(^{-1}\) Carophyll Pink\(^{TM}\) (synthetic astaxanthin)
Diet 2 = 50 mg kg\(^{-1}\) NatuRose\(^{TM}\) (Haematococcus pluvialis)
Diet 3 = 70 mg kg\(^{-1}\) NatuRose\(^{TM}\)
4.3.2 Diet preparation

The composition of the basal diets for trial 4.1 (Table 4.3) and trial 4.2 (Table 4.4) were designed to promote optimal growth, under simulated practical conditions.

Table 4.3 Basal diet formulation for trial 4.1.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Inclusion (g kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT-94 Fish-meal a</td>
<td>540.0</td>
</tr>
<tr>
<td>Wheat-meal b</td>
<td>185.3</td>
</tr>
<tr>
<td>Soybean (Solvent Extracted) c</td>
<td>150.0</td>
</tr>
<tr>
<td>Fish Oil d</td>
<td>110.0</td>
</tr>
<tr>
<td>Vitamin Premix d</td>
<td>5.0</td>
</tr>
<tr>
<td>Mineral Premix d</td>
<td>4.0</td>
</tr>
<tr>
<td>Chromium Oxide er</td>
<td>2.5</td>
</tr>
<tr>
<td>Choline Chloride e</td>
<td>2.0</td>
</tr>
<tr>
<td>Yttrium Oxide er</td>
<td>1.0</td>
</tr>
<tr>
<td>Inositol e</td>
<td>0.2</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
</tr>
</tbody>
</table>

a Norsildmel, Bergen, Norway; b Pascoe’s Ltd., Bridgewater, Somerset, UK (Astaxanthin was added at the expense of extruded wheat); c Cherwell Valley Silos Ltd. Twyford, Oxon, UK; d Biomar Ltd., Grangemouth, UK; e Sigma Chemical Co., Poole, Dorset, UK; f inert marker.

For trial 4.1, each diet was produced in 3 kg batches (University of Plymouth) mixed using a commercial (3 kg capacity) blender (Hobart A-120, London, UK) and cold pressed using 3-mm dies (Hobart A-120, London, UK). The synthetic astaxanthin (Carophyll Pink™) contained 8% (dry weight) astaxanthin immobilised in a gelatine-starch matrix that was pre-treated, by adding 25 ml distilled water to 1.875 g and 0.375 g of the Carophyll Pink™ product for diets 1 (50 mg kg⁻¹ astaxanthin) and 2 (10 mg kg⁻¹ astaxanthin), respectively. The Carophyll Pink™ solution was heated to 50°C using a temperature controlled water bath (Sonimatic, Langford Ultrasonics, West Midlands, UK) in order to dissolve its gelatine-starch matrix. The Carophyll Pink™ solution was then added to the feed mix prior to being cold pressed. The diets containing Haematococcus pluvialis derived astaxanthin were mixed into the appropriate diets in its native form prior to cold pressing. The commercial source of H. pluvialis (NatuRose™) added to diets 3 and 4 had a total carotenoid content of 1.5% (dry weight). Thus, 10 g and 2 g of NatuRose™ was added to
the dietary ingredients to produce 3 kg of diets 3 (50 mg kg\(^{-1}\) astaxanthin) and 4 (10 mg kg\(^{-1}\) astaxanthin) respectively. The experimental *H. pluvialis* produced by Liverpool John Moores University had a total carotenoid content of 1.00%. Thus, 15 g of LJM *H. pluvialis* was added to the dietary ingredients to produce 3 kg of diet 5 (50 mg kg\(^{-1}\) astaxanthin).

The formulation of the basal diet used in trial 4.2 was based on a commercial diet, being determined by work carried out by Biomar Ltd.

Table 4.4 Basal diet formulation for trial 4.2.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Inclusion (g kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT-94 Fish-meal(^{a})</td>
<td>380.0</td>
</tr>
<tr>
<td>Norsemink Fish-meal(^{a})</td>
<td>170.0</td>
</tr>
<tr>
<td>Wheat Gluten(^{b})</td>
<td>50.0</td>
</tr>
<tr>
<td>Wheat-meal(^{bc})</td>
<td>196.5</td>
</tr>
<tr>
<td>Fish Oil(^{b})</td>
<td>190.0</td>
</tr>
<tr>
<td>Vitamin Premix(^{b})</td>
<td>7.5</td>
</tr>
<tr>
<td>Mineral Premix(^{b})</td>
<td>6.0</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
</tr>
</tbody>
</table>

\(^{a}\) Norsildmel, Bergen, Norway; \(^{b}\) Biomar Ltd., Grangemouth, UK; \(^{c}\) Astaxanthin was added at the expense of extruded wheat

The diets for trial 4.2 were produced in 25 kg batches using an experimental (1000 kg capacity) commercial extruder (Biomar, Grangemouth, UK). Diets 1 targeting 50 mg kg\(^{-1}\) synthetic astaxanthin (Carophyll Pink\(^{TM}\)) was pre-treated by heating, in order to dissolve its starch-gelatine matrix. 16.63 g of the Carophyll Pink\(^{TM}\) product was added to 200 ml distilled water and heated to 50°C to form an astaxanthin solution. The Carophyll Pink\(^{TM}\) solution was then added to the feed mix prior to being extruded. The diets containing *H. pluvialis* derived astaxanthin were mixed into the appropriate diets in its native form prior to extrusion. The NatuRose\(^{TM}\) added to diets 2 and 3 had a total carotenoid content of 1.25% (dry weight). Thus, 12 g and 16.8 g of NatuRose\(^{TM}\) was added to diets 2 (50 mg kg\(^{-1}\) astaxanthin) and 3 (70 mg kg\(^{-1}\) astaxanthin) respectively. The carotenoid (astaxanthin) content of all the experimental diets was extracted using the Soxtherm method (section
2.5.1) and then analysed by HPLC. The fish in both trials followed a fixed feeding regime (section 2.4.3).

4.3.3 Image analysis
Images of the fillets from trial 4.1 sampled at week 12 (on termination of the trial), were acquired via a Hitachi HV-C20 3CCD camera and Canon TV zoom lens positioned 1 m above the right hand fillet. Illumination was provided by a 100 w tungsten bulb set at 45° at a distance of 500 mm (Fig. 3.12). Prior to analysis of the fillets, a macro was used to control the camera’s responsiveness to both chromaticity and brightness, and the SalmoFan™ was placed under the camera and used as a calibrant. The camera was linked to the image analyser (Quantimet Q570, Cambridge instruments, Cambridge, UK) via three leads one for each colour component red (R), green (G) and blue (B). The macro was then used to set both gain (contrast, c) and offset (brightness, b) for each of the three colour components of the camera, R, G and B. This adjustment of the three contrasts resulted in the same chromaticity for every run. The six settings (Rc, Rc, Gc, Gb, Bc and Bb) used in this first macro were then incorporated into a second macro. The second macro acquired each colour component separately at the correct gain and offset for analysing the fillet. The analysis was performed by taking six transects down the fillet at evenly spaced intervals from anterior to posterior. The resulting grey levels for each of the three images were then saved to a floppy disk. Each file represented one of the three colour (RGB) components and had approximately six transects of 185 pixels giving 1110 rows of data. These three files for each fillet were merged into an excel spreadsheet for statistical analysis.

4.3.4 Determination of exudative moisture loss (drip-loss)
The unskinned right hand fillet from each fish sampled at week 12 in trial 4.1 was weighed and placed into a re-sealable plastic bag containing a pre-weighed commercial absorbent pad (J. Sainsbury PLC, Plymouth, UK) and then frozen at -20°C (T350WS, Lec
Refrigeration PLC, UK). The fillets were defrosted for 24 hours in a domestic refrigerator (R250WS, Lec Refrigeration PLC, UK) at 4°C. After thawing the fillets were re-weighed once daily for 7 days, and stored at 4°C. The recorded weights were used to calculate the percentage moisture loss (Baker, 1996).

4.3.5 Scanning electron microscopy

The observation area of three aluminium stubs were coated with double sided adhesive carbon tape, to which 0.5 mg experimental *H. pluvialis* (Liverpool John Moores University), 0.5 mg of commercial *H. pluvialis* (NatuRose™) and 0.5 mg Carophyll Pink™ were applied to stubs 1, 2 and 3 respectively. The stubs were placed under vacuum and sputter coated with gold (K550, Emitech, UK). The surface topography of all three astaxanthin sources were observed using scanning electron microscopy at 25 kV (Jeol JSM – 5200, Japan). The images were recorded digitally using Semafore software (Jeol, Japan).

4.3.6 Statistical analysis

The statistical analyses used for growth, Roche colour scores and the retention of astaxanthin in white epaxial muscle is described in section 2.8. The statistical analyses for image analysis and drip loss were carried out using Minitab 12 statistics computer software. The data was subjected to a nested one way analysis of variance, with a significance level of P<0.05. Results are presented as n = 2 tanks ± standard deviation, unless otherwise stated. The means for dietary treatments were ranked by Duncans Multiple Range Test.
4.4 Results

4.4.1 Trial 4.1

The stability of synthetic and algal astaxanthin in cold pressed diets and their efficacy for pigmenting rainbow trout flesh were analysed in this trial. Dietary carotenoid content of the six experimental diets tested is presented in Table 4.5, being assessed as free astaxanthin content for diets 1 (50 mg kg\(^{-1}\) Carophyll Pink\(^{\text{TM}}\)) and 2 (10 mg kg\(^{-1}\) Carophyll Pink\(^{\text{TM}}\)) and as total carotenoid content for diets 3 (50 mg kg\(^{-1}\) NatuRose\(^{\text{TM}}\)), 4 (10 mg kg\(^{-1}\) NatuRose\(^{\text{TM}}\)), 5 (50 mg kg\(^{-1}\) Liverpool John Moores experimental \textit{Haematococcus pluvialis}) and 6 (unpigmented control). The diets were analysed at weeks zero, four, eight and twelve, reflecting the sampling of the fish. All the diets remained stable with no storage degradation of astaxanthin being detected over the twelve-week storage period. The three sources of astaxanthin (LJM \textit{H. pluvialis}, NatuRose\(^{\text{TM}}\) \textit{H. pluvialis} and synthetic Carophyll Pink\(^{\text{TM}}\)) used in the present experiment were observed using scanning electron microscopy. Plates 4.1 and 4.2, display scanning electron micrographs of an experimental \textit{Haematococcus pluvialis} cell produced by John Moores Liverpool University (LJM) and NatuRose\(^{\text{TM}}\) \textit{Haematococcus pluvialis} cells, respectively. The topography of LJM \textit{H. pluvialis} cells appears very regular and smooth without any disruption, whereas the topography of the NatuRose\(^{\text{TM}}\) cells appears irregular and perforated with several large fissures and cavities. Plate 4.3 displays a scanning electron micrograph of the surface topography of a single Carophyll Pink\(^{\text{TM}}\) bead, which clearly shows the bead to be composed of many small beadlets.
Table 4.5. Dietary content of total carotenoids for NatuRose™ and Liverpool John Moores experimental *Haematococcus pluvialis*; and free astaxanthin for Carophyll Pink™; n=3, ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Week</th>
<th>CP 50 (mg kg⁻¹)</th>
<th>CP 10 (mg kg⁻¹)</th>
<th>NR 50 (mg kg⁻¹)</th>
<th>NR 10 (mg kg⁻¹)</th>
<th>LJM (mg kg⁻¹)</th>
<th>Control (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50.84 ±1.54</td>
<td>9.52 ±0.51</td>
<td>51.58 ±1.26</td>
<td>9.78 ±0.63</td>
<td>0.15 ±0.07</td>
<td>0.16 ±0.03</td>
</tr>
<tr>
<td>4</td>
<td>51.32 ±2.05</td>
<td>10.31 ±0.73</td>
<td>51.34 ±1.40</td>
<td>10.61 ±0.84</td>
<td>0.16 ±0.04</td>
<td>0.18 ±0.06</td>
</tr>
<tr>
<td>8</td>
<td>49.46 ±0.79</td>
<td>10.48 ±1.02</td>
<td>50.32 ±1.05</td>
<td>10.23 ±0.93</td>
<td>0.17 ±0.05</td>
<td>0.17 ±0.04</td>
</tr>
<tr>
<td>12</td>
<td>51.08 ±1.25</td>
<td>9.79 ±0.32</td>
<td>50.83 ±0.81</td>
<td>10.46 ±1.17</td>
<td>0.17 ±0.03</td>
<td>0.14 ±0.06</td>
</tr>
</tbody>
</table>

*a 50 mg kg⁻¹ Carophyll Pink™; b 10 mg kg⁻¹ Carophyll Pink™; c 50 mg kg⁻¹ NatuRose™; d 10 mg kg⁻¹ NatuRose™; e 50 mg kg⁻¹ Liverpool John Moores experimental *Haematococcus pluvialis*; f Unpigmented basal diet*
Plate 4.1. A scanning electron micrograph of an *Haematococcus pluvialis* cell produced under experimental conditions at Liverpool John Moores University (Liverpool, Merseyside, UK) (magnification x 3500); the surface topography appears smooth and intact.
Plate 4.2. A scanning electron micrograph of NatuRose™ (Cyanotech, Kona, Hawaii, USA) *Haematococcus pluvialis* cells (magnification x 2000); the surface topography appears irregular and several cavities can clearly be distinguished.
Plate 4.3. A scanning electron micrograph of a single Carophyll Pink™ (Hoffmann-La Roche, Basel, Switzerland) bead (magnification x 350); which can clearly seen to be made up of small beadlets.
The growth performance of juvenile rainbow trout for each bi-weekly period over the course of the experimental feeding trial is presented in Table 4.6. The fish doubled in weight by the end of the twelve-week experimental period in accordance with their expected growth performance. Both specific growth rate (SGR) and feed conversion ratio (FCR) for rainbow trout were calculated over the 78 days of actual feeding. Both indices were indicative of an adequate growth performance and a satisfactory utilisation efficiency of the basal diet formulation employed for the delivery of the test pigmentation sources as compared to previous work by Lagocki (1995). These values were typically SGR (0.85-0.92) and FCR values (1.56-1.61) (Table 4.6), both indices showed no significant differences (P>0.05) between the diets.

The monthly deposition of colour (for each of the dietary treatments) in the flesh of rainbow trout assessed by the SalmoFan™ colour score index is summarised in Table 4.7. At week zero (initial sampling) all of the 20 randomly sampled fish were so pale (no noticeable pink colouration) that they could not be scored using the Roche colour score index (Plate 4.4). This was also true of the fish sampled at week four for all the dietary regimes. A notable visual pink colouration only became apparent in the fish fed diets 1 (Carophyll Pink™ 50 mg kg⁻¹), 2 (Carophyll Pink™10 mg kg⁻¹) and 3 (50 mg kg⁻¹ NatuRose™) after week eight and was slightly enhanced at week twelve. At week eight, diets 1 (CP 50), 2 (CP 10), and 3 (NR 50) obtained Roche Colour Scores of 22.8, 20.1 and 21.5 respectively. Diet 1 scored significantly (P<0.05) higher than the other pigmented diets (2 and 3). The fillets of those fish fed diets 4 (NR 10), 5 (LJM 10) and 6 (Control) were so pale that the SalmoFan™ was not applicable. At week twelve (representing the final sampling of fish for flesh colour assessment), those fish fed diets 2 and 4 (containing 10 mg kg⁻¹) obtained Roche Colour Scores (RCS) of 21.9, and 20.2 respectively (Plate 4.5).
Table 4.6. Mean weight (g) of fish, measured bi-weekly to assess growth, n=2 (tanks), ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1&lt;sup&gt;a&lt;/sup&gt; (g)</th>
<th>Diet 2&lt;sup&gt;b&lt;/sup&gt; (g)</th>
<th>Diet 3&lt;sup&gt;c&lt;/sup&gt; (g)</th>
<th>Diet 4&lt;sup&gt;d&lt;/sup&gt; (g)</th>
<th>Diet 5&lt;sup&gt;e&lt;/sup&gt; (g)</th>
<th>Diet 6&lt;sup&gt;f&lt;/sup&gt; (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90.84 ±2.36</td>
<td>91.31 ±2.86</td>
<td>91.58 ±3.02</td>
<td>92.17 ±2.91</td>
<td>91.04 ±1.87</td>
<td>92.36 ±2.58</td>
</tr>
<tr>
<td>2</td>
<td>101.76 ±2.59</td>
<td>101.48 ±1.72</td>
<td>104.34 ±3.47</td>
<td>102.81 ±2.44</td>
<td>103.03 ±2.64</td>
<td>101.89 ±2.60</td>
</tr>
<tr>
<td>4</td>
<td>120.46 ±4.27</td>
<td>117.91 ±2.33</td>
<td>118.32 ±5.05</td>
<td>123.17 ±3.23</td>
<td>122.59 ±4.77</td>
<td>122.79 ±3.41</td>
</tr>
<tr>
<td>6</td>
<td>131.58 ±5.79</td>
<td>135.53 ±5.61</td>
<td>132.83 ±5.81</td>
<td>134.46 ±4.88</td>
<td>132.51 ±5.38</td>
<td>134.84 ±4.06</td>
</tr>
<tr>
<td>8</td>
<td>147.81 ±5.37</td>
<td>147.22 ±3.75</td>
<td>151.65 ±7.23</td>
<td>151.64 ±8.21</td>
<td>145.09 ±6.17</td>
<td>150.74 ±7.20</td>
</tr>
<tr>
<td>10</td>
<td>164.58 ±3.11</td>
<td>163.87 ±5.98</td>
<td>168.11 ±9.11</td>
<td>170.35 ±6.83</td>
<td>160.45 ±6.09</td>
<td>169.37 ±5.82</td>
</tr>
<tr>
<td>12</td>
<td>181.35 ±7.74</td>
<td>180.52 ±6.41</td>
<td>184.57 ±9.37</td>
<td>189.05 ±8.60</td>
<td>175.82 ±7.96</td>
<td>188.01 ±9.59</td>
</tr>
</tbody>
</table>

SGR<sup>g</sup> 0.89 ±0.02 0.88 ±0.02 0.90 ±0.02 0.92 ±0.05 0.85 ±0.04 0.91 ±0.04
FCR<sup>h</sup> 1.58 ±0.03 1.59 ±0.03 1.58 ±0.05 1.56 ±0.06 1.61 ±0.02 1.56 ±0.04

<sup>a</sup> 50 mg kg<sup>−1</sup> Carophyll Pink™; <sup>b</sup> 10 mg kg<sup>−1</sup> Carophyll Pink™; <sup>c</sup> 50 mg kg<sup>−1</sup> NatuRose™; <sup>d</sup> 10 mg kg<sup>−1</sup> NatuRose™; <sup>e</sup> 50 mg kg<sup>−1</sup> Liverpool John Moores experimental Haematococcus pluvialis; <sup>f</sup> Unpigmented basal diet; <sup>g</sup> Specific growth rate calculated for the over all twelve-week experimental period; <sup>h</sup> Feed conversion ratio calculated for the over all twelve-week experimental period.
Table 4.7. Mean Roche Colour Scores for unskinned fillets, using the SalmoFan™, n=2 (tanks), ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1&lt;sup&gt;a&lt;/sup&gt; (CSU)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Diet 2&lt;sup&gt;b&lt;/sup&gt; (CSU)</th>
<th>Diet 3&lt;sup&gt;c&lt;/sup&gt; (CSU)</th>
<th>Diet 4&lt;sup&gt;d&lt;/sup&gt; (CSU)</th>
<th>Diet 5&lt;sup&gt;e&lt;/sup&gt; (CSU)</th>
<th>Diet 6&lt;sup&gt;f&lt;/sup&gt; (CSU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N/A&lt;sup&gt;h&lt;/sup&gt;</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>22.78 ±0.19</td>
<td>20.14 ±0.63</td>
<td>21.53 ±0.58</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>24.67 ±0.39</td>
<td>21.85 ±0.13</td>
<td>22.31 ±0.31</td>
<td>20.16 ±0.23</td>
<td>N/A</td>
<td>N/A&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> 50 mg kg<sup>-1</sup> Carophyll Pink™; <sup>b</sup> 10 mg kg<sup>-1</sup> Carophyll Pink™; <sup>c</sup> 50 mg kg<sup>-1</sup> NatuRose™; <sup>d</sup> 10 mg kg<sup>-1</sup> NatuRose™; <sup>e</sup> 50 mg kg<sup>-1</sup> Liverpool John Moores experimental Haematococcus pluvialis; <sup>f</sup> Unpigmented basal diet; <sup>g</sup> Colour Score Units (arbitrary units); <sup>h</sup> Not Applicable.
Plate 4.4. A representation of the typical flesh colour of a fish (rainbow trout) receiving an unpigmented (basal) diet at week 0 (all fish) and week 12 for those receiving 50 mg kg\(^{-1}\) Liverpool John Moores experimental *Haematococcus pluvialis.*
Plate 4.5. A representation of the typical flesh colour of a fish (rainbow trout) receiving a diet pigmented with either 10 mg kg\(^{-1}\) Carophyll Pink\(^{TM}\) (synthetic astaxanthin) or 10 mg kg\(^{-1}\) NatuRose\(^{TM}\) (\textit{Haematococcus pluvialis}) at week 12.
However, those fish fed diets 1 and 3 (containing 50 mg kg$^{-1}$) obtained an RCS of 24.67 and 22.3 respectively (Plate 4.6) The score for diet 1 was significantly (P<0.01) higher than the other diets. Also, the fish receiving diet 3 scored significantly (P<0.01) higher than diet 4 fed fish. The fish fed diets 5 (LJM 50) and 6 (control) remained unpigmented at week 12 on conclusion of the study.

Table 4.8 displays the mean colour (red component only) of the fillets (sampled at week 12), measured using image analysis for the duplicate groups of fish receiving each test diet. Results of the six transects are displayed sequentially with transects one and six representing the anterior (head) and posterior (tail) respectively. The results are expressed as grey values, which range from 0-256, where grey values of 0 and 256 are black and pure white, respectively. Several noticeable trends were apparent for all diets. The first and most noticeable trend being, the mean grey value for each successive transect decreased from the anterior to the posterior of each fillet. The second trend being, that no significant differences (P>0.05) were observed between transect 1, 2 or 3. The third trend, was that transects 5 and 6 were not significantly different (P>0.05) from each other but were significantly (P<0.05) lower than transects 1, 2 and 3. The grey value obtained for the mean of all six transects (overall mean) resulted in 187.03, 220.39, 206.43, 225.25, 239.88 and 242.20 for diets 1 (CP 50), 2 (CP 10), 3 (NR 50), 4 (NR 10), 5 (LJM 50) and 6 (control) respectively. The overall mean grey value for diet 1 was significantly (P<0.01) lower than all the other diets. Diet 3 obtained an over all grey value that was significantly (P<0.05) lower than diets 2, 4, 5 and 6. Likewise, the overall mean value for diet 2 was significantly (P<0.05) lower than diets 4, 5 and 6. Again, diet 4 obtained a significantly (P<0.05) lower overall grey value than diets 5 and 6. However, no significant differences (P>0.05) were observed for the overall mean grey values between diets 5 and 6.
Plate 4.6. A representation of the typical flesh colour of a fish (rainbow trout) receiving a diet pigmented with either 50 mg kg\(^{-1}\) Carophyll Pink™ (synthetic astaxanthin) or 50 mg kg\(^{-1}\) NatuRose™ (*Haematococcus pluvialis*) at week 12.
Table 4.8. Mean grey values (red component only) for the six transects measured from head to tail along the fillets of all the fish sampled at week 12, n=2 (tanks), ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Transect</th>
<th>Diet 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Diet 2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Diet 3&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Diet 4&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Diet 5&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Diet 6&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;g&lt;/sup&gt;</td>
<td>222.84 ±8.36</td>
<td>235.98 ±3.55</td>
<td>228.44 ±1.83</td>
<td>246.90 ±4.85</td>
<td>249.58 ±1.30</td>
<td>252.35 ±7.72</td>
</tr>
<tr>
<td>2</td>
<td>215.92 ±2.59</td>
<td>227.08 ±5.06</td>
<td>221.18 ±1.81</td>
<td>238.62 ±3.56</td>
<td>245.11 ±2.64</td>
<td>249.17 ±8.96</td>
</tr>
<tr>
<td>3</td>
<td>209.14 ±4.27</td>
<td>223.26 ±9.15</td>
<td>215.26 ±4.34</td>
<td>229.77 ±9.37</td>
<td>242.45 ±5.82</td>
<td>244.71 ±10.49</td>
</tr>
<tr>
<td>4</td>
<td>177.62 ±16.66</td>
<td>217.74 ±6.48</td>
<td>205.59 ±7.63</td>
<td>217.85 ±1.56</td>
<td>237.92 ±16.13</td>
<td>238.87 ±4.06</td>
</tr>
<tr>
<td>5</td>
<td>154.73 ±18.37</td>
<td>211.97 ±7.38</td>
<td>189.34 ±5.38</td>
<td>211.97 ±6.33</td>
<td>234.41 ±5.17</td>
<td>235.25 ±3.12</td>
</tr>
<tr>
<td>6&lt;sup&gt;h&lt;/sup&gt;</td>
<td>141.91 ±25.64</td>
<td>206.33 ±4.52</td>
<td>178.78 ±4.75</td>
<td>206.38 ±4.60</td>
<td>229.80 ±6.09</td>
<td>232.84 ±2.87</td>
</tr>
<tr>
<td>Mean&lt;sup&gt;i&lt;/sup&gt;</td>
<td>187.03</td>
<td>220.39</td>
<td>206.43</td>
<td>225.25</td>
<td>239.88</td>
<td>242.20</td>
</tr>
<tr>
<td>RCS&lt;sup&gt;j&lt;/sup&gt;</td>
<td>26</td>
<td>22</td>
<td>23</td>
<td>21</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

<sup>a</sup> 50 mg kg<sup>−1</sup> Carophyll Pink™; <sup>b</sup> 10 mg kg<sup>−1</sup> Carophyll Pink™; <sup>c</sup> 50 mg kg<sup>−1</sup> NatuRose™; <sup>d</sup> 10 mg kg<sup>−1</sup> NatuRose™; <sup>e</sup> 50 mg kg<sup>−1</sup> Liverpool John Moores experimental Haematococcus pluvialis; <sup>f</sup> Unpigmented basal diet; <sup>g</sup> Located at the head of each fillet; <sup>h</sup> Located at the tail of each fillet; <sup>i</sup> Mean value of all six transects (for the whole fillet); <sup>j</sup> Roche Colour Score (RCS) corresponding to the grey value mean.
Table 4.9 displays the mean white muscle astaxanthin concentrations of the duplicate groups of fish receiving each test diet. At week zero (initial sampling) none of the 20 randomly sampled fish had any detectable carotenoid present in their white epaxial muscle. At week four, diets 1 (Carophyll Pink™ 50 mg kg⁻¹), 2 (Carophyll Pink™ 10 mg kg⁻¹), 3 (50 mg kg⁻¹ NatuRose™) and 4 (10 mg kg⁻¹ NatuRose™) retained 0.09, 0.04, 0.09 and 0.04 mg kg⁻¹ astaxanthin in their white muscle, respectively. No carotenoids were detected in the white muscle of those fish fed either diet 5 (50 mg kg⁻¹ LJM H. pluvialis) or 6 (Control). The astaxanthin concentration of white muscle proved to be significantly different (P<0.001) between the 6 diets. A dose response was observed at weeks eight and twelve between diets 1 (Carophyll Pink™ 50 mg kg⁻¹) and 2 (Carophyll Pink™ 10 mg kg⁻¹). This resulted in an epaxial white muscle astaxanthin concentration of 1.68 and 0.31 mg kg⁻¹ at week eight, and 2.53 and 0.76 mg kg⁻¹ at week twelve for diets 1 and 2 respectively. Thus, fish fed diet 1 retained a significantly (P<0.01) higher concentration of astaxanthin than the fish fed diet 2 at weeks 8 and 12. A similar dose response also occurred between diets 3 (50 mg kg⁻¹ NatuRose™) and 4 (10 mg kg⁻¹ NatuRose™) at weeks eight and twelve. This resulted in a flesh astaxanthin concentration of 0.90 and 0.16 mg kg⁻¹ at week eight, and 1.52 and 0.48 mg kg⁻¹ at week twelve for diets 3 and 4 respectively. Thus, fish fed diet 3 (50 mg kg⁻¹ NatuRose™) retained a significantly (P<0.01) higher concentration of astaxanthin than the fish fed diet 4 (10 mg kg⁻¹ NatuRose™) at weeks 8 and 12. At both inclusion levels, the fish receiving Carophyll Pink™ had significantly (P<0.05) higher concentrations of astaxanthin in their epaxial white muscle than the fish receiving NatuRose™ (H. pluvialis). Diet 5, containing 50 mg kg⁻¹ of the experimental H. pluvialis produced by Liverpool John Moores University caused no detectable carotenoids to be retained within the white muscle of nine of the ten fish sampled. However, one fish contained 0.09 mg kg⁻¹ astaxanthin yielding a surprisingly noticeable flesh colouration as an outlier. As expected the fish fed the unpigmented control diet (6) had no detectable carotenoids within their white muscle at weeks 8 and 12, respectively. The net apparent
retention (NAR) of the test pigments in diets 1 (Carophyll Pink™ 50 mg kg⁻¹), 2 (Carophyll Pink™ 10 mg kg⁻¹), 3 (NatuRose™ 50 mg kg⁻¹) and 4 (NatuRose™ 10 mg kg⁻¹) reflected the final astaxanthin concentration measured in the flesh at week 12 (Table 4.9), retaining 9.33, 13.77, 5.78 and 8.13 % of the test pigments added to diets 1 to 4 respectively. This was also the case for the unpigmented control diet (6) that had a NAR of 0 %, whereas the NAR for those fish fed diet 5 (50 mg kg⁻¹ LJ M. pluvialis) was higher than anticipated, retaining 0.36 % of the total carotenoid in their flesh. In terms of a dose response, the NAR for diet 1 (CP 50) was significantly (P<0.01) lower than the NAR for diet 2 (CP 10). Likewise, NAR for diet 3 (NR 50) was significantly (P<0.01) lower than the NAR for diet 4 (NR 10). At both inclusion levels, the fish receiving Carophyll Pink™ gained significantly (P<0.01) higher NAR values than the fish receiving NatuRose™ (H. pluvialis). Diets 1, 2, 3 and 4 all gained significantly (P<0.001) higher NAR values than diets 5 (LJM 50) and 6 (control). The concentrations of astaxanthin in the flesh did not reflect the same significant differences observed for the Roche colour scores.

Table 4.10 displays the percentage drip-loss (exudative moisture loss) from those fillets sampled at week twelve and stored for seven days at 4°C. A general trend occurred for all diets where the amount of drip-loss increased with time, resulting in approximately 20 % drip-loss after seven days of storage, with the greatest loss occurring at day 1. However, no significant differences (P>0.05) were observed between any of the diets at any of the sampling sessions.
Table 4.9 White muscle astaxanthin concentration (mg kg\(^{-1}\)), n=2 (tanks), ± standard deviation of the mean, measured at strategic times to assess net apparent retention (NAR) of the test pigments.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1(^a) (mg kg(^{-1}))</th>
<th>Diet 2(^b) (mg kg(^{-1}))</th>
<th>Diet 3(^c) (mg kg(^{-1}))</th>
<th>Diet 4(^d) (mg kg(^{-1}))</th>
<th>Diet 5(^e) (mg kg(^{-1}))</th>
<th>Diet 6(^f) (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N/D (^g)</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>4</td>
<td>0.09 ±0.03</td>
<td>0.04 ±0.03</td>
<td>0.09 ±0.06</td>
<td>0.04 ±0.03</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>8</td>
<td>1.68 ±0.32</td>
<td>0.31 ±0.07</td>
<td>0.90 ±0.14</td>
<td>0.16 ±0.06</td>
<td>0.09 ±0.03</td>
<td>N/D</td>
</tr>
<tr>
<td>12</td>
<td>2.53 ±1.06</td>
<td>0.76 ±0.40</td>
<td>1.52 ±0.03</td>
<td>0.48 ±0.24</td>
<td>0.09 ±0.03</td>
<td>N/D</td>
</tr>
<tr>
<td>NAR (^h)</td>
<td>9.33 ±0.54</td>
<td>13.77 ±1.08</td>
<td>5.78 ±0.29</td>
<td>8.13 ±0.23</td>
<td>0.36 ±0.25</td>
<td>0 ±0.00</td>
</tr>
</tbody>
</table>

\(^a\) 50 mg kg\(^{-1}\) Carophyll Pink\(^TM\); \(^b\) 10 mg kg\(^{-1}\) Carophyll Pink\(^TM\); \(^c\) 50 mg kg\(^{-1}\) NatuRose\(^TM\); \(^d\) 10 mg kg\(^{-1}\) NatuRose\(^TM\); \(^e\) 50 mg kg\(^{-1}\) Liverpool John Moores experimental Haematococcus pluvialis; \(^f\) Unpigmented basal diet; \(^g\) Non detected; \(^h\) Net apparent retention, calculated for the overall twelve-week experimental period, expressed as a percentage.
Table 4.10 Percentage drip-loss from unskinned fillets measured over a seven day period at 4°C, n=2 (tanks), ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Day</th>
<th>Diet 1&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>Diet 2&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>Diet 3&lt;sup&gt;c&lt;/sup&gt; (%)</th>
<th>Diet 4&lt;sup&gt;d&lt;/sup&gt; (%)</th>
<th>Diet 5&lt;sup&gt;e&lt;/sup&gt; (%)</th>
<th>Diet 6&lt;sup&gt;f&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>5.58 ±2.36</td>
<td>2.33 ±1.74</td>
<td>4.98 ±2.41</td>
<td>3.02 ±1.06</td>
<td>5.77 ±2.41</td>
<td>1.98 ±0.96</td>
</tr>
<tr>
<td>2</td>
<td>9.15 ±1.59</td>
<td>7.18 ±1.97</td>
<td>9.13 ±3.01</td>
<td>10.34 ±3.14</td>
<td>10.70 ±2.06</td>
<td>9.75 ±1.93</td>
</tr>
<tr>
<td>3</td>
<td>12.15 ±2.03</td>
<td>11.03 ±2.29</td>
<td>11.58 ±2.07</td>
<td>12.60 ±2.11</td>
<td>14.23 ±1.65</td>
<td>11.98 ±2.03</td>
</tr>
<tr>
<td>4</td>
<td>14.61 ±2.16</td>
<td>13.10 ±1.74</td>
<td>13.69 ±1.61</td>
<td>14.83 ±1.56</td>
<td>16.24 ±1.24</td>
<td>14.05 ±1.82</td>
</tr>
<tr>
<td>5</td>
<td>16.09 ±1.59</td>
<td>14.55 ±1.39</td>
<td>18.48 ±0.84</td>
<td>16.41 ±1.24</td>
<td>20.58 ±4.03</td>
<td>15.25 ±2.15</td>
</tr>
<tr>
<td>6</td>
<td>17.11 ±1.61</td>
<td>18.31 ±1.70</td>
<td>19.93 ±2.14</td>
<td>20.70 ±1.06</td>
<td>22.25 ±2.14</td>
<td>19.10 ±1.36</td>
</tr>
<tr>
<td>7</td>
<td>19.58 ±1.59</td>
<td>19.69 ±4.21</td>
<td>22.54 ±1.24</td>
<td>22.39 ±2.28</td>
<td>24.43 ±3.08</td>
<td>20.05 ±1.73</td>
</tr>
</tbody>
</table>

<sup>a</sup> 50 mg kg<sup>-1</sup> Carophyll Pink;  
<sup>b</sup> 10 mg kg<sup>-1</sup> Carophyll Pink;  
<sup>c</sup> 50 mg kg<sup>-1</sup> NatuRose;  
<sup>d</sup> 10 mg kg<sup>-1</sup> NatuRose;  
<sup>e</sup> 50 mg kg<sup>-1</sup> Liverpool John Moores experimental Haematococcus pluvialis;  
<sup>f</sup> Unpigmented basal diet.
4.4.2 Trial 4.2

The stability of synthetic and algal astaxanthin during the production of commercially extruded diets and their efficacy for pigmenting rainbow trout flesh were analysed in this trial. Dietary carotenoid content is presented in Table 4.11, being assessed as total carotenoid content for the algal astaxanthin (NatuRose™) and free astaxanthin content for the synthetic source (Carophyll Pink™). The diets were analysed at week minus four (the start of the acclimation period) when the diets were received from the manufacturer and then at weeks zero (the start of the experimental period), four, eight and twelve (on termination of the experiment). Diet 1 (Carophyll Pink™ 50 mg kg⁻¹) remained stable throughout the sixteen-week storage period. Diets 2 (NatuRose™ 50 mg kg⁻¹) and 3 (NatuRose™ 70 mg kg⁻¹) were less stable, showing a 19% and 27% loss of total carotenoid content for NR 50 and NR 70 respectively, over the sixteen week storage period.

Table 4.11. Dietary content measured as free astaxanthin for diet 1 and total carotenoid content for diets 2 and 3; n=3, ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1 * (mg kg⁻¹)</th>
<th>Diet 2 b (mg kg⁻¹)</th>
<th>Diet 3 c (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-4</td>
<td>29.73 ±0.62</td>
<td>54.89 ±1.79</td>
<td>80.67 ±1.77</td>
</tr>
<tr>
<td>0</td>
<td>30.58 ±1.15</td>
<td>50.92 ±1.35</td>
<td>72.49 ±1.59</td>
</tr>
<tr>
<td>4</td>
<td>29.80 ±0.91</td>
<td>48.32 ±2.04</td>
<td>63.53 ±0.92</td>
</tr>
<tr>
<td>8</td>
<td>31.26 ±1.09</td>
<td>46.01 ±1.53</td>
<td>60.21 ±1.20</td>
</tr>
<tr>
<td>12</td>
<td>32.52 ±1.26</td>
<td>44.40 ±1.07</td>
<td>58.63 ±1.81</td>
</tr>
</tbody>
</table>

* 50 mg kg⁻¹ Carophyll Pink™ (Hoffmann La Roche, Basel, Switzerland);  b 50 mg kg⁻¹ NatuRose™ (Cyanotech, Kona, Hawaii, USA);  c 70 mg kg⁻¹ NatuRose™

The growth performance of juvenile rainbow trout for each bi-weekly period over the course of the experimental feeding trial is presented in Table 4.12. At the end of the twelve week experimental period the fish increased their weight by approximately 200%. Both specific growth rate (SGR) and feed conversion ratio (FCR) for rainbow trout were calculated over the 78 days of actual feeding and was indicative of an excellent growth performance as compared to work by Bjerkeng et al. (1997) and a high utilisation efficiency of the basal diet formulation employed for the delivery of the test pigmentation.
sources. These values were typically SGR (1.47-1.52) and FCR (0.98-1.00) (Table 4.12), both indices showed no significant differences (P>0.05) between the diets.

Table 4.12. Mean weight (g) of fish, measured bi-weekly to assess growth, n=4 (tanks) ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Week</th>
<th>CP 50a (g)</th>
<th>RPAN 50b (g)</th>
<th>RPAN 70c (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>99.31 ±5.35</td>
<td>99.43 ±2.46</td>
<td>102.03 ±3.17</td>
</tr>
<tr>
<td>2</td>
<td>139.55 ±5.10</td>
<td>139.46 ±4.71</td>
<td>142.05 ±3.96</td>
</tr>
<tr>
<td>4</td>
<td>178.05 ±7.52</td>
<td>178.81 ±3.93</td>
<td>182.12 ±5.25</td>
</tr>
<tr>
<td>6</td>
<td>212.65 ±8.39</td>
<td>208.76 ±5.72</td>
<td>212.12 ±7.48</td>
</tr>
<tr>
<td>8</td>
<td>242.32 ±11.58</td>
<td>238.61 ±7.03</td>
<td>243.68 ±5.94</td>
</tr>
<tr>
<td>10</td>
<td>280.26 ±16.03</td>
<td>274.72 ±10.45</td>
<td>283.38 ±7.83</td>
</tr>
<tr>
<td>12</td>
<td>326.87 ±18.69</td>
<td>315.06 ±12.75</td>
<td>322.00 ±10.37</td>
</tr>
</tbody>
</table>

SGRd 1.52 ±0.04 1.47 ±0.02 1.48 ±0.01  
FCRe 0.98 ±0.03 1.00 ±0.02 0.98 ±0.02  

a 50 mg kg⁻¹ Carophyll Pink™ (Hoffmann La Roche, Basel, Switzerland); b 50 mg kg⁻¹ NatuRose™ (Cyanotech, Kona, Hawaii, USA); c 70 mg kg⁻¹ NatuRose™; d Specific growth rate calculated for the over all twelve-week experimental period; e Feed conversion ratio calculated for the over all twelve-week experimental period.

The results for the monthly deposition of carotenoid in the flesh of rainbow trout as assessed by the SalmoFan™ system are presented in Table 4.13. It is obvious that apart from the initial sampling (week zero) a visual pink colouration became apparent in the fish fed diets 1 (Carophyll Pink™ 50 mg kg⁻¹), 2 (NatuRose™ 50 mg kg⁻¹) and 3 (NatuRose™ 70 mg kg⁻¹) after week four, becoming enhanced at week eight and was slightly further enhanced after week twelve. As in trial 4.1, at week zero (initial sampling) all of the 20 randomly sampled fish were so pale that they could not be scored using the Roche colour score index. At week four the fish fed diets 1, 2 and 3 obtained Roche Colour Scores of 23.4, 22.7, and 24.7, respectively. Where diet 3 (NR 70) scored significantly (P<0.05) higher than the other diets, and diet 1 (CP 50) scored significantly (P<0.05) higher than diet 2 (NR 50). At week 8, diets 1, 2 and 3 obtained Roche Colour Scores of 27.2, 27.5, and 27.9, respectively. This demonstrated that there were no significant differences (P>0.05) between the diets in terms of perceived pigmentation. At week twelve, representing the final sampling of fish for flesh colour assessment, the fish fed CP 50, NR
50 and NR 70 obtained mean Roche Colour Scores of 27.9, 28.6, and 29.4, respectively. The fish receiving diet 3 (NR 70) scored significantly (P<0.05) higher than those fish fed diets 1 (CP 50) and 2 (NR 50). Also the fish fed NR 50 scored significantly (P<0.05) higher than the fish fed CP 50.

Table 4.13. Mean Roche Colour Scores for unskinned fillets, using the SalmoFan™, n=4 (tanks), ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1 a (CSU) d</th>
<th>Diet 2 b (CSU)</th>
<th>Diet 3 c (CSU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N/A e</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>23.42 ±0.22</td>
<td>22.67 ±0.09</td>
<td>24.72 ±0.35</td>
</tr>
<tr>
<td>8</td>
<td>27.19 ±0.17</td>
<td>27.45 ±0.25</td>
<td>27.92 ±0.92</td>
</tr>
<tr>
<td>12</td>
<td>27.92 ±0.19</td>
<td>28.59 ±0.12</td>
<td>29.43 ±0.13</td>
</tr>
</tbody>
</table>

a 50 mg kg⁻¹ Carophyll Pink™ (Hoffmann La Roche, Basel, Switzerland); b 50 mg kg⁻¹ NatuRose™ (Cyanotech, Kona, Hawaii, USA); c 70 mg kg⁻¹ NatuRose™; Colour Score Units (arbitrary units); d Colour Score Units (arbitrary units); e Not Applicable.

Table 4.14 displays the epaxial white muscle astaxanthin concentrations of the quadruplicate groups of fish receiving each test diet. At week 0 (initial sampling) none of the 20 randomly sampled fish had any detectable carotenoids in their white muscle. A dose response seemed to occur at weeks four, eight and twelve between diets 2 (NatuRose™ 50 mg kg⁻¹) and 3 (NatuRose™ 70 mg kg⁻¹). This resulted in a mean flesh astaxanthin concentration of 1.92 mg kg⁻¹ and 2.54 mg kg⁻¹ at week four, and 2.91 and 3.23 mg kg⁻¹ at week eight, and 3.61 and 3.81 mg kg⁻¹ at week twelve for diets 2 and 3 respectively. However, the epaxial white muscle astaxanthin concentrations for the fish fed diet 2 (NR 50) and 3 (NR 70) were not significantly different (P>0.05) at any of the sampling sessions. The fish receiving diet 2 (NR 50) displayed similar epaxial white muscle astaxanthin concentrations relative to the Carophyll Pink™ reference (control) fish (diet 1) throughout the experimental period, which retained 2.54, 3.23 and 3.81 mg kg⁻¹, at weeks four, eight and twelve respectively. This resulted in no significant differences (P>0.05) being observed between the diets 1 and 2, or 1 and 3 at any of the sampling sessions. The net apparent retention (NAR) of the test pigments in diets 2 (NatuRose™ 50 mg kg⁻¹) and 3
(NatuRose™ 50 mg kg⁻¹) reflects the final astaxanthin concentration measured in the flesh at week twelve (Table 4.13). As the fish receiving diets 2 and 3 retained 6.17 % and 4.65 % respectively (of the total carotenoid added to the basal diet) as astaxanthin in their white epaxial muscle. Whereas the NAR for those fish fed the control diet (1) containing Carophyll Pink™ (50 mg kg⁻¹) was higher than anticipated, retaining 8.79 % of the available astaxanthin in their flesh. The NAR values for each diet were significantly different (P<0.05); where the NAR value for diet 2 was significantly (P<0.05) higher than diet 3 and the NAR value for diet 1 was significantly (P<0.05) higher than the NAR values for diets 2 and 3. Again, the concentrations of astaxanthin in the flesh did not reflect the same significant differences observed for the Roche colour scores.

Table 4.14. Mean white muscle astaxanthin concentration (mg kg⁻¹), n=4 (tanks), ± standard deviation of the mean, measured at strategic times to assess net apparent retention (NAR) of the test pigments.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1 ⁠(mg kg⁻¹)</th>
<th>Diet 2 ⁠(mg kg⁻¹)</th>
<th>Diet 3 ⁠(mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>4</td>
<td>2.33 ±0.38</td>
<td>1.92 ±0.36</td>
<td>2.54 ±0.45</td>
</tr>
<tr>
<td>8</td>
<td>2.83 ±0.29</td>
<td>2.91 ±0.25</td>
<td>3.23 ±0.92</td>
</tr>
<tr>
<td>12</td>
<td>3.26 ±0.19</td>
<td>3.61 ±0.12</td>
<td>3.81 ±0.30</td>
</tr>
<tr>
<td>NAR</td>
<td>8.79 ±0.43</td>
<td>6.17 ±0.05</td>
<td>4.65 ±0.36</td>
</tr>
</tbody>
</table>

⁠a 50 mg kg⁻¹ Carophyll Pink™ (Hoffmann La Roche, Basel, Switzerland); ⁠b 50 mg kg⁻¹ NatuRose™ (Cyanotech, Kona, Hawaii, USA); ⁠c 70 mg kg⁻¹ NatuRose™; ⁠d Non detected; ⁠e Net apparent retention, calculated for the overall twelve-week experimental period, expressed as a percentage.
4.5 Discussion

The results reported for trials 4.1 and 4.2 were based on a feeding regime that employed an initial acclimation phase where fish were fed on a standard (unpigmented) commercial salmonid feed (Trouw standard 40). This was to ensure that the trout were disease free and in good health, so reducing the risk of stress and mortality the fish may experience during the experimental period (Mork, pers comm.). This also ensures that all fish receiving each treatment fed consistently and uniformly prior to the experimental period. In trial 4.1 the fish only approximately doubled in weight during the experimental period. The overall feed conversion ratio (FCR) for the experimental period was similar for all diets at approximately 1.5. FCR is defined as the feed intake per unit of live weight gain, i.e. values < 1 demonstrate a high utilisation of the feed, with the weight increase of the fish being greater than the feed administered. This is possible because the calculation of FCR uses wet weight, where approximately 60 % of the mass of a rainbow trout is attributed to muscle, of which approximately 80 % is water (Lagocki, unpublished data). Hence, as a trout's mass increases, the major component of its weight gain is actually due to retained water. Trial 4.2 was dependent on a correctly balanced modern high energy trout feed produced using extrusion technology that supported enhanced growth rates, enabling the rainbow trout to triple their weight in the 12 week experimental period. The associated feed utilisation efficiency is in accordance with that of Bjerkeng et al. (1997) using a similar basal diet formulation. All dietary treatments in trial 4.2 performed similarly in this respect, so the degree of pigmentation would be of a similar potential. However, the FCR values for trial 4.1 were > 1, which are fairly consistent with the feed utilisation associated with the type of experimental diets (cold pressed) employed. As the basal diet was cold pressed it could only retain a low oil content (11%). Hence, the major energy component of the diet was protein, which is metabolically more expensive than oil. Since both growth rate and FCR are dependent upon diet composition (Torrissen, 1985), the aquaculture industry has elected to use extruded high oil content diets which provide FCR values of < 1.
under commercial conditions (Campbell, *pers comm.*). The decision to use cold pressed low oil content diets for trial 4.1 was based on the fact that commercially produced extruded high oil content diets do not guarantee homogeneity of the pigment throughout the diet or its final pigment level. This is due to the harsh conditions (high temperatures and pressures) which the pigment is subjected to during the extrusion process. Additionally, cold pressed diets produced in small batches provide adequate blending of the ingredients (macro and micro) to form an homogenous mixture, and are formed under mild conditions (low temperature and pressure). Although cold pressed experimental diets can only retain a low oil level, they have the advantage of minimising degradation of pigment, as demonstrated from the results of experiment 3.2.

The lack of significant differences (P>0.05) in growth rate and FCR between fish fed different diets in both trials (4.1 and 4.2) provides further support to the hypothesis proposed by Christiansen *et al.* (1995b) that astaxanthin is not an essential nutrient for salmonids. Moreover the results indicate that *H. pluvialis* had no adverse effects on the palatability of the diet. Given that rates of growth for all fish were similar within each trial, their degree of pigmentation should also be similar, since the deposition of astaxanthin is dependent on the rate of growth in salmonids (Nickell and Bromage, 1998b).

Two independent assessments of flesh pigmentation were used in both feeding trials. Firstly, the Roche colour score (SalmoFan™) was routinely employed as a qualitative indicator of colour perception (Skrede *et al.*, 1990). Secondly, the direct analytical (HPLC) determination of the astaxanthin content of epaxial white muscle tissue extracts from freshly excised fillets. This method is an efficient way of quantitatively determining the carotenoid concentration in this type of material (No and Storebakken, 1991; Foss *et al.*, 1987; Bjerkeng, 1997; Nickell and Bromage, 1998a).

As described in section 3.7.1 the Roche colour score (SalmoFan™) was developed for the salmonid industry to enable farmers to assess the colour of salmon flesh routinely and inexpensively (Christiansen *et al.*, 1995b). Roche colour score values are arbitrary, being
quoted on the basis of whole numbers (20 - 34), where a score of 20 colour score units (CSU) represents very pale pink flesh and a score of 34 CSU a very intense pink/red flesh colour.

The Roche colour score (SalmoFan™) data for fish sampled at week zero for both trials were not of significance, since the white muscle remained unpigmented at this stage. This is rationalised by the fact that week zero was the effective start of the trial evaluation when fish were conditioned to the experimental diets. Thus, fish evaluated at this stage (week zero) represented the base level of pigmentation for comparison of each treatment on termination (week twelve) of each trial. In trial 4.1, at week four, flesh samples remained pale and no colour score could be applied. However, by week eight pigmentation of the fillets was evident for the trout fed the diets containing the synthetic astaxanthin (Carophyll Pink™) at both inclusion levels (50 and 10mg kg⁻¹) and the diet containing the high inclusion level (50 mg kg⁻¹) of the *H. pluvialis* (NatuRose™). Furthermore the fillets of fish fed the higher inclusion rate of synthetic astaxanthin scored significantly (P<0.05) higher values than the fish fed the lower inclusion level. For a given inclusion level a significant difference (P<0.05) was detected between the two pigment sources, with the synthetic astaxanthin yielding the higher values. The pigmentation of the fillets became more enhanced after week twelve with pigmentation of fish fed the lower inclusion level of *H. pluvialis* (NatuRose™) also becoming apparent. A definite numerical value was difficult to apply to the fish fed the diet containing the experimental LJH *H. pluvialis* and the unpigmented control diet, throughout the trial period, due to the pale (white) flesh appearance of the fish. So, no colour score could be applied to these groups of fish. Thus, significant differences (P<0.01) were observed in the final colour score between the two inclusion levels for both pigment sources and between pigment sources at both inclusion levels.

In trial 4.2 a visual colouration was evident at week four for the diets, which became more apparent after weeks eight and twelve. At week four, the fish receiving the pigmented
control diet (50 mg kg⁻¹ Carophyll Pink™) scored significantly (P<0.05) higher values than the diet containing the low inclusion (50 mg kg⁻¹) level of *H. pluvialis* (NatuRose™). The fish receiving the diet containing the high inclusion level (70 mg kg⁻¹) of *H. pluvialis* (NatuRose™) scored (P<0.05) significantly higher values than the diets containing 50 mg kg⁻¹ astaxanthin. At week eight the pigmentation of the fillets became more enhanced for all the dietary treatments, resulting in increased scores. However, the mean scores for each dietary treatment were not significantly different (P>0.05). The final muscle samples taken on conclusion of the study are of importance since they relate to rainbow trout of marketable size (average weight 300g) and are of direct interest to the aquaculturist, fish processor and consumer (Schiedt, 1988). Colour scores increased slightly by week twelve for all test diets. The fish fed the diet containing the low inclusion level of *H. pluvialis* resulted in a significantly (P<0.05) higher visual score than the fish receiving the pigmented control diet. The fillets of the fish receiving the high inclusion level of *H. pluvialis* scored significantly (P<0.05) higher than the fish receiving the other diets. Comparing the results gained in this study with those of previous studies is extremely difficult. This is due to refinement of the Roche colour score technique, which saw the introduction of the SalmoFan™ in 1998 (Roche, 1999b). It should be noted that previous studies used the Roche Colour Card™, which incorporated two scoring systems (Smith *et al.*, 1992). Caution must therefore be maintained, as errors may arise due to differences in illumination, presentation of the samples and the subjective nature of colour perception among individuals. Despite these problems the SalmoFan™ colour assessment generally supported the muscle astaxanthin concentration of all the groups from weeks four to twelve.

It must be noted that for both trials (4.1 and 4.2) no significant differences (P>0.05) in white muscle astaxanthin concentration were observed between tanks receiving the same diet, showing that tank position did not have any effect on the feeding performance of the fish, *i.e.* there was no occurrence of 'tank effect'. The fillets from fish fed the unpigmented
control diet contained no detectable carotenoids, demonstrating that the carotenoids present within the other feed ingredients (macro and micro) do not have any noticeable effect on the pigmentation of the epaxial white muscle of the rainbow trout. In both trials (4.1 cold pressed diets and 4.2 extruded diets) the mean values for the astaxanthin concentration of the white muscle suggest a dose response. In trial 4.1 at week eight, the fish fed the diet containing the synthetic astaxanthin (Carophyll Pink™) at 50 mg kg\(^{-1}\) retained over five times more astaxanthin than the fish receiving 10 mg kg\(^{-1}\) Carophyll Pink™. A similar dose response also occurred at week eight for the diets containing the commercial \textit{H. pluvialis} (NatuRose™), as the fish receiving 50 mg kg\(^{-1}\) retained almost six times more astaxanthin than the fish receiving 10 mg kg\(^{-1}\). However, at week twelve this trend was less pronounced; the fish fed the higher inclusion level (50 mg kg\(^{-1}\)) for both pigment sources, only retained approximately three times more astaxanthin than the fish receiving the lower inclusion level (10 mg kg\(^{-1}\)). In trial 4.2, at week four, the fish fed the diet containing the commercial \textit{H. pluvialis} (NatuRose™), at 70 mg kg\(^{-1}\) retained over 1.3 times more astaxanthin than the fish receiving 50 mg kg\(^{-1}\) NatuRose™. However, at week eight this trend was less pronounced; the fish fed the higher inclusion level (70 mg kg\(^{-1}\)), only retained approximately 1.1 times more astaxanthin than the fish receiving the lower inclusion level (50 mg kg\(^{-1}\)). At week twelve the difference in epaxial white muscle astaxanthin concentration between the two inclusion levels of \textit{H. pluvialis} continued to decrease, where the fish fed the higher inclusion level only retained approximately 1.05 times more astaxanthin than the fish receiving the lower inclusion level (50 mg kg\(^{-1}\)). However, the epaxial white muscle astaxanthin concentrations of the fish fed \textit{H. pluvialis} at the different inclusion rates were not significantly different (P>0.05) at any of the sample sessions. At weeks four, eight and twelve the epaxial white muscle astaxanthin concentration of those fish receiving the pigmented control diet (50 mg kg\(^{-1}\) Carophyll Pink™) were not significantly different (P>0.05) to those receiving either of the diets containing \textit{H. pluvialis}. 
The apparent dose response observed in both trials may be due to the epaxial white muscle becoming saturated with astaxanthin. Also, the rate at which new binding sites are formed due to the production of more muscle during growth decreases with increasing body mass. This is because the rate at which rainbow trout grow, decreases as they increase in mass (Lagocki, 1995). Only one of the ten fish that received the diet containing 50 mg kg\(^{-1}\) experimental Liverpool John Moores (LJM) \(H.\) *pluvialis* had a detectable quantity of astaxanthin in the epaxial white muscle. This may be due to this fish having a genetic aberration or being of a different strain, thus enabling it to utilise the experimental LJM \(H.\) *pluvialis*. Previous studies have shown that different strains of salmon are able to utilise the same source of astaxanthin with different rates of efficiency (Aas, *pers comm*.). Nevertheless, the results for white muscle astaxanthin concentration for the fish fed commercial \(H.\) *pluvialis* (NatuRose\textsuperscript{TM}) are in accordance with those reported by Sommer *et al.* (1992), for rainbow trout fed diets containing *Haematococcus pluvialis* at inclusion rates of 40, 60 and 80 mg kg\(^{-1}\). Although the dose response they reported was of a lower magnitude (2 times) for rainbow trout fed diets containing *Haematococcus pluvialis* at different inclusion rates.

The net apparent retention (NAR) of astaxanthin in the epaxial white muscle, was calculated for fish receiving different diets in both trials. In trial 4.1, the NAR values for diets 1, 2, 3 and 4 reflected the astaxanthin concentration measured in the epaxial white muscle of those fish fed the respective diets, where diet 2 > diet 1 > diet 4 > diet 3 > diet 5 > diet 6. However, the NAR for diet 5 was much lower than anticipated, as the concentration of astaxanthin retained in the epaxial white muscle of those fish receiving diet 5 was very low compared to the inclusion rate (50 mg kg\(^{-1}\)) of LJM \(H.\) *pluvialis*. The dose response noted for the retention of astaxanthin in the flesh of those fish receiving diets 1 and 2 containing Carophyll Pink\textsuperscript{TM}, and those receiving diet 3 and 4 containing NatuRose\textsuperscript{TM} demonstrated NAR values consummate with their inclusion level. Both diet 2 (10 mg kg\(^{-1}\) Carophyll Pink\textsuperscript{TM}) and 4 (10 mg kg\(^{-1}\) NatuRose\textsuperscript{TM}) had NAR values
approximately 40% (significantly, P<0.01) higher than diet 1 (50 mg kg⁻¹ Carophyll Pink™) and 3 (50 mg kg NatuRose™) respectively. The lower NAR of astaxanthin for diets 1 and 3 as compared to diets 2 and 4 is vindicated by the fact that diets 1 and 3 contained approximately 500% more pigment than diets 2 and 4, and dietary pigment concentration is part of the denominator in the NAR equation. Comparing the NAR values with the astaxanthin concentrations measured in the white epaxial muscle for treatments 1 and 2, plus 3 and 4, suggests that a lower inclusion level (10 mg kg⁻¹) of pigment in the diet is more efficiently retained in the flesh. Thus, it appears that the inclusion level of pigment in the diet is a critical factor in gaining high NAR values. The control diet (6: basal diet) had an NAR of 0%, this rationalized by the fact that diet 6 was unpigmented and none of the fish had any detectable carotenoids in their flesh at any of the sampling sessions. In trial 4.2 the NAR values for all the diets did not reflect the astaxanthin concentration retained in the flesh of those fish receiving their respective diets. Diet 1 targeting 50 mg kg⁻¹ Carophyll Pink™ obtained an NAR value 42% (significantly, P<0.05) higher than diet 2 that also targeted 50 mg kg⁻¹ astaxanthin (NatuRose™). This is explained by the fact that diet 1 suffered a 34% loss of its astaxanthin, and as discussed, a decrease in astaxanthin dietary content would probably result in an elevated NAR value. This was also demonstrated by diet 3 (70 mg kg⁻¹ NatuRose™) that obtained an NAR 25% lower than that of diet 2 (50 mg kg⁻¹ NatuRose™). Comparing the NAR values for those diets that targeted 50 mg kg⁻¹ NatuRose™ from trials 4.1 and 4.2, it seems as if the extruded diet provided superior NAR of astaxanthin in flesh. This is probably due to the extruded diet having a higher oil content which provided better growth and more muscle mass to pigment.

Dietary carotenoid content of trial 4.1 feeds was calculated on addition of astaxanthin to the diets during production, as the results from experiment 3.2 showed no astaxanthin losses occurred at any stage of production of cold pressed diets. However, the undetectable concentration of astaxanthin in the white muscle of 90% of those fish fed the experimental
LJM *H. pluvialis* was due to the fact that the experimental LJM *H. pluvialis* had intact cell coverings, as illustrated in plate 4.1. The electron micrograph of the LJM *H. pluvialis* cell shows their surface to be intact and very smooth. This is a stark contrast to the electron micrograph of NatuRose™ *H. pluvialis* cells (plate 4.2), the cell walls of which appear to have been breached numerous times, creating deep fissures and cavities. Thus, it seems the intact cell walls of the LJM *H. pluvialis* cells caused its carotenoid content to have an extremely low bioavailability in the fish. This also resulted in the diet having no detectable carotenoids that had the potential to pigment rainbow trout flesh. The synthetic astaxanthin product Carophyll Pink™ that is described as being produced as free flowing beads, when observed under the scanning electron microscope appear to be composed of many smaller beadlets, which are probably bound together by the starch-gelatine matrix.

Trial 4.1 showed *Haematococcus pluvialis* to be a less efficient pigment source compared to synthetic astaxanthin (Carophyll Pink™) for rainbow trout, when presented to the fish at the same inclusion levels in cold pressed low oil content diets. The fish receiving the diet containing 50 mg kg\(^{-1}\) synthetic astaxanthin retained a significantly (P<0.05) higher (66%) concentration of astaxanthin in their white muscle than the fish fed the diet containing 50 mg kg\(^{-1}\) *Haematococcus pluvialis*. A similar difference (63%) was also apparent at the lower astaxanthin inclusion rate. Previous work by Sommer *et al.* (1991) has also shown that *Haematococcus pluvialis* derived astaxanthin is a less efficient pigment source than the synthetic form, when present in the diet at an inclusion rate of 40 mg kg\(^{-1}\); pigment retention was 71% higher for the synthetic pigment. The lower efficacy of *Haematococcus pluvialis* compared to the synthetic astaxanthin (Carophyll Pink™) is most likely due to the chemical form of the source pigment. NatuRose™ *Haematococcus* meal contains approximately 70% astaxanthin mono-esters, 10% astaxanthin di-esters, 5% free astaxanthin and the remaining 15% consists of a mixture of β-carotene, lutein, canthaxanthin, zeaxanthin and other carotenoids (Lorenz, 1999). This compares with the synthetic source (Carophyll Pink™) being guaranteed to contain 8% free astaxanthin with a
1 : 3, cis : trans isomeric ratio, where the trans isomer contains a racemic mixture of the three stereo isomers (3S,3'S; 3R,3'S meso and 3R,3'R) in a ratio of 1:2:1 (Schiedt, 1988). The trans isomers have been shown to have better retention in epaxial white muscle than the cis isomers (Bjerkeng et al., 1997), regardless of the trans stereo isomer form (Meyers, 1994). It has also been demonstrated that free astaxanthin is deposited unaltered into salmonid flesh, whereas esterified astaxanthin e.g. (astaxanthin dipalmitate) is hydrolysed in the intestinal lumen, and therefore produces less pigmented flesh (Schiedt, 1988). This may be due to catabolism of the esterified astaxanthin being the rate limiting step, such that the transient time in the lumen is not sufficient for total hydrolysis. Furthermore, the calculated weight of *H. pluvialis* required to obtain the desired pigment concentration of 10 and 50 mg kg\(^{-1}\) was based on total carotenoid concentration of the *H. pluvialis* product and not its total astaxanthin concentration. Total carotenoid concentration was used because the other carotenoids present in *H. pluvialis* may have exerted some affect on white muscle astaxanthin concentration. Canthaxanthin, for example, has been widely demonstrated to have the ability to pigment salmonids (Bauernfeind, 1976, Choubert and Blanc, 1985; Torrissen, 1985; Bjerkeng et al., 1990; Storebakken and No, 1992), and lutein may be metabolised to astaxanthin. Also, both synthetic and algal astaxanthin produce a dose response, as the carotenoid content of the diet is reflected in the concentration of astaxanthin retained in the flesh. However, the results from trial 4.2 showed *H. pluvialis* to pigment epaxial white muscle with the same efficacy as synthetic astaxanthin (Carophyll Pink\(^{TM}\)), at the same inclusion levels, when presented to fish in expanded commercial high oil content diets. These results may be due to partial de-esterification of the astaxanthin esters present in *H. pluvialis* during the extrusion process, where the dietary ingredients are exposed to high temperatures and pressures.

Dietary carotenoid content of the feeds for trial 4.2 was calculated on the free astaxanthin content of synthetic astaxanthin Carophyll Pink\(^{TM}\) and the total carotenoid content of the NatuRose\(^{TM}\) *H. pluvialis*. As stated, Carophyll Pink\(^{TM}\) is guaranteed to contain 8% free
astaxanthin with a 1 : 3, *cis : trans* isomeric ratio. The control diet targeted 50mg kg$^{-1}$ Carophyll Pink™, but on analysis only yielded approximately 33 mg kg$^{-1}$, even though it remained stable throughout the sixteen week storage period. This 34% loss may be due to those incurred during the addition of the Carophyll Pink™ into the extruder along with the other dietary ingredients, also, astaxanthin has been demonstrated to be thermally labile (Latscha, 1990). However, NatuRose™ showed no degradation during the extrusion process. This is probably due to the protection afforded by the cell wall, as demonstrated for light degradation in experiment 3.6. The astaxanthin derived from Carophyll Pink™ (synthetic) and NatuRose™ (natural, *H. pluvialis*) remained stable during the experimental period because both pigments were bound within the pellet matrix. It seems that the pellet matrix is quite dense, protecting astaxanthin from ultra violet radiation and elevated temperatures. NatuRose™ (*H. pluvialis*) used in the experimental diets was reported to contain 1.43% free astaxanthin by weight (Sabatier, *pers comm.*).

Image analysis was only used in trial 4.1, solely being applied to the fillets of those fish sampled at week 12 (on termination of the trial). This technique measured all three colour components (red, green and blue) of the fillets as grey values. Greyness values are a measure of reflectance, where no reflectance gives a zero grey value being black on the greyness scale and total reflectance (a grey value of 256) being pure white. The blue and green components for every fillet in all treatments showed remarkable similarity to each other. Statistical analysis of the different colour components (in all treatments) showed both the green and blue components to be significantly (*P<0.001*) lower than the red component, but not significantly different from each other. In fact, for all treatments the levels of green and blue were so low as compared to the red component that removing them from the total colour proved to have a negligible effect. So it seems fillet colour is primarily due to a change in the red component, being measured as a change in its corresponding grey values, such that only the red component of the fillets were shown in Table 4.8. As only the red component was used in the results, a direct association between
grey value and redness occurs, where the higher the grey value the lighter the red hue. The mean grey value for all six transects, for all treatments were compared with the SalmoFan™. These demonstrated a comparable increase in redness with an increased inclusion rate of pigment in the diet. In all treatments a general pattern emerged when comparing the mean grey values for each transect, whereby their grey values decreased as the transect number increased i.e. grey values decrease from the anterior (head) transect one to the posterior (tail) transect six. This correlates to the redness of the fillet increasing at the tail, indicating a higher concentration of pigment being present in this region of the fillet. This observation confirms work carried out by Nickell and Bromage (1998b) which demonstrated differences in muscle astaxanthin concentration in different regions of the fillet, where the epaxial anterior muscle had an astaxanthin concentration of 4.7 mg kg$^{-1}$ and the posterior muscle contained 14.2 mg kg$^{-1}$. The mean grey value for the red component for all treatments lies between transects three and four. This area of the fillet directly corresponds to the region under the dorsal fin from which a sample of epaxial muscle was excised for analysis to quantify the concentration of astaxanthin within the fillet. These results further suggest that taking muscle samples from under the dorsal fin for carotenoid analysis does provide a good indication of the overall carotenoid concentration of a fillet. It must also be noted that the grey values within a transect may vary considerably along its length. These large variations are due to the presence of intramuscular fat that is deposited in between the myotomes of muscle, which do not contain astaxanthin as demonstrated in experiment 3.2. Hence, the pixels within a transect where intramuscular fat is being measured can be identified and omitted from the data to enable a more representative muscle colour to be determined.

The percentage drip-loss measured for the fillets sampled at week twelve in trial 4.1 showed no significant differences ($P>0.05$) between the diets and did not reflect the dose response observed in the retention of astaxanthin in the epaxial white muscle between diets 1 and 2, or diets 3 and 4. It seems the highest amount of drip-loss measured in a 24 hour
period was at day 1. This may be explained by the fact that the fillets were frozen prior to the experiment. Water has a partly ordered structure where clusters of molecules continually undergo the formation and breakage of hydrogen bonds, but during the freezing process, the degree of order and the size of the clusters increases until all potential hydrogen bonds are formed (Stryer, 1988). As a result ice forms a highly regular crystalline structure that is less dense than water and has a greater volume. Hence, as the fillets froze ice crystals were probably formed within the cytoplasm causing the cell to enlarge, eventually rupturing the cells plasma membrane. This of course had noticeable effect whilst the fillets remained frozen, however on thawing the cells contents were released on to the absorbent pad, increasing the rate of drip-loss at day 1. The problem of excessive moisture loss on day one could possibly be eliminated by excluding the freezing from the drip-loss protocol.

In conclusion, the results reported for trial 4.1 (cold pressed diets) display quite a slow development in pigmentation of the flesh and external integument of the fish for all diets containing carotenoid supplementation, when compared to the results reported for these parameters for trials 4.2 (extruded diets). Nevertheless, the industry tends to only feed rainbow trout pigmented diets for 8-12 weeks, and as seen in trial 4.1 it required 8 weeks of feeding all the pigmented test diets before any colour score could be applied to the fillets. This was probably due to the use of a cold pressed low oil content basal diet, which only allowed the fish to double in weight. In comparison the fish in trial 4.2 received a commercially produced expanded high oil content basal diet that not only provided a stable environment for astaxanthin during storage, but also enabled the fish to triple their weight and become more pigmented. This accelerated growth resulted in an increase in muscle mass and an increase in the number of hydrophobic pockets to which astaxanthin could bind to. Consequently, increased weight leads to an increase in food consumption, exposing the fish to higher quantities of astaxanthin, resulting in an elevated epaxial white muscle astaxanthin concentration. It also seems from the results of trial 4.1 that the
presence of astaxanthin in rainbow trout flesh does not have any affect on exudative moisture loss. Analysis of the diets showed *H. pluvialis* to be a stable source of astaxanthin that has no affect on growth. *H. pluvialis* derived astaxanthin showed a similar dose response to the synthetic form of the pigment confirming that it was assimilated from the diet. However, natural astaxanthin was not retained in the epaxial white muscle to the same level as the synthetic astaxanthin form. Nevertheless, trial 4.1 demonstrated that the *Haematococcus pluvialis* cell wall has to be disrupted for the fish to utilise the carotenoids (namely astaxanthin) present within the protoplast. Thus, from trials 4.1 and 4.2 it seems that the efficacy of *H. pluvialis* as a product to pigment salmonid flesh has promise, but further work needs to be carried out.
CHAPTER 5

Evaluation of processing and extraction of *Haematococcus pluvialis* derived astaxanthin fractions on rainbow trout pigmentation. Decysted astaxanthin (Trial 5.1) and astaxanthin ester extracts (Trial 5.2)
5.1 Introduction

Initial studies undertaken by Sommer et al. (1991) seem to suggest Haematococcus pluvialis could be an economical natural alternative to synthetic astaxanthin for the pigmentation of salmonids. However, the results of the feeding trials carried out in chapter 4 demonstrated H. pluvialis to be an inferior pigment source when compared to its synthetic counterpart, Carophyll Pink™ (Hoffmann-La Roche, Basel, Switzerland). Nevertheless, the results from both feed trials (4.1 and 4.2) strongly suggest H. pluvialis does have the potential to be used as a pigment source by the aquafeed industry. These results also suggest that the presence of a cell wall limits the bioavailability of H. pluvialis derived astaxanthin in the gastrointestinal tract of rainbow trout.

Elliot (1934) demonstrated that the encystment of H. pluvialis cells and the biosynthesis of astaxanthin are directly correlated. This is due to H. pluvialis evolving protection systems that are activated when the organism becomes stressed. These systems enable H. pluvialis to inhabit ephemeral pools, the conditions of which can readily change, creating habitats that encompass harsh environmental conditions such as intense light and extreme temperatures. The production of astaxanthin protects H. pluvialis against the effects of intense light, primarily the photobleaching of its chlorophyll. The formation of an outer cyst protects the H. pluvialis cells from desiccation (Hoek et al., 1998). This is due to the cyst being composed of sporopollenin, a lipid polymer that is impermeable to water (Kolattukudy, 1983). Sporopollenin is a polyester containing many monomers, principally carotenoids and apocarotenoids, of which zeaxanthin and β-carotene are most prevalent, and also fatty acids (Juniper and Jeffree, 1983). These facts rationalise Elliot’s (1934) results, as astaxanthin and sporopollenin are both classed as secondary metabolites with both being further sub-classed as secondary carotenoids that are produced from reactions within the same metabolic pathway (Wiermann, 1984).

Chapter 4 introduced several steps and rationales to initially determine the efficacy of a food additive such as astaxanthin. However, when dealing with natural sources of
astaxanthin other considerations have to be taken into account, primarily whether the medium in which the astaxanthin resides requires processing prior to being added to a diet, to render the astaxanthin bioavailable to the fish. Trial 4.1 compared the efficacy of *H. pluvialis* cells that had intact cell walls with processed cells, the cell walls of which had been ruptured. The results of this trial demonstrated that the cell wall of the *H. pluvialis* cells has to be breached for the fish to exploit the astaxanthin retained within the protoplast matrix. This result also posed the question: would complete removal of the cyst from encysted cells, increase the bioavailability of *H. pluvialis* derived astaxanthin? Comparing the data gained from both feeding trials in chapter 4, it is obvious that the growth and deposition of astaxanthin within the white muscle, is more rapid for those fish fed commercially extruded diets (trial 4.2) as compared to those fish fed cold pressed diets (trial 4.1). Thus, the use of commercially produced expanded diets for all impending trials was deemed necessary. However, it is also obvious from this data that significant losses of astaxanthin, especially the synthetic source (*Carophyllum Pink™*) occurred during the production of expanded diets (trial 4.2). This led to the development of a simple method of top-coating unpigmented extruded diets (basal diet) with either synthetic astaxanthin or natural (*H. pluvialis* cells). Combining these results with the question raised by trial 4.1, led to the primary aim of trial 5.1. This was to test the efficacy of decysted *H. pluvialis* cells with respect to the bioavailability of their astaxanthin as compared to processed (partially degraded cysts) *H. pluvialis* cells (*NatuRose™*, Cyanotech, Kona, Hawaii, USA) using a commercially manufactured expanded diet as a carrier.

Schiedt (1988) demonstrated only free astaxanthin was retained in the flesh of rainbow trout, even though the diet they received contained astaxanthin esterified to two palmitate (fatty acid) molecules. Similar results were gained in trials 4.1 and 4.2 where only free astaxanthin was detected in the flesh of those fish receiving diets containing *H. pluvialis*, even though the majority of *H. pluvialis* derived astaxanthin is esterified to either one or two fatty acids. These results seem to suggest that the bioavailability of *H. pluvialis*
derived astaxanthin is further limited by the majority being esterified to fatty acid moieties. Therefore, a possible solution to increase the bioavailability of *H. pluvialis* derived astaxanthin is to decyst the cells by total or partial removal of the cell wall and liberate the astaxanthin from the fatty acid moieties to which it is esterified.

Hence, the aim of trial 5.2 is to test the efficacy of pure extracts of both types of astaxanthin ester (mono- and di-) to reveal which, if any, causes retarded bioavailability of astaxanthin from *H. pluvialis* cells as compared to the synthetic source (Carophyll Pink™), using a commercially manufactured expanded diet as a carrier. Both trials (5.1 and 5.2) used rainbow trout (*Oncorhynchus mykiss*) as a model for salmonid species.
5.2 Aims

The main aims of the present experiments were:

1. Assess the efficacy of an immobilised total carotenoid extract derived from *Haematococcus pluvialis* presented as a top-coat on a commercially produced diet (Trial 5.1).

2. Compare the efficacy of the total carotenoid extract against a commercial source of *Haematococcus pluvialis* and a commercial immobilised synthetic source of astaxanthin (Trial 5.1).

3. Assess the efficacy of a mono-esterified astaxanthin extract, di-esterified astaxanthin extract and total carotenoid extract derived from *Haematococcus pluvialis* and presented as top coat on a commercially produced basal diet (Trial 5.2).

4. Assess the stability of mono-esterified astaxanthin extract, di-esterified astaxanthin extract and total carotenoid extract presented in commercially produced diets (Trial 5.2).
5.3 Materials and Methods

5.3.1 Experimental designs

5.3.1.1 Trial 5.1

Three hundred and sixty rainbow trout were divided into 9 tanks (40 fish tank⁻¹, utilising two experimental systems: section 2.3). The fish were fed one of three diets containing different sources of astaxanthin (Table 5.1) with three replicates (tanks) per diet (Fig. 5.1).

Table 5.1 Desired (target) astaxanthin of the experimental diets.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Astaxanthin source</th>
<th>Target [Astaxanthin] (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Synthetic&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>Haematococcus&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>Total Carotenoid Extract&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60</td>
</tr>
</tbody>
</table>

<sup>a</sup> Carophyll Pink™ (Hoffmann-La Roche, Basel, Switzerland).<sup> b</sup> NatuRose™ (Cyanotech Corporation, Hawaii, USA).<sup> c</sup> Extracted from NatuRose™ Haematococcus pluvialis (John Moores University, Liverpool, UK) containing astaxanthin in its free, mono- and di-esterified forms and also several other carotenoids, also referred to as Decysted NatuRose™ (Fig 5.1).

Rainbow trout (Oncorhynchus mykiss), each with an initial mean weight of 98 ±9g were used for the present feeding trial. One hundred and fifty five fish were used as a representative sample for the period of the trial. Fish were sampled at three intervals during the experimental period, 20 fish were used in sample one (week 0), 45 (5 fish tank⁻¹) in sample two (at week 4), and 90 (10 fish tank⁻¹) in sample three (week 10) on termination of the trial. At each sampling time the perceived colour of the fillets of each of the randomly selected fish was recorded using the SalmoFan™ (section 2.5.3) and the concentration of astaxanthin in the epaxial white muscle was quantified by HPLC (section 2.5.5) against external standards (section 2.6). This data was used to calculate the feed conversion ratio (section 2.4.3), specific growth rate (section 2.4.3) and net apparent retention of astaxanthin (section 2.7).
Fig. 5.1 A schematic plan of the two recirculation systems used trial 5.1, in relationship to the distribution of experimental diets amongst the tanks. Where:

- Diet 1 = 60 mg kg\(^{-1}\) Carophyll Pink\(^{TM}\) (synthetic astaxanthin)
- Diet 2 = 60 mg kg\(^{-1}\) NatuRose\(^{TM}\) (Haematococcus pluvialis)
- Diet 3 = 60 mg kg\(^{-1}\) Decysted NatuRose\(^{TM}\)
- SF = Stock fish (to maintain the biomass in the system)
5.3.1.2 Trial 5.2

Four hundred and eighty rainbow trout were divided into 12 tanks (40 fish tank\(^{-1}\), utilising two experimental systems: section 2.3). The fish were fed one of four diets containing different sources of astaxanthin (Table 5.2) with three replicates (tanks) per diet (Fig. 5.2).

Table 5.2 Desired (target) astaxanthin content of the experimental diets.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Astaxanthin source</th>
<th>Target [Astaxanthin] (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Free(^a)</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>Mono-ester(^b)</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>Di-ester(^b)</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>TC extract(^bc)</td>
<td>30</td>
</tr>
</tbody>
</table>

\(^a\) Carophyll Pink\(^\text{TM}\) (Hoffmann-La Roche, Basel, Switzerland) control diet. \(^b\) Extracted from *Haematococcus pluvialis* NatuRose\(^\text{TM}\) (Cyanotech, Hawaii, USA). \(^c\) *Haematococcus pluvialis* total carotenoid extract contains astaxanthin in its free, mono- and di-esterified forms and also several other carotenoids.

Rainbow trout (*Oncorhynchus mykiss*), each with an initial mean weight of 141 ±3g were used for the present feeding trial. Fish were sampled at two intervals during the experimental period, 20 fish were used in sample one (week 0) and 180 (15 fish tank\(^{-1}\)) used in sample two (week 6) on termination of the trial. At each sampling time the perceived colour of the fillets of each of the randomly selected fish was recorded using the SalmoFan\(^\text{TM}\) (section 2.5.3) and the concentration of astaxanthin in the epaxial white muscle was quantified by HPLC (section 2.5.5) against external standards (section 2.6). This data was used to calculate the feed conversion ratio (section 2.4.3), specific growth rate (section 2.4.3) and net apparent retention of astaxanthin (section 2.7). Image analysis (section 4.3.3) was carried out on the fillets of those fish sampled at week 6, on termination of the trial. No unpigmented control diet was required for trials 5.1 and 5.2, as the fish in trial 4.1 after 12 weeks of receiving diet 6 (unpigmented control) retained no detectable carotenoids in their flesh.
Fig. 5.2 A schematic plan of the two recirculation systems used trial 5.2, in relationship to the distribution of experimental diets amongst the tanks. Where:

- **Diet 1** = 30 mg kg\(^{-1}\) Carophyll Pink\(^{TM}\) (synthetic free astaxanthin)
- **Diet 2** = 30 mg kg\(^{-1}\) *Haematococcus pluvialis* Mono-ester extract
- **Diet 3** = 30 mg kg\(^{-1}\) *Haematococcus pluvialis* Di-ester extract
- **Diet 4** = 30 mg kg\(^{-1}\) *Haematococcus pluvialis* Total carotenoid extract
5.3.2 Diet preparation

The composition of the basal diets for trials 5.1 and 5.2 (Table 5.3) were designed to promote optimal growth being determined by work conducted by Biomar UK Ltd (Grangemouth, UK).

Table 5.3 Basal diet formulation for trials 5.1 and 5.2.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Inclusion (g kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT-94 Fish-meal a</td>
<td>380.00</td>
</tr>
<tr>
<td>Norsemink Fish-meal a</td>
<td>170.00</td>
</tr>
<tr>
<td>Wheat Gluten b</td>
<td>50.00</td>
</tr>
<tr>
<td>Wheat-meal b</td>
<td>196.50</td>
</tr>
<tr>
<td>Cod Liver Oil c</td>
<td>102.75</td>
</tr>
<tr>
<td>Fish Oil b</td>
<td>90.00</td>
</tr>
<tr>
<td>Vitamin Premix b</td>
<td>7.50</td>
</tr>
<tr>
<td>Mineral Premix b</td>
<td>6.00</td>
</tr>
<tr>
<td>Yttrium oxide b</td>
<td>0.25</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
</tr>
</tbody>
</table>

a Norsildmel, Bergen, Norway; b Biomar Ltd., Grangemouth, UK; c Carrier oil for all top coated pigment sources (Seven Seas limited, Hull, UK).

The diets for trial 5.1 were produced in one 150 kg batch using a pilot scale (1000 kg capacity) commercial extruder (Biomar, Brande, Denmark), whereas, the diets for trial 5.2 were produced in one 100 kg batch using a pilot scale (1000 kg capacity) commercial extruder (Trouw, Wincham, UK) both being produced as the basal diet for their respective trials. The application of the different pigment sources in both trials was carried out post extrusion, with each source being added to the basal diet (Table 5.4). In trial 5.1, the synthetic astaxanthin (Carophyll Pink™) added to diet 1 (60 mg kg⁻¹) contained 8.00 % (dry weight) astaxanthin immobilised in a gelatine-starch matrix. *H. pluvialis* (NatuRose™) added to diet 2 (60 mg kg⁻¹) consisted of freeze dried cells with a total carotenoid content of 1.01 % (dry weight). The total carotenoid extract (obtained from NatuRose™, *H. pluvialis*) added to diet 3 (60 mg kg⁻¹), contained 2.01 % (dry weight) astaxanthin immobilised in gelatine, produced by Liverpool John Moores University. The immobilised pigments were both pre-treated, by adding 25 ml distilled water to 2.25 g
Carophyll Pink™ and 8.96 g total carotenoid extract. The pigment solutions were heated to 50°C using a temperature controlled water bath (Sonimatic, Langford Ultrasonics, West Midlands, UK) in order to dissolve their immobilising matrix. The pigment solution was then added to 102.75 g cod liver oil and homogenised (Silverson, Buckinghamshire, UK) for 15 s to form an emulsion. 17.822 g of NatuRose™ (H. pluvialis derived astaxanthin) in its native form was added to 102.75 g cod liver oil and homogenised using a magnetic stirrer (B292, J. Bibby Science Products Limited, Staffordshire, UK) for 15 minutes to form a NatuRose™ oil suspension.

Table 5.4 The weight (Wt) of basal diet required to produce 3 kg batches of pigmented diet for the respective experimental pigments used in trial 5.1 and trial 5.2.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Diet</th>
<th>Pigment</th>
<th>Basal diet Wt required (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>1</td>
<td>Synthetic&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.665</td>
</tr>
<tr>
<td>5.1</td>
<td>2</td>
<td>Haematococcus&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.683</td>
</tr>
<tr>
<td>5.1</td>
<td>3</td>
<td>TC Extract&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>2.649</td>
</tr>
<tr>
<td>5.2</td>
<td>1</td>
<td>Free&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.666</td>
</tr>
<tr>
<td>5.2</td>
<td>2</td>
<td>Mono-ester&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>2.692</td>
</tr>
<tr>
<td>5.2</td>
<td>3</td>
<td>Di-ester&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>2.692</td>
</tr>
<tr>
<td>5.2</td>
<td>4</td>
<td>TC extract&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>2.692</td>
</tr>
</tbody>
</table>

<sup>a</sup> Carophyll Pink™ (Hoffmann-La Roche, Basel, Switzerland). <sup>b</sup> NatuRose™ (Cyanotech Corporation, Hawaii, USA). <sup>c</sup> Extracted from NatuRose™ Haematococcus pluvialis (John Moores University, Liverpool, UK). <sup>d</sup> Haematococcus pluvialis total carotenoid extract contains astaxanthin in its free, mono- and di-esterified forms and also several other carotenoids.

In trial 5.2, the free astaxanthin (Carophyll Pink™) added to diet 1 (30 mg kg<sup>-1</sup>) was pre-treated as described above. The mono-esterified astaxanthin added to diet 2 (30 mg kg<sup>-1</sup>), di-esterified astaxanthin added to diet 3 (30 mg kg<sup>-1</sup>) and total carotenoid extract added to diet 4 (30 mg kg<sup>-1</sup>) were prepared by Liverpool John Moores University as freeze dried products that were mixed with cod liver oil containing the antioxidant ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline, added at an inclusion rate of 100 mg kg<sup>-1</sup>) to form an oil/pigment mixture. Prior to the addition of either of the pigment emulsions, the NatuRose™ oil suspension or any of the oil/pigment mixtures, 2.69 kg of basal diet was weighed into a mixing bowl (3 kg capacity, Hobart, London, UK) and incubated.
(Convection oven, Pickerstone Ovens, UK) at 40°C for 30 minuets. After incubation the mixing bowl was attached to a commercial blender (Hobart A-120, London, UK) and each pigment emulsion and NatuRose™ oil suspension was slowly poured over the basal diet pellets (over a 10 min period) which were concomitantly agitated by a mixing paddle. After addition of a pigment, each diet was agitated for 30 min to ensure a homogeneous distribution of pigment and cod liver oil over all the pellets. The carotenoid (astaxanthin) content of all the experimental diets was extracted using the enzyme method (section 2.5.2) and then analysed by HPLC. The fish in both trials followed a fixed feeding regime (section 2.4.3).

5.3.3 Statistical analysis

The statistical analyses used for growth, Roche colour scores and the retention of astaxanthin in white epaxial muscle is described in section 2.8. The data from the image analysis (trial 5.2) was also subjected to statistical analysis as described in section 4.3.6.
5.4 Results

5.4.1 Trial 5.1

Dietary carotenoid content is presented in Table 5.5, being assessed as free astaxanthin content for diet 1 (Carophyll Pink™) and as total carotenoid content for diets 2 (NatuRose™) and 3 (Decysted \textit{H. pluvialis}). The diets were analysed at week zero (the start of the experimental period) and at weeks four and ten (termination of the experiment), reflecting the sampling of the fish. Diet 2 remained stable throughout the experimental period showing no significant degradation. Diet 3 showed a total carotenoid loss of 10.3 % at week four with a further 11.4 % loss by week ten. Diet 1 proved to be the least stable of the astaxanthin sources used, demonstrating a 22.3 % loss of free astaxanthin at week four and further 6.2 % at week ten.

Table 5.5. Mean dietary content of astaxanthin measured as free astaxanthin content for diet 1 and total carotenoid content for diets 2 and 3 (mg kg$^{-1}$); $n=3$, ±standard deviation.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1$^a$ (mg kg$^{-1}$)</th>
<th>Diet 2$^b$ (mg kg$^{-1}$)</th>
<th>Diet 3$^{bc}$ (mg kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>52.73 ±2.54</td>
<td>52.79 ±0.53</td>
<td>51.53 ±2.00</td>
</tr>
<tr>
<td>4</td>
<td>40.99 ±2.27</td>
<td>51.28 ±1.01</td>
<td>46.20 ±1.15</td>
</tr>
<tr>
<td>10</td>
<td>38.44 ±0.96</td>
<td>51.86 ±1.09</td>
<td>40.93 ±0.30</td>
</tr>
</tbody>
</table>

$^a$ Control (Carophyll Pink™, Hoffmann-La Roche, Basel, Switzerland); $^b$ NatuRose™ (Cyanotech, Kona, Hawaii, USA); $^c$ Decysted cells (\textit{H. pluvialis} carotenoid extract) immobilised in a gelatine matrix (RPAN, Commentry, France).

The down stream processing used to obtain the total carotenoid extract was carried out by the trial (5.1) sponsor (Rhone Poulenc Animal Nutrition, Paris, France). During the production of the decysted \textit{H. pluvialis} (total carotenoid extract) the \textit{H. pluvialis} cells were exposed to quite harsh conditions, employing immobilised enzymes being incubated at approximately 55°C (Sabatier, \textit{pers comm.}). However, the complete protocol for decysting and immobilising the \textit{H. pluvialis} extract cells was deemed confidential. The carotenoid profile of the test pigments top-coated on to the basal diet are displayed in Table 5.6, as determined by Bowen (Liverpool John Moores University, Lancashire, UK). Carophyll Pink™ only contains free astaxanthin whereas the NatuRose™ product and the decysted \textit{H. pluvialis} contain both free astaxanthin and additional carotenoids.
*pluvialis* cells contain at least seven carotenoids, with astaxanthin being the most abundant carotenoid in both products. No significant differences (P > 0.05) were observed between the carotenoid profiles of NatuRose™ and decysted *H. pluvialis* cells.

Table 5.6 The carotenoid profiles of the three pigments added to the basal diet. n=3, ± standard deviation of the mean (Bowen, pers comm.).

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>CP (%)</th>
<th>NR (%)</th>
<th>TC extract (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Ax</td>
<td>100.00 ±0.00</td>
<td>4.62 ± 0.96</td>
<td>4.72 ± 0.85</td>
</tr>
<tr>
<td>Ax Mono-ester</td>
<td>0</td>
<td>57.68 ± 2.70</td>
<td>58.79 ± 2.56</td>
</tr>
<tr>
<td>Ax Di-ester</td>
<td>0</td>
<td>19.62 ± 1.52</td>
<td>21.00 ± 1.91</td>
</tr>
<tr>
<td>Astacene</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Canthaxanthin</td>
<td>0</td>
<td>7.56 ± 1.71</td>
<td>6.04 ± 1.69</td>
</tr>
<tr>
<td>Lutein</td>
<td>0</td>
<td>5.63 ± 0.34</td>
<td>5.41 ± 0.41</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>0</td>
<td>1.76 ± 0.25</td>
<td>2.35 ± 0.32</td>
</tr>
<tr>
<td>Adonirubin</td>
<td>0</td>
<td>3.13 ± 1.24</td>
<td>1.69 ± 0.29</td>
</tr>
</tbody>
</table>

* Carophyll Pink™ (Hoffmann-La Roche, Basel, Switzerland); b NatuRose™ (Cyanotech, Kona, Hawaii, USA);c Total carotenoid extract, decysted cells immobilised in a gelatine matrix (RPAN, Commentry, France).

Table 5.7 shows the growth performance of juvenile rainbow trout for each period over the course of the experimental feeding trial. There was a satisfactory growth increase of approximately 200% for fish at the end of the ten-week experimental period. The specific growth rate (SGR) and feed conversion ratio (FCR) for rainbow trout was calculated over the 68 days of actual feeding and was indicative of an excellent growth performance and a high utilisation efficiency of the basal diet formulations employed for the delivery of the test pigmentation sources. These values were typically SGR (1.56-1.62) and FCR values (0.83-0.85) (Table 5.7), both indices showed no significant differences (P>0.05) between the diets.
Table 5.7. Mean weight (g) of fish, measured at strategic time intervals to assess growth, n=3 (tanks), ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1^a (g)</th>
<th>Diet 2^b (g)</th>
<th>Diet 3^c (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>97.88 ± 9.26</td>
<td>97.56 ± 9.41</td>
<td>98.78 ± 7.92</td>
</tr>
<tr>
<td>4</td>
<td>165.99 ±2.50</td>
<td>167.91 ±3.02</td>
<td>167.73 ±8.18</td>
</tr>
<tr>
<td>10</td>
<td>282.43 ±4.16</td>
<td>290.52 ±5.84</td>
<td>287.21 ±5.19</td>
</tr>
<tr>
<td>SGR^d</td>
<td>1.56 ±0.07</td>
<td>1.62 ±0.06</td>
<td>1.57 ±0.08</td>
</tr>
<tr>
<td>FCR^e</td>
<td>0.85 ±0.03</td>
<td>0.83 ±0.03</td>
<td>0.84 ±0.04</td>
</tr>
</tbody>
</table>

^a Control (Carophyll Pink™, Hoffmann-La Roche, Basel, Switzerland); ^b NatuRose™ (Cyanotech, Kona, Hawaii, USA); ^c Decysted cells (H. pluvialis carotenoid extract) immobilised in a gelatine matrix (PRAN, Commentry, France); ^d Specific growth rate calculated for the overall ten-week experimental period; ^e Feed conversion ratio calculated for the overall ten-week experimental period.

The results for the deposition of carotenoid in the flesh of rainbow trout are presented in Table 5.8, using the colour score index as assessed by the SalmoFan™ system. It is obvious that apart from the initial sampling (week zero) a significant visual pink colouration became apparent at week four and was enhanced at week ten; this trend was applicable for all diets. At week four, diets 1, 2 and 3 obtained Colour Score Units (CSU) of 22.9, 22.8 and 21.8. Although subjective, at week four, diet 1 (Carophyll Pink) scored significantly (P<0.05) higher than diet 3 (Decysted H. pluvialis), but no significant differences (P>0.05) were observed between diets 1 and 2, or 2 and 3. At week ten, representing the final sampling of fish for flesh colour assessment, diets 1 (Plate 5.1), 2 (Plate 5.2) and 3 (Plate 5.3) obtained colour scores of 26.9, 27.1, and 26.7 CSU respectively. However, at week ten no significant differences (P>0.05) were observed between the diets.

Table 5.8 Mean Roche Colour Scores (RCS) for unskinned fillets, using the SalmoFan™, n=3 (tanks), ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1^a (RCS)</th>
<th>Diet 2^b (RCS)</th>
<th>Diet 3^c (RCS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>22.91 ±0.73</td>
<td>22.82 ±0.52</td>
<td>21.87 ±0.70</td>
</tr>
<tr>
<td>10</td>
<td>26.94 ±0.14</td>
<td>27.15 ±0.29</td>
<td>26.75 ±0.13</td>
</tr>
</tbody>
</table>

^a Control (Carophyll Pink™, Hoffmann-La Roche, Basel, Switzerland); ^b NatuRose™ (Cyanotech, Kona, Hawaii, USA); ^c Decysted cells (H. pluvialis carotenoid extract) immobilised in a gelatine matrix (PRAN, Commentry, France); ^d Not Applicable.
Plate 5.1. A representation of the typical flesh colour of a fish (rainbow trout) receiving diet 1 top-coated with 50 mg kg$^{-1}$ Carophyll Pink$^{TM}$ (free astaxanthin) at week 10.
Plate 5.2. A representation of the typical flesh colour of a fish (rainbow trout) receiving diet 2 top-coated with 50 mg kg\(^{-1}\) NatuRose\textsuperscript{TM} \textit{(Haematococcus pluvialis)} at week 10.
Plate 5.3. A representation of the typical flesh colour of a fish (rainbow trout) receiving diet 3 top-coated with 50 mg kg$^{-1}$ decysted *Haematococcus pluvialis* at week 10.
Table 5.9 displays the astaxanthin concentrations in each respective group of fish receiving each test diet in triplicate. The values are derived from the mean of three groups of fish sampled at each time period over the duration of the trial. At week zero, the values were the same for all the diets as this is the representation of the background pigment concentration in the fish epaxial white muscle at the start of the experimental period. At week four, the astaxanthin concentration had increased to 1.31, 1.27 and 1.18 mg kg\(^{-1}\) for diets 1, 2 and 3 respectively. This resulted in no significant differences (P>0.05) in epaxial white muscle astaxanthin concentration. At week ten, the epaxial white muscle astaxanthin concentration had considerably increased for all diets to 6.15, 5.98 and 5.17 mg kg\(^{-1}\) for diets 1, 2 and 3 respectively. The fish receiving diet 1 (Carophyll Pink\textsuperscript{TM}) retained a significantly (P<0.05) higher astaxanthin concentration in their white epaxial muscle than those fish receiving diet 3 (Decysted \textit{H. pluvialis}). The fish fed diet 2 (NatuRose\textsuperscript{TM}) displayed similar final tissue concentrations of pigment relative to the Carophyll Pink\textsuperscript{TM} reference fish. Thus, no significant differences (P>0.05) were observed between diets 1 and 2, or diets 2 and 3. These carotenoid levels generally supported the visual assessments observed at weeks four and ten. The net apparent retention (NAR) of the test pigments reflects the final astaxanthin concentration measured in the epaxial white muscle at week ten (Table 5.9) for all diets. The fish fed the control diet (1) containing 60 mg kg\(^{-1}\) Carophyll Pink\textsuperscript{TM} obtained the highest NAR at 8.91 % compared to those fed diet 2 containing 60 mg kg\(^{-1}\) NatuRose\textsuperscript{TM} that only retained 7.11% of the available pigment. The fish fed diet 3 (60 mg kg\(^{-1}\) decysted \textit{H. pluvialis}) retained 7.16 % of the pigment they consumed, similar to those fish receiving diet 2. The NAR value for diet 1 was significantly (P<0.05) higher than the NAR values for diets 2 and 3, whereas the NAR values for diets 2 and 3 were not significantly different (P>0.05).
Table 5.9. Mean white muscle astaxanthin concentration (mg kg\(^{-1}\)), n=3 (tanks), \(\pm\) standard deviation of the mean.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1(^a) (mg kg(^{-1}))</th>
<th>Diet 2(^b) (mg kg(^{-1}))</th>
<th>Diet 3(^c) (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.07 (\pm)0.01</td>
<td>0.07 (\pm)0.01</td>
<td>0.07 (\pm)0.01</td>
</tr>
<tr>
<td>4</td>
<td>1.31 (\pm)0.32</td>
<td>1.27 (\pm)0.16</td>
<td>1.18 (\pm)0.11</td>
</tr>
<tr>
<td>10</td>
<td>6.15 (\pm)0.96</td>
<td>5.98 (\pm)0.89</td>
<td>5.17 (\pm)0.30</td>
</tr>
<tr>
<td>NAR</td>
<td>8.91 (\pm)0.89</td>
<td>7.11 (\pm)0.86</td>
<td>7.16 (\pm)0.35</td>
</tr>
</tbody>
</table>

\(^a\) Control (Carophyll Pink\(^TM\), Hoffmann-La Roche, Basel, Switzerland); \(^b\) NatuRose\(^TM\) (Cyanotech, Kona, Hawaii, USA); \(^c\) Decysted cells (\(H.\) pluvialis carotenoid extract) immobilised in a gelatine matrix (RPAN, Commentry, France); \(^d\) Net apparent retention calculated for the overall six-week experimental period, being expressed as a percentage.

5.4.2 Trial 5.2

Dietary carotenoid content is presented in Table 5.10, being assessed as free astaxanthin content for diet 1 (Carophyll Pink) and as total carotenoid content for diets 2 (astaxanthin mono-ester), 3 (astaxanthin di-ester) and 4 (\(H.\) pluvialis total carotenoid extract). The diets were analysed at week zero (at the start of the experimental period) and at week six (termination of the experiment), reflecting the sampling of the fish. All the diets remained stable throughout the experimental period showing no significant degradation. Diets 1, 2 and 3 attained the target inclusion level of 30 mg kg\(^{-1}\), whereas diet 4 contained an elevated (36.4 \%) total carotenoid content.

Table 5.10. Mean dietary content of astaxanthin measured as free astaxanthin content for diet 1 and total astaxanthin content for diets 2, 3 and 4 (mg kg\(^{-1}\)), n=3, \(\pm\) standard deviation.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1(^a) (mg kg(^{-1}))</th>
<th>Diet 2(^b) (mg kg(^{-1}))</th>
<th>Diet 3(^c) (mg kg(^{-1}))</th>
<th>Diet 4(^d) (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>31.65 (\pm)3.21</td>
<td>30.28 (\pm)3.33</td>
<td>32.01 (\pm)3.49</td>
<td>40.92 (\pm)1.26</td>
</tr>
<tr>
<td>6</td>
<td>29.54 (\pm)0.32</td>
<td>32.03 (\pm)1.12</td>
<td>33.03 (\pm)0.35</td>
<td>41.57 (\pm)2.49</td>
</tr>
</tbody>
</table>

\(^a\) Control (Carophyll Pink\(^TM\), Hoffmann-La Roche, Basel, Switzerland); \(^b\) Astaxanthin mono-esters extracted from NatuRose\(^TM\) (Cyanotech, Kona, Hawaii, USA) \(Haematococcus\) pluvialis; \(^c\) Astaxanthin di-esters extracted from NatuRose\(^TM\); \(^d\) NatuRose\(^TM\) total carotenoid extract.

Table 5.11 displays the growth performance of juvenile rainbow trout for each period over the course of the experimental feeding trial. There was a satisfactory growth increase of approximately 77 \% for fish at the end of the six-week experimental period. The specific
growth rate (SGR) and feed conversion ratio (FCR) for rainbow trout was calculated over the 42 days of actual feeding and was indicative of a good growth performance and a high utilisation efficiency of the basal diet formulations employed for the delivery of the test pigmentation sources. These values were typically SGR (1.40-1.46): and FCR values (0.99-1.02) (Table 5.11), both indices showed no significant differences (P>0.05) between the diets.

Table 5.11. Mean weight (g) of fish, measured at strategic time intervals to assess growth, n=3 (tanks), ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1 (g)</th>
<th>Diet 2 (g)</th>
<th>Diet 3 (g)</th>
<th>Diet 4 (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>140.37 ± 3.23</td>
<td>141.92 ± 3.43</td>
<td>142.03 ± 2.62</td>
<td>139.69 ± 2.07</td>
</tr>
<tr>
<td>3</td>
<td>204.10 ± 6.20</td>
<td>206.93 ± 3.38</td>
<td>206.79 ± 7.01</td>
<td>202.27 ± 7.41</td>
</tr>
<tr>
<td>6</td>
<td>246.70 ± 12.82</td>
<td>249.03 ± 13.64</td>
<td>252.06 ± 9.71</td>
<td>251.32 ± 11.14</td>
</tr>
</tbody>
</table>

SGR ^e 1.42 ±0.08 1.40 ±0.09 1.43 ±0.05 1.46 ±0.08
FCR ^f 1.02 ±0.03 1.02 ±0.03 0.99 ±0.05 1.00 ±0.05

^a Control (Carophyll Pink™, Hoffmann-La Roche, Basel, Switzerland); ^b Astaxanthin mono-esters extracted from NatuRose™ (Cyanotech, Kona, Hawaii, USA) Haematococcus pluvialis; ^c Astaxanthin di-esters extracted from NatuRose™; ^d NatuRose™ total carotenoid extract; ^e Specific growth rate calculated for the overall six-week experimental period; ^f Feed conversion ratio calculated for the overall six-week experimental period.

The results for the perceived colour due to the deposition of carotenoid in the flesh of rainbow trout are presented in Table 5.12, using the colour score index as assessed by the SalmoFan™ system. At the initial sampling (week zero) a visual pink colouration was apparent, which became enhanced at week six, this trend was applicable for all diets. At week zero, due to twenty fish being randomly sampled prior to being randomly designated to their tanks, all four diets obtained a mean colour score of 21.4 Colour Score Units (CSU). At week six, representing the final sampling of fish for flesh colour assessment, diets 1, 2, 3 and 4 obtained colour scores of 26.8, 27.4, 25.6 and 26.4 CSU respectively. Although subjective, diets 1, (Carophyll Pink™), 2 (astaxanthin mono-esters) and 4 (total carotenoid extract) scored significantly (P<0.05) higher than diet 3 (astaxanthin di-esters). Yet, no significant differences (P>0.05) were observed between diets 1, 2 and 4.
Table 5.12. Mean Roche Colour Scores (RCS) for unskinned fillets, using the SalmoFan™, n=3 (tanks), ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1^a (RCS)</th>
<th>Diet 2^b (RCS)</th>
<th>Diet 3^c (RCS)</th>
<th>Diet 4^d (RCS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>21.43 ± 1.38</td>
<td>21.43 ± 1.38</td>
<td>21.43 ± 1.38</td>
<td>21.43 ± 1.38</td>
</tr>
<tr>
<td>6</td>
<td>26.81 ± 1.47</td>
<td>27.40 ± 1.54</td>
<td>25.56 ± 1.35</td>
<td>26.37 ± 1.68</td>
</tr>
</tbody>
</table>

^a Control (Carophyll Pink™, Hoffmann-La Roche, Basel, Switzerland); ^b Astaxanthin mono-esters extracted from NatuRose™ (Cyanotech, Kona, Hawaii, USA) Haematococcus pluvialis; ^c Astaxanthin di-esters extracted from NatuRose™; ^d NatuRose™ total carotenoid extract.

Table 5.13 displays the mean colour (red component only) of the fillets (sampled at week 6), measured using image analysis for the triplicate groups of fish receiving each test diet. Results of the six transects are displayed sequentially with transects one and six representing the anterior (head) and posterior (tail) respectively. The results are expressed as grey values, which range from 0-256, where grey values of 0 and 256 are black and pure white, respectively. A noticeable trend was apparent for all the diets, whereby the mean grey value for each successive transect decreased from the anterior to the posterior of each fillet. Diets 1, 2 and 4 demonstrated the mean grey value for each successive transect to be significantly (P<0.01) lower than its predecessor. No significant differences (P>0.05) were observed between the mean grey values for transects 1 and 2 for those fish receiving diet 2. However, the mean grey values for transects 3, 4, 5 and 6 were all significantly (P<0.05) lower than transects 1 and 2. Transects 3, 4, 5 and 6 of diet 3 fed fish followed a similar trend as seen in diets 1, 2 and 4, in that the mean grey value for each successive transect was significantly lower (P<0.01) than its predecessor. The grey value obtained for the mean of all six transects (overall mean) resulted in 144.71, 134.84 170.66 and 149.51 for diets 1 (Carophyll Pink™), 2 (astaxanthin mono-esters), 3 (astaxanthin di-esters) and 4 (total carotenoid extract), respectively. The overall mean grey value for diet 2 was significantly (P<0.05) lower than all the other diets, whereas the over all grey value for diet 3 was significantly (P<0.05) higher than all the other diets. However, no significant differences (P>0.05) were observed for the overall mean grey values between diets 1 and 4.
Table 5.13 Mean grey values (red component only) for the six transects measured from head to tail along the fillets of all the fish sampled at week 6, using image analysis, n=3 (tanks), ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Transect</th>
<th>Diet 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Diet 2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Diet 3&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Diet 4&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>204.68 ± 4.29</td>
<td>199.61 ± 8.13</td>
<td>215.84 ± 1.97</td>
<td>209.32 ± 3.19</td>
</tr>
<tr>
<td>2</td>
<td>179.32 ± 6.82</td>
<td>168.08 ± 4.80</td>
<td>211.32 ± 7.35</td>
<td>181.59 ± 6.54</td>
</tr>
<tr>
<td>3</td>
<td>167.99 ± 5.73</td>
<td>151.79 ± 5.61</td>
<td>197.98 ± 8.51</td>
<td>162.94 ± 9.20</td>
</tr>
<tr>
<td>4</td>
<td>138.53 ± 8.30</td>
<td>127.18 ± 3.68</td>
<td>158.53 ± 4.29</td>
<td>141.89 ± 3.77</td>
</tr>
<tr>
<td>5</td>
<td>103.82 ± 4.19</td>
<td>97.48 ± 5.99</td>
<td>121.82 ± 6.22</td>
<td>105.72 ± 6.81</td>
</tr>
<tr>
<td>6&lt;sup&gt;f&lt;/sup&gt;</td>
<td>73.89 ± 5.74</td>
<td>70.97 ± 7.62</td>
<td>108.46 ± 3.91</td>
<td>95.75 ± 2.47</td>
</tr>
</tbody>
</table>

Mean<sup>g</sup> | 144.71 | 135.84 | 170.66 | 149.53 |

RCS<sup>h</sup> | 28 | 29 | 27 | 28 |

<sup>a</sup> Control (Carophyll Pink™, Hoffmann-La Roche, Basel, Switzerland);  
<sup>b</sup> Astaxanthin mono-esters extracted from NatuRose™ (Cyanotech, Kona, Hawaii, USA) Haematococcus pluvialis;  
<sup>c</sup> Astaxanthin di-esters extracted from NatuRose™;  
<sup>d</sup> NatuRose™ total carotenoid extract;  
<sup>e</sup> Located at the head of each fillet;  
<sup>f</sup> Located at the tail of each fillet;  
<sup>g</sup> Mean of all six transects (for the whole fillet);  
<sup>h</sup> Roche Colour Score (RCS) corresponding to the overall mean grey value.

Table 5.14 displays the astaxanthin concentration retained in the epaxial white muscle of each respective group of fish receiving each test diet in triplicate. The values are derived from the mean of three groups of fish sampled at each time period over the duration of the trial. At week zero the values were the same for all the diets as this is the representation of the background pigment concentration in the fish’s epaxial white muscle at the start of the experimental period. Twenty fish randomly sampled prior to being randomly designated to their tanks resulted in an initial (week zero) mean epaxial white muscle astaxanthin concentration of 0.62 mg kg<sup>-1</sup> for all four diets. At week six the astaxanthin concentration had increased to 3.95, 4.34, 3.46 and 3.94 mg kg<sup>-1</sup> for diets 1, 2, 3 and 4 respectively. These resulted in the fish receiving diet 2 (astaxanthin mono-ester) retaining a significantly (P < 0.05) higher astaxanthin concentration in their white epaxial muscle than those fish receiving diet 3 (astaxanthin di-ester). The fish fed diets 2, 3 and 4 all displayed similar final tissue concentrations of pigment relative to the Carophyll Pink™ (diet 1) reference fish. Thus, no significant differences (P > 0.05) were observed between diets 1, 2 and 4, or diets 1, 3 and 4. These carotenoid levels generally supported the visual assessments observed at weeks zero and six. The net apparent retention (NAR) of the test pigments...
reflects the final astaxanthin concentration measured in the epaxial white muscle at week six (Table 5.14) for diets 1, 2 and 3; whereas diet 4 was lower than anticipated. The fish fed diet 2 retained 18.73% (of the astaxanthin mono-ester added to the basal feed) in their epaxial white muscle, compared to only 13.22% (of the astaxanthin di-ester added to the basal feed) for the fish receiving diet 3. The NAR of astaxanthin in the white epaxial muscle of those fish fed the control diet (1) containing Carophyll pink™ was similar (16.13%) to diet 2. Likewise, the NAR of astaxanthin in the white muscle of those fish receiving diet 4 containing the *H. pluvialis* total carotenoid extract was similar (11.86%, lower than anticipated) to diet 3. No significant differences (*P* > 0.05) were observed for the NAR values between diets 1 and 2, or diets 1, 3 and 4. However, the NAR values for diets 3 and 4 were significantly (*P* < 0.01) lower than the NAR value for diet 2.

Table 5.14 Mean epaxial white muscle astaxanthin concentrations (mg kg⁻¹), n=3 (tanks), ± standard deviation of the mean, measured at strategic times to assess net apparent retention (NAR) of the test pigments.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1 a (mg kg⁻¹)</th>
<th>Diet 2 b (mg kg⁻¹)</th>
<th>Diet 3 c (mg kg⁻¹)</th>
<th>Diet 4 d (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.62 ± 0.41</td>
<td>0.62 ± 0.41</td>
<td>0.62 ± 0.41</td>
<td>0.62 ± 0.41</td>
</tr>
<tr>
<td>6</td>
<td>3.95 ± 0.98</td>
<td>4.34 ± 0.53</td>
<td>3.46 ± 0.32</td>
<td>3.94 ± 1.47</td>
</tr>
<tr>
<td>NAR  e</td>
<td>16.13 ± 1.84</td>
<td>18.73 ± 1.45</td>
<td>13.22 ± 1.18</td>
<td>11.86 ± 2.56</td>
</tr>
</tbody>
</table>

a Control (Carophyll Pink™, Hoffmann-La Roche, Basel, Switzerland); b Astaxanthin mono-esters extracted from NatuRose™ (Cyanotech, Kona, Hawaii, USA) *Haematococcus pluvialis*; c Astaxanthin di-esters extracted from NatuRose™; d NatuRose™ total carotenoid extract; e Net apparent retention calculated for the overall six-week experimental period, being expressed as a percentage.
5.5 Discussion

It is evident from the results of both feeding trials (5.1 and 5.2) that the rainbow trout performed according to expectations within the respective experimental periods, i.e. ten weeks for trials 5.1 and six weeks for trial 5.2. The 200% increase in weight gain and feed conversion ratio of <1 demonstrated by the fish in trial 5.1 confirmed that the feed utilisation efficiency was consistent with optimal farmed conditions using the present basal diet formulation. In contrast, the fish in trial 5.2 only managed a feed conversion ratio of approximately 1, gaining 77% in weight over the experimental period, even though the basal diet in both trials had the same formulation. However, the feed utilisation efficiency for trial 5.2 was still consistent with reasonably good farm conditions. In both trials all dietary treatments performed similarly with respect to growth and weight gain, showing no significant differences (P>0.05) between the diets within each trial. In this context, the degree of pigmentation within each would be of a similar potential given the fact that the deposition of astaxanthin is dependent on the rate of growth in salmonids. The results reported for both trials (5.1 and 5.2) were based on the same feeding regime as trials 4.1 and 4.2, that employed an initial acclimation phase where fish were fed on a standard (unpigmented) commercial feed (Trouw standard 40). This was to ensure that the trout were not stressed, were disease free and of general good health. This also ensures that all fish receiving each treatment fed consistently and uniformly prior to the experimental period, during which muscle (fillet) samples were excised and analysed for their respective levels of carotenoid (astaxanthin). As stated in chapter 4, two independent assessments were used to measure the colour of the fillets from both trials. The first, involved the SalmoFan™ (Roche colour reference) as a subjective indicator of colour. The SalmoFan™ is routinely employed by the salmon industry as a simple method to grade the quality of salmon and trout. The second, by the direct analytical determination of the astaxanthin content in tissue extracts from freshly excised fillets using high performance liquid chromatography. The results from both trials (5.1 and 5.2) for these parameters, displayed
trends that were indicative of an expeditious development of flesh pigmentation, for all the diets. In trial 5.1 the Roche colour score data for the 20 randomly sampled fish at week zero were not of significance. As a definite numerical value was difficult to apply at this stage, since no CSU could be applied for the initial fish due to their pale (white) flesh appearance (visually unpigmented). This is rationalised by the fact that week zero was the effective start of the trial evaluation, when fish were conditioned to the experimental diets. Thus, fish evaluated at this stage represented the base level of pigmentation for comparison of each treatment on termination. Visual colouration was evident at week four for the Carophyll Pink™ (diet 1), NatuRose™ (diet 2) and decysted *H. pluvialis* (diet 3) fed trout. This became more apparent after week ten. At week four, the fish fed diets 1 and 2 had increased their colour by at least three colour score units (CSU) to 23 and those fed diet 3 only increased by 2 CSU to 22. CSU values are quoted on the basis of the whole numbers constituting the Roche colour score (SalmoFan™ system). The final muscle samples taken on conclusion of the study (week ten) are of importance since they relate to rainbow trout of marketable size (approximately 300 g) and are of direct interest to the aquaculturist fish processor and the consumer. At week ten, all the treatments (diets 1, 2 and 3) had increased by at least four CSU's to 27, leading to no significant differences (P>0.05) between the diets. It should be noted that the Roche colour score (RCS) data for fish sampled at week zero in trial 5.2 were of significance. A numerical RCS of 21.4 was applied for all treatments at this stage due to the fish having slightly pigmented flesh, even though the fish for all the trials were obtained from the same source (Hatchlands Trout Farm, Rattery, Devon, UK). This may be rationalised by the fact that at week zero (the effective start of the trial evaluation) the fish in trial 5.2 had a mean weight of 140 g, this weight being at least 40% heavier than the mean initial weight of the fish used in trials 4.1, 4.2 and 5.1. At this increased start weight, even though the fish (obtained from a farm) had only ever received an unpigmented diet, it is conceivable that they had been foraging for natural prey such as gammarus and other aquatic invertebrates that contain natural sources of
carotenoids which they are able to assimilate. Another possibility may be that the unpigmented diet they received on the farm could have been contaminated with astaxanthin during production. Astaxanthin contamination of unpigmented diets occurs due to the fact that the vast majority of commercial diets comprise of up to 10% remix, i.e. the remnants and waste of other diets, some which may be pigmented. Nevertheless, fish evaluated at this stage represented the base level of pigmentation for comparison of each treatment on termination. The final muscle samples taken on conclusion of the study (week six) were again of importance since they to were of a marketable size at approximately 250 g. At week six, diet 3 (30 mg kg\(^{-1}\) astaxanthin di-esters) increased by four CSU's to 25, diets 1 (30 mg kg\(^{-1}\) Carophyll Pink™) and 4 (30 mg kg\(^{-1}\) total carotenoid extract) had increased by five CSU'S to 26 and diet 2 (30 mg kg\(^{-1}\) astaxanthin mono-esters) had increased by six CSU'S to 27. This led to diets 1, 2 and 4 scoring significantly (P<0.05) higher than diet 3, whilst no significant differences (P>0.05) were observed between diets 1, 2 and 4. Nevertheless, the Roche colour score system is subjective, and distinguishing perceived colour may be impeded by the presence of muscle fat deposits (Kestin, pers comm.). Likewise, the perceived colour may be enhanced by the presence of blood due to impaired bleeding (Storebakken and No, 1992; Foss et al., 1987).

It must be noted that for both trials (5.1 and 5.2) no tank effect was observed for the astaxanthin concentration measured in white epaxial muscle from fish fed the same diet but residing in different tanks. Trials 5.1 and 5.2 followed the same trend observed in trials 4.1 and 4.2; i.e. tank position did not have an effect on the feeding performance of the fish. In trial 5.1, white muscle astaxanthin concentration generally supported the visual assessment of all the groups at all the sampling sessions at weeks zero, four and ten. At week zero no astaxanthin was detected in any of the 20 randomly sampled fish. By week four the white muscle astaxanthin concentrations for all diets (1, 2 and 3) were similar, displaying appreciable retention. It seems from the final sampling at week ten that the pigmentation of all groups increased dramatically by over 400 % (which equates to at least 3.9 mg kg\(^{-1}\))
over the six week period from the previous samples taken at week four. In trial 5.2, white muscle astaxanthin concentration generally supported the visual assessment of all the groups from weeks 0 to 6. The white muscle astaxanthin concentrations at week 0 for all treatments was 0.62 mg kg\(^{-1}\). It seems from the final sampling at week 6 that the pigmentation of all groups increased dramatically by over 550 % (which equates to at least 3.45mg kg\(^{-1}\)) during the six week experimental period. The large increase in pigmentation observed in both trials over the final six weeks may be due to the addition of cod liver oil as the carrier for the application of the pigments to the diets. As cod liver oil is highly palatable and is readily assimilated by salmonids, probably due to its fatty acid profile and high lipid soluble vitamin content, namely vitamin E, which is a potent antioxidant that may have a sparing effect on the utilisation of astaxanthin as an antioxidant. Hence, cod liver oil may provide increased uptake of astaxanthin from the trout gut. Under the experimental conditions of trial 5.1, decysted *H. pluvialis* was demonstrated to be an inferior pigment source than Carophyll Pink™ when fed to rainbow trout at an inclusion rate of 60mg kg\(^{-1}\) astaxanthin equivalence. In comparison, the fish fed NatuRose™ (diet 2) displayed similar final tissue concentrations of pigment relative to the Carophyll Pink™ reference fish (diet 1). The results also demonstrate that removing the cell wall from *H. pluvialis* cells has little effect on the bioavailability of astaxanthin from this source, this in-turn has little effect on retention of astaxanthin in the white epaxial muscle of rainbow trout. The net apparent retention (NAR) of the test pigments reflected the final astaxanthin concentration measured in the epaxial white muscle at week ten for all diets, where diet 1 > diet 3 > diet 2. The fish fed the control diet (1) containing free astaxanthin (Carophyll Pink™) retained approximately 25 % more pigment in their flesh than the fish receiving either NatuRose™ *H. pluvialis* or decysted *H. pluvialis*. However, the fish receiving NatuRose™ *H. pluvialis* or decysted *H. pluvialis* obtained very similar NAR values, that were not significantly different (P>0.05). The present study confirms that the presence of a cell wall, although damaged as in the case of NatuRose™ *H. Pluvialis* cells, does not affect
the bioavailability of free, mono-esterified or di-esterified astaxanthin. The addition of NatuRose™ and the decysted *H. pluvialis* were calculated on total astaxanthin content, which for both pigment sources was 85% of their total carotenoid content. This is rationalised by the fact that decysted *H. pluvialis* was produced from NatuRose™. The decision to use decysted NatuRose™ was taken as total carotenoid content of *Haematococcus pluvialis* varies depending on the species (Grung *et al.*, 1992). The analysis of the carotenoid profiles of NatuRose™ and decysted *H. pluvialis* (Table 5.6) showed both sources to contain the same carotenoids. More importantly, no significant differences (P>0.05) were observed between the content of any of the carotenoids identified in either source (Bowen, *pers comm.*). This demonstrates the method elected to decyst the cells had no effect on their carotenoid profile or content. This also rationalises the fact that diet 2 (NatuRose™) and diet 3 (decysted *H. pluvialis*) obtained similar NAR values.

Under the experimental conditions of trial 5.2, the di-esterified astaxanthin extract (diet 3) was demonstrated to be an inferior pigment source than mono-esterified astaxanthin extract (diet 2) when fed to rainbow trout at an inclusion rate of 30mg kg⁻¹ astaxanthin equivalence. Thus, the concentration of astaxanthin measured in the white epaxial muscle tissue of the fish fed di-esterified astaxanthin was significantly (P<0.05) lower than that of the fish fed mono-esterified astaxanthin.

This to some extent may be rationalised by work carried out by Schiedt (1988) who demonstrated that free astaxanthin is deposited unaltered into salmonid flesh whereas esterified astaxanthin is hydrolysed in the intestinal lumen. Schiedt (1988) proposed that astaxanthin esters (e.g. astaxanthin dipalmitate) are hydrolysed in the intestinal lumen, and as a consequence produce less pigmented flesh. The present study makes a further suggestion that the degree of esterification (i.e. mono- or di-ester) could be a critical factor to the bioavailability to the different forms of astaxanthin. This may provide some credence to the suggestion made in chapter 4 that catabolism of the astaxanthin esters may
be the rate limiting step, such that the transient time in the lumen is not sufficient for their total hydrolysis. The results from trial 5.2 suggest the rate limiting step may be in particular due to the catabolism of the di-esters. This difference could be linked to the profile of the non-carotenoid moiety of astaxanthin di-esters, as non specific esterases will probably demonstrate distinct affinities for the different astaxanthin di-esters, being dependent on to which R group the astaxanthin is ester bonded. The lower retention of the astaxanthin di-ester compared to the mono-ester form may also be linked to the cis/trans ratio of astaxanthin molecules. As Bjerkeng et al. (1997) demonstrated, all-E-trans astaxanthin had a better retention in epaxial white muscle than astaxanthin cis-Z-isomers. Meyers (1994) further suggested there to be no significant differences in the retention of the three astaxanthin trans isomeric (3R, 3'R; 3R, 3S meso; 3S, 3'S) forms. No significant differences (P>0.05) were observed between the concentration of astaxanthin retained in the flesh of those fish receiving diets 2 (astaxanthin mono-ester) and 4 (H. pluvialis total carotenoid extract). This may be explained by the carotenoid profile of NatuRose™ Haematococcus meal from which extracts were prepared to pigment diets 2, 3 and 4. NatuRose™ Haematococcus meal contains approximately 70% astaxanthin mono-esters, 10% astaxanthin di-esters, 5% free astaxanthin and the remaining 15% consisting of a mixture of β-carotene, lutein, canthaxanthin, zeaxanthin and other carotenoids (Lorenz, 1999). Nevertheless, lutein, canthaxanthin have been demonstrated to pigment salmonid species (Latscha, 1990), but the results of trial 4.1 suggests the levels found in the species of H. pluvialis used in these trials had negligible affect on pigmenting rainbow trout epaxial white muscle. Thus, the similar bioavailabilities of astaxanthin mono-esters and H. pluvialis total carotenoid extract is probably due to the majority of astaxanthin in H. pluvialis being in the mono-ester form. This may also simply be explained by the fact that diets 1, 2 and 3 achieved the target inclusion level of 30 mg kg\(^{-1}\), whereas diet 4 had an elevated inclusion level of 41 mg kg\(^{-1}\). However, no significant differences (P>0.05) in the final tissue concentrations of pigment were observed between the Carophyll Pink™
reference fish (diet 1) and those of any of the other treatments (diets 2, 3 or 4), although Carophyll Pink™ has been demonstrated to be a superior pigment source than *H. pluvialis* as demonstrated in trial 4.1. The net apparent retention (NAR) values for diets 1, 2 and 3 reflected the astaxanthin concentration measured in the epaxial white muscle of those fish fed the respective diets, where diet 2 > diet 1 > diet 3 > diet 4. However, the concentration of astaxanthin in the epaxial white muscle of those fish fed diet 4 seemed higher than the astaxanthin concentration measured in the muscle of diet 3 fed fish. The lower net apparent retention of astaxanthin for diet 4 is explained by the fact that diet 4 contained approximately 30 % more pigment than the other diets. As discussed in chapter 4, it appears that the inclusion level of pigment in the diet is a critical factor in gaining high NAR values. Comparing the NAR values with the astaxanthin concentrations measured in the white epaxial muscle for all the treatments, suggests that a lower inclusion level (30 mg kg$^{-1}$) of pigment in the diet is more efficiently retained in the flesh. However, the cost benefit to the farmer in using lower inclusion levels of pigment must be determined against the time required to attain an astaxanthin concentration in the flesh that would be acceptable to the consumer.

Image analysis was only used in trial 5.2, solely being applied to the fillets of those fish sampled at week 6 (on termination of the trial). As discussed in chapter 4, this technique measured all three colour components (red, green and blue) of the fillets as grey values. The blue and green components for every fillet in all treatments showed remarkable similarity to each other, as demonstrated in trial 4.1. Again, statistical analysis of the different colour components (in all treatments) showed both the green and blue components to be significantly (*P<0.001*) lower than the red component, but not significantly different (*P>0.05*) from each other, having negligible effect on the overall colour of the fillets. Hence, only the corresponding grey values for the red component were shown in Table 5.13. The use of image analysis allows a direct association between grey value and redness, where the higher the grey value the lighter the red hue, all of which are
comparable with the SalmoFan™. In all treatments a general pattern emerged when comparing the mean grey values for each transect, whereby their grey values decreased as the transect number increased i.e. grey values decrease from the anterior (head) transect one to the posterior (tail) transect six. This result correlates with the results gained in chapter 4, where the redness of the fillet increased at the tail, indicating a higher concentration of pigment being present in this region of the fillet. Differences in muscle astaxanthin concentration in different regions of a fillet are due to the shape of rainbow trout and the fillets they yield. Starting at the anterior (head) of a fillet, the distance from the dorsal edge to the ventral edge continuously decreases as one moves towards the posterior (tail) of the fillet. However, the number of muscle fibres remains continuous from anterior to posterior, such that towards the posterior of the fillet the muscle fibres become more tightly packed; the degree of packing of muscle fibres is termed muscle fibre density. Combining the fact that the number of astaxanthin binding sites per actomyosin unit is relatively constant with the fact that muscle fibre density increases towards the tail, results in the muscle located in the tail region not only to appear more red but to also contain a higher pigment concentration per kg (as compared to muscle located in the head region of the fillet). Again, the overall mean grey value for the red component for all treatments lies between transects three and four, as demonstrated in trial 4.1. This provides further evidence that taking muscle samples from under the dorsal fin for carotenoid analysis does provide a good indication of the overall carotenoid concentration of a fillet. The overall mean grey values for all treatments reflects the results gained at week six for the mean epaxial white muscle astaxanthin concentration.

It would seem from the results gained in this study (trial 5.2) that commercially, the production of a mono-ester extract is not a cost effective route. Introducing downstream processes to remove (decyst) the cell wall from *H. pluvialis* and separate the astaxanthin mono-esters would lead to an increase in production costs which would inevitably be passed on to the feed manufacturer, fish farmer and consumer with no other direct benefits.
Likewise, the results from trial 5.1, suggest commercial production of decysted cells would not be cost effective as the introduction of downstream processes to remove (decyst) the cell wall from *H. pluvialis* would also lead to increased production costs with no other direct benefits.

Dietary carotenoid content of the feeds used in trial 5.1 was calculated on the free astaxanthin content of the synthetic source and the total astaxanthin (free, mono and di-esters) of the natural sources (NatuRose™ and decysted *H. pluvialis*). Carophyll Pink™ manufactured by Hoffmann-La Roche is guaranteed to contain 8 % free astaxanthin (dry weight) as confirmed by Bowen (*pers comm.*), with a 1 : 3, *cis : trans* isomeric ratio. The *trans* isomers have been shown to have better retention in epaxial white muscle than the *cis* isomers (Bjerkeng *et al.*, 1997), regardless of the *trans* stereo isomer form (Meyers, 1994).

NatuRose™ and decysted *H. pluvialis* had total carotenoid contents of 1.01 and 2.27 % (dry weight) respectively, as measured by Bowen (*pers comm.*). The astaxanthin content of all the diets in trial 5.2 remained stable throughout the six-week storage period, even though the pigments were add to the basal diet using a top coating method. This technique was adopted, as the results from trial 4.2 demonstrated that adding Carophyll Pink™ with a target inclusion of 50 mg kg\(^{-1}\) to a diet prior to being extruded incurred approximately 35 % degradation of its astaxanthin content. Nevertheless, all the diets in trial 5.1 targeted 60 mg kg\(^{-1}\), which was achieved at week 0 by employing the top-coating technique, except diet 1 top-coated with Carophyll Pink™ resulted in a 27 % loss during the ten week storage period. The stability of Carophyll Pink™ in trial 4.2 proved to be better than that observed during trial 5.1, and this may be due to the fact that astaxanthin residing within the pellet matrix is protected from photo-degradation and oxidation, concomitantly providing thermal insulation against high temperatures. A similar loss (21 %) was also observed for the decysted *H. pluvialis* over the same storage period. Yet, the NatuRose™ pigment remained stable throughout the same storage period. The difference in stability between these two natural sources of astaxanthin is probably due to the fact that the pigment in the
decysted *H. pluvialis* was exposed. In comparison, the pigment residing in the encysted cells of NatuRose™ were afforded the same protection as that discussed for Carophyll Pink™ residing within the pellet matrix. This increase in the stability of Carophyll Pink™ and *H. pluvialis* extracts observed in trial 5.2 may be due to the addition of the antioxidant ethoxyquin to the oil pigment solutions, which acts to protect the astaxanthin from oxidation.

In conclusion, the decysted *H. pluvialis* product tested in trial 5.1 performed effectively and provided very similar flesh colouration characteristics when compared to free astaxanthin (Carophyll Pink™) at the final assessment (week ten). The degree of perceived pigmentation, as evaluated by the SalmoFan™, was adequate and favourable resulting in a deep pink colour for the fillets. There were no apparent obvious differences between the groups to the trained observer. Additionally, direct measurement of the astaxanthin in the flesh by chromatographic techniques (HPLC) confirmed that the trout were able to absorb and assimilate the different astaxanthin sources to a similar degree. In trial 5.2 the total carotenoid and the astaxanthin mono-esters and di-esters extracted from *H. pluvialis* (NatuRose™) also performed effectively, providing very similar flesh colouration compared to free astaxanthin (Carophyll Pink™) at the final assessment (week six). This resulted in a desirable pink colour for all fillets. However, there was a slight apparent difference between the groups to the trained observer. The direct measurements of the astaxanthin in the flesh by HPLC again confirmed that the trout were able to absorb and assimilate the different astaxanthin sources to a similar degree. In both trials, the efficacy of utilisation of the extracted *H. pluvialis* products was surprisingly good, given the fact that *Haematococcus pluvialis* algae and other naturally occurring carotenoids are generally inferior to synthetic forms, with respect to their bioavailability from the gastrointestinal tract of salmonid fish. There were no apparent problems with respect to the health of the rainbow trout at the end of either trial and no indication of any internal lesions or abnormalities associated with supplementation of the diets with the algal products. It may
therefore be conclude that decysted *H. pluvialis* and astaxanthin mono-esters are effective substitutes for Carophyll Pink™ under the conditions of the present investigations. In the future (providing efficient, cost effective methods of down stream processing can be developed) decysted *H. pluvialis* and astaxanthin mono-esters could be recommended as sources for the pigmentation of salmonids destined for the table market.
CHAPTER 6

Evaluation of Corbinol (2-palmitoylamino-propionic acid) on the pigmentation of rainbow trout fed synthetic astaxanthin (Trial 6.1)
6.1 Introduction

The formation of a mesoderm in triploblastic organisms allows them to increase in size and develop a specific alimentary canal (Green et al., 1990a). The specialised nature of different tissues that form organs such as the heart, liver and various regions of the alimentary canal is due to compartmentalisation of cells which affords their autonomy whilst allowing specific control of differentiated functions. This may be due to specific metabolic reactions being unduly inhibited by the presence of a manifold of organic and inorganic compounds. Cells in turn are subject to internal compartmentalisation to form organelles. Compartmentalisation occurs at both the cellular and sub-cellular level due to the presence of membranes. The primary role of the plasma membrane is to protect the cell from the hostile environment in which they are present, and to maintain enzymes, ions and other compounds essential to maintain homeostasis and life (Racker, 1976). Many biochemical, physiochemical and electron microscopic investigations have enabled the basic structure of the cell membrane to be elucidated (Devlin, 1992). All membranes are composed of lipids arranged into two (inner and outer) leaflets (more commonly described as a lipid bilayer) which is disrupted by the presence of globular proteins the majority of which span the membrane being in contact with the aqueous environment on either side (Devlin, 1992). The major component of all membranes are amphipathic phospholipids which contain a hydrophilic (polar) head and a hydrophobic (non-polar) tail, these form the thermodynamically stable bilayer with the polar head groups facing the aqueous phase and the non-polar tails projecting internally (Carroll, 1989). Membranes are not rigid structures that remain static; workers using a fluorescence photobleaching recovery technique demonstrated rapid lateral movement of lipids and proteins in the plane of the membrane (Stryer, 1988). The use of electron spin resonance spectroscopy has demonstrated phospholipids to undergo transverse diffusion from one leaflet to the other in a flip-flop motion (Alberts et al., 1989). Yet, flip flopping rarely occurs, being energetically unfavourable as the polar head must transit through the non-polar interior of the membrane.
This is probably the reason why proteins have never been observed to flip-flop, because they generally contain greater numbers of polar regions in their tertiary structures. Singer and Nicholson (1972) proposed the fluid mosaic model to describe the structure of membranes, which suggested globular proteins were integral, penetrating the bi-lipid layer as well as being loosely bound to their surface. After extensive studies the model proposed by Singer and Nicholson has become widely accepted, and is referred to by the name which they used to describe membranes i.e. the ‘fluid mosaic model’ (Devlin, 1992). Thus, the proteins “float in a sea” of lipid, which act like a solvent providing the membrane with fluidity. Nevertheless, the architecture of membranes is highly variable, depending on the specialised nature of the cell or organelle (Darnell et al., 1990). Immense variation not only occurs between the ratio of lipid to protein in a particular membrane but also the ratio of different lipids and the degree of double bonding within a lipid, all of which have a direct effect on membrane fluidity (Devlin, 1992). The vast majority of lipids within all membranes belong to one of three groups, phospholipids, sphingolipids and cholesterol, all of which interact forming non-covalent bonds to form a continuous sheet that form closed boundaries that are highly selectively permeable (Stryer, 1988). The formation of hydrophobic interior makes all membranes extraordinarily impermeable to polar molecules and ions and prevents the cell’s contents from escaping (Alberts et al., 1989). Similarly it also makes the entry of uncharged polar molecules (such as astaxanthin) into the cell difficult. Yet, in theory if given enough time virtually any molecule will diffuse across a protein free (synthetic) membrane down its concentration gradient (Alberts et al., 1989). In terms of carotenoid bioavailability in rainbow trout, the membrane of the pyloric region of the gastrointestinal tract has been identified as the primary site for astaxanthin absorption (White, unpublished). Absorption of astaxanthin is probably restricted by several factors, namely due to it being a polar molecule combined with a highly variable residence time within the gut (which is dependent on fish size and feeding rate). In trials 4.1, 4.2, 5.1 and 5.2, respectively the low values obtained for the net
apparent retention of astaxanthin in the fillets of fish fed diets containing either the Carophyll Pink™ or NatuRose™ provided some indication of its poor bioavailability to salmonids. The dose response observed in trial 4.1 for Carophyll Pink™ is limited, as the retention of astaxanthin in white epaxial muscle has a hyperbolic relationship to the concentration added to the diet, where a dietary content of 100 mg kg⁻¹ lies on the plateau (Storebakken and No, 1992). Therefore, increasing the inclusion rate above 100 mg kg⁻¹ is of no practical benefit and will serve to increase feed costs. Hence, an economical alternative could be to increase the bioavailability of astaxanthin by the addition of a novel additive. One such additive, Corbinol (2-palmitoylaminopropionic acid) has been advocated as an aid for the uptake of astaxanthin during in vitro studies (Garnett, pers comm.). If this technology is transferable to the in vivo condition, it has the possibility of increasing astaxanthin bioavailability, which in theory could increase muscle retention. Corbinol has the added advantage of being thermally labile making it suitable for use in commercially extruded feeds (Garnett, 1998). The aim of the present experiment was to test in vivo (using rainbow trout as a model for salmonid species) the efficacy of adding Corbinol to commercially produced diets, in terms of its effect on the bioavailability of synthetic astaxanthin, using the retention of astaxanthin within the epaxial white muscle as an indicator.
6.2 Aims

The main aims of the present experiment were:

1. Assess the effect of 2-palmitoylaminopropionic acid (Corbinol) on the bioavailability of synthetic astaxanthin (Carophyll Pink™) in commercially produced expanded diets.

2. Assess the effect of 2-palmitoylaminopropionic acid (Corbinol) on the bioavailability of background carotenoids present in commercially produced expanded diets.
6.3 Materials and Methods

6.3.1 Experimental design

6.3.1.1 Trial 6.1

Three hundred and sixty rainbow trout were divided into 12 tanks (30 fish tank⁻¹ utilising 2 experimental systems: section 2.3) and three replicate groups were fed one of four diets (Fig. 6.1), containing synthetic astaxanthin (Carophyll Pink™) and/or 2-palmitoylaminoproprionic acid (Corbinol) (Table 6.1) with a positive and negative control, for 12 weeks.

| Table 6.1 Desired (target) astaxanthin content of the experimental diets. |
|-----------------|-----------------|-----------------|-----------------|
| Diet | Astaxanthin source | Target [Astaxanthin] (mg kg⁻¹) | Corbinol e (mg kg⁻¹) |
| 1   | N/A c | 0 | 0 |
| 2   | N/A | 0 | 1000 |
| 3 b | Synthetic d | 40 | 0 |
| 4   | Synthetic | 40 | 1000 |

a Negative control; b Positive control; c Not applicable; d Carophyll Pink™ (Hoffmann-La Roche, Basel, Switzerland); e 2-palmitoylaminoproprionic acid (Lovesgrove Research, Machynlleth, UK).

Fish were sampled at four intervals during the experimental period, 20 fish were used in sample one (week 0), 36 in sample two (at week 4), 36 in sample three (week 8) and 120 used in sample four (week 12) on termination of the trial. At each sampling time the fish were weighed, the perceived colour of the fillets of each of the randomly selected fish was recorded using the SalmoFan™ (section 2.5.3) and the concentration of astaxanthin in the epaxial white muscle was quantified by HPLC (section 2.5.5) against external standards (2.6). This data was used to calculate the feed conversion ratio (section 2.4.3), specific growth rate (section 2.4.3) and net apparent retention of astaxanthin (section 2.7).
Fig. 6.1 A schematic plan of the two recirculation systems used in trial 6.1, in relationship to the distribution of experimental diets amongst the tanks. Where:

Diet 1 = Unpigmented control
Diet 2 = 1000 mg kg\(^{-1}\) Corbinol
Diet 3 = 40 mg kg\(^{-1}\) Carophyll Pink\(^{\text{TM}}\) (synthetic astaxanthin)
Diet 4 = 40 mg kg\(^{-1}\) Carophyll Pink\(^{\text{TM}}\) & 1000 mg kg\(^{-1}\) Corbinol
6.3.2 Diet preparation

The composition of the basal diets for trial 6.1 (Table 6.2) was designed to promote optimal growth (based on a commercial diet), being determined by work carried out by Biomar Ltd.

Table 6.2 Basal diet formulation for trial 6.1.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Inclusion (g kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT-94 Fish-meal a</td>
<td>380.0</td>
</tr>
<tr>
<td>Norsemink Fish-meal a</td>
<td>170.0</td>
</tr>
<tr>
<td>Wheat Gluten b</td>
<td>50.0</td>
</tr>
<tr>
<td>Wheat-meal b</td>
<td>196.5</td>
</tr>
<tr>
<td>Fish Oil b</td>
<td>190.0</td>
</tr>
<tr>
<td>Vitamin Premix b</td>
<td>7.5</td>
</tr>
<tr>
<td>Mineral Premix b</td>
<td>6.0</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
</tr>
</tbody>
</table>

a Norsildmel, Bergen, Norway, b Biomar Ltd., Grangemouth, UK.

The diets for trial 6.1 were produced in 25kg batches using a pilot scale (1000 kg capacity) commercial extruder (Biomar, Brande, Denmark). The synthetic astaxanthin (Carophyll Pink™) added to Diets 3 and 4 targeting 40 mg kg⁻¹ was pre-treated, by heating in order to dissolve its starch gelatine matrix. 12.5 g of the Carophyll Pink™ product was added to 200 ml distilled water and heated to 50°C to form an astaxanthin solution. The Carophyll Pink™ solution was then added to the feed mix prior to being extruded. Any degradation of astaxanthin due to extrusion was compensated for by mixing extra astaxanthin solution with oil and added to the diet post-extrusion as a top-coat (applied by spraying the extruded pellets with the oil astaxanthin solution). To protect this astaxanthin the pellets were top coated a second time with oil. Due to its stability the Corbinol (1000 mg kg⁻¹) incorporated into diets 2 and 4 was added in its native form prior to extrusion (Garnett, 1998). Diet 1 (negative control) was unpigmented and did not contain Corbinol (basal diet). The fish followed a fixed feeding regime (2.4.3). The carotenoid (astaxanthin)
content of all the experimental diets was extracted using the Soxtherm method (2.5.1) and then analysed by HPLC.

6.3.3 Statistical analysis

The statistical analyses used for growth, Roche colour scores and the retention of astaxanthin in white epaxial muscle is described in section 2.8.
6.4 Results

The effect of 2-palmitoylaminoproprionic acid (Corbinol) on the bioavailability of synthetic astaxanthin (Carophyll Pink™) in commercially produced expanded feeds, was analysed in this trial. Dietary carotenoid content is presented in Table 6.3, being assessed as total carotenoid content. The diets were analysed at week zero (the start of the experimental period) when the diets were received from the manufacturer and then at weeks four, eight and twelve, on termination of the experiment. All the diets remained stable throughout the twelve-week storage period. However, diets 3 and 4 that targeted 40 mg kg\(^{-1}\) Carophyll Pink™, at week zero showed a 11.65 % and 10.19 % loss of carotenoid, respectively.

### Table 6.3. Mean dietary content measured as total carotenoid content for diets 1 and 2, and free astaxanthin for diets 3 and 4; n=3, ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1 (mg kg(^{-1}))(^b)</th>
<th>Diet 2 (mg kg(^{-1}))(^b)</th>
<th>Diet 3 (mg kg(^{-1}))(^c)</th>
<th>Diet 4 (mg kg(^{-1}))(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.52 ±0.08</td>
<td>0.51 ±0.44</td>
<td>35.34 ±0.54</td>
<td>35.96 ±0.47</td>
</tr>
<tr>
<td>4</td>
<td>0.55 ±0.28</td>
<td>0.49 ±0.23</td>
<td>35.89 ±0.71</td>
<td>35.68 ±1.93</td>
</tr>
<tr>
<td>8</td>
<td>0.46 ±0.13</td>
<td>0.54 ±0.17</td>
<td>36.02 ±1.24</td>
<td>34.83 ±1.06</td>
</tr>
<tr>
<td>12</td>
<td>0.58 ±0.20</td>
<td>0.52 ±0.11</td>
<td>34.65 ±2.87</td>
<td>35.55 ±0.79</td>
</tr>
</tbody>
</table>

\(^a\) Negative control \(^b\) 2-palmitoylaminoproprionic acid (Lovesgrove Research, Machynlleth, UK); \(^c\) 40 mg kg\(^{-1}\) Carophyll Pink™ (Hoffmann-La Roche, Basel, Switzerland); \(^d\) Positive control.

The growth performance of juvenile rainbow trout for each bi-weekly period over the course of the experimental feeding trial is presented in Table 6.4. At the end of the twelve-week experimental period the fish increased their weight by approximately 200%. Both specific growth rate (SGR) and feed conversion ratio (FCR) for rainbow trout was calculated over the 78 days of actual feeding and was indicative of a good growth performance and a high utilisation efficiency of the basal diet formulations employed for the delivery of the test pigmentation sources, as compared with work carried out by Bjerken et al (1997). These values were typically SGR (1.60-1.65): and FCR values (1.00-1.08) (Table 6.4), both indices showed no significant differences (P>0.05) between the diets.
Table 6.4. Mean weight (g) of fish, measured biweekly to assess growth, n=3 (tanks), ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1a(g)</th>
<th>Diet 2b(g)</th>
<th>Diet 3c(g)</th>
<th>Diet 4d(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>92.11±3.41</td>
<td>95.79±3.40</td>
<td>94.79±2.36</td>
<td>89.98±7.21</td>
</tr>
<tr>
<td>2</td>
<td>130.48±6.00</td>
<td>133.74±5.82</td>
<td>132.29±4.97</td>
<td>126.34±10.80</td>
</tr>
<tr>
<td>4</td>
<td>167.02±9.74</td>
<td>171.44±6.40</td>
<td>168.49±9.92</td>
<td>163.48±9.98</td>
</tr>
<tr>
<td>6</td>
<td>212.06±15.72</td>
<td>215.68±4.39</td>
<td>212.68±13.88</td>
<td>206.47±7.26</td>
</tr>
<tr>
<td>8</td>
<td>242.60±12.21</td>
<td>252.55±4.11</td>
<td>255.48±17.97</td>
<td>239.75±10.90</td>
</tr>
<tr>
<td>10</td>
<td>290.44±14.37</td>
<td>292.31±6.43</td>
<td>297.69±19.82</td>
<td>276.67±11.83</td>
</tr>
<tr>
<td>12</td>
<td>331.76±19.11</td>
<td>331.42±8.79</td>
<td>337.66±22.05</td>
<td>315.67±9.39</td>
</tr>
</tbody>
</table>

SGR e 1.64±0.03 1.60±0.02 1.62±0.06 1.65±0.04
FCR f 1.03±0.04 1.04±0.05 1.00±0.05 1.08±0.03

a Negative control; b 2-palmitoylaminopropionic acid (Lovesgrove Research, Machynlleth, UK); c 40 mg kg⁻¹ Carophyll Pink™ (Hoffmann-La Roche, Basel, Switzerland); d Positive control; e Specific growth rate calculated for the overall twelve week experimental period; f Feed conversion ratio calculated for the overall twelve week experimental period.

The results for the perceived deposition of carotenoid (colour score) in the flesh of rainbow trout as assessed by the SalmoFan™ system are presented in Table 6.5. It is obvious that apart from the initial sampling (week 0) a notable visual pink colouration became apparent and was enhanced after week four for diets 3 (positive control, Carophyll Pink 40mg kg⁻¹) and 4 (Carophyll Pink 40 mg kg⁻¹ and Corbinol 1000 mg kg⁻¹) becoming more enhanced after weeks eight and twelve. These trends seemed to tail off until the end of the trial, for the fish fed diet 3, but continued for fish fed diet 4. At week 0 (initial sampling) all of the 20 randomly sampled fish were so pale (no noticeable pink colouration) that they could not be scored using the Roche colour score index. The lack of colouration was also noted at weeks four eight and twelve in the fillets of those fish fed diets 1 (negative control) and 2 (Corbinol 1000 mg kg⁻¹). However, the fillets of those fish fed either of the pigmented diets scored 22.9 and 22.3 CSU's at week four and 27.5 and 25.3 CSU's at week eight for diet 3 and 4, respectively. At week twelve, representing the final sampling of fish for flesh colour assessment, the fish receiving diets 3 (Carophyll Pink™ 40mg kg⁻¹) and 4 (40mg kg⁻¹ Carophyll Pink™ + 100mg kg⁻¹ Corbinol) scored 27.9 and 28.2 respectively. No significant differences (P>0.05) were observed between the two pigmented diets (3 and 4)
or between the two unpigmented diets (1 and 2) throughout the experimental period. Nevertheless, the fillets of those fish receiving either of the two pigmented diets (3 and 4), obtained significantly higher (P< 0.001) scores than the fillets of those fish fed either of the non-pigmented diets (1 and 2).

Table 6.5. Mean Roche Colour Scores (RCS) for unskinned fillets, using the SalmoFan™, n=3(tanks), ± standard deviation of the mean.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1 (^a) (RCS)</th>
<th>Diet 2 (^b) (RCS)</th>
<th>Diet 3 (^cd) (RCS)</th>
<th>Diet 4 (^bc) (RCS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N/A (^c)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>N/A</td>
<td>N/A</td>
<td>22.89 ±2.28</td>
<td>22.26 ±0.98</td>
</tr>
<tr>
<td>8</td>
<td>N/A</td>
<td>N/A</td>
<td>27.48 ±1.68</td>
<td>25.26 ±2.06</td>
</tr>
<tr>
<td>12</td>
<td>N/A</td>
<td>N/A</td>
<td>27.90 ±1.33</td>
<td>28.22 ±1.24</td>
</tr>
</tbody>
</table>

a Negative control; \(^b\) 2-palmitoylaminoproprionic acid (Lovesgrove Research, Machynlleth, UK); \(^c\) 40 mg kg\(^{-1}\) Carophyll Pink™ (Hoffmann-La Roche, Basel, Switzerland); \(^d\) Positive control; \(^e\) Not Applicable.

Table 6.6 displays the astaxanthin concentrations retained in the epaxial white muscle of the triplicate groups of fish receiving each test diet. At week zero (initial sampling) none of the 20 randomly sampled fish had any detectable carotenoids in their flesh. At week four and eight no significant differences (P > 0.05) were observed between the astaxanthin concentration retained in the flesh of those fish fed diets 3 (Carophyll Pink™ 40 mg kg\(^{-1}\)) and 4 (Carophyll Pink™ 40 mg kg\(^{-1}\) and Carbinol 1000 mg kg\(^{-1}\)). This resulted in a mean flesh astaxanthin concentration of 2.84 and 2.80 mg kg\(^{-1}\) at week four, and 3.65 and 2.92 mg kg\(^{-1}\) at week eight, for diets 3 and 4 respectively. The fish fed the unpigmented diet (2) containing Carbinol (1000 mg kg\(^{-1}\)) retained (0.13, 0.21 and 0.15 mg kg\(^{-1}\) at weeks 4, 8 and 12 respectively) significantly (P<0.001) lower epaxial white muscle carotenoid concentrations than fish receiving the two pigmented diets (3 and 4), throughout the experimental period. However, diet 2 fed fish retained a significantly (P<0.01) higher astaxanthin concentration than those fish receiving diet 1 (negative control, basal diet) throughout the experimental period. As no carotenoids were detectable in the epaxial white muscle of those fed diet 1, throughout the experimental period. At week twelve
representing the final sampling session, no significant difference (P>0.05) between epaxial white muscle astaxanthin concentrations was observed in the fish receiving diets 3 (Carophyll Pink™ 40mg kg⁻¹) and 4 (40mg kg⁻¹ Carophyll Pink™ + 1000 mg kg⁻¹ Corbinol) that retained 4.14 and 3.96 mg kg⁻¹ respectively. These flesh astaxanthin concentrations generally support the visual assessment of the fillets at all the sampling sessions (weeks 0, 4, 8 and 12). The net apparent retention (NAR) of astaxanthin in the epaxial white muscle of those fish fed diet 2 was significantly (P<0.01) higher than all the other diets at 21.27 %. No significant differences (P>0.05) were observed between the NAR values for diets 3 and 4, which obtained values of 10.37 % and 9.36 %, respectively, reflecting the final astaxanthin concentration of flesh as measured at week 12. The NAR for the basal diet (negative control, diet 1) was determined to be inapplicable.

Table 6.6 Mean white muscle astaxanthin concentration (mg kg⁻¹), n=3 (tanks), ± standard deviation of the mean, measured at strategic times to assess net apparent retention (NAR) of the test pigments.

<table>
<thead>
<tr>
<th>Week</th>
<th>Diet 1a (mg kg⁻¹)</th>
<th>Diet 2b (mg kg⁻¹)</th>
<th>Diet 3c (mg kg⁻¹)</th>
<th>Diet 4d (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>ND e</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
<td>0.13 ±0.07 l</td>
<td>2.84 ±1.53</td>
<td>2.80 ±1.06</td>
</tr>
<tr>
<td>8</td>
<td>ND</td>
<td>0.21 ±0.11 l</td>
<td>3.65 ±0.85</td>
<td>2.92 ±1.57</td>
</tr>
<tr>
<td>12</td>
<td>ND</td>
<td>0.15 ±0.12 l</td>
<td>4.14 ±1.75</td>
<td>3.96 ±0.83</td>
</tr>
<tr>
<td>NAR f</td>
<td>N/A g</td>
<td>21.27 ±0.17</td>
<td>10.37 ±1.96</td>
<td>9.36 ±1.81</td>
</tr>
</tbody>
</table>

a Negative control; b 2-palmitoylaminoproprionic acid (Lovesgrove Research, Machynlleth, UK); c 40 mg kg⁻¹ Carophyll Pink™ (Hoffmann-La Roche, Basel, Switzerland); d Positive control; e No detectable astaxanthin; f Net apparent retention calculated for the overall twelve-week experimental period; g Not applicable; l Suspected to be Zeaxanthin.
6.5 Discussion

As with the previous feeding trials discussed in chapters 4 and 5, the result of trial 6.1 employed a similar feeding regime, which incorporated an initial acclimation phase to ensure that the trout were disease free and in good health. It is evident from the results of the feeding trial the rainbow trout performed according to expectations within the twelve-week experimental period. The 200 % increase in weight gain and feed conversion ratio of approximately one confirmed that the feed utilisation efficiency was consistent with good farm conditions. All dietary treatments performed similarly in this respect and the degree of pigmentation would be expected to be of a similar potential given the fact that the deposition of astaxanthin is dependent on the rate of growth in salmonids, as demonstrated in chapter 4. No significant differences (P>0.05) were observed between any of the diets at any stage of the experiment, in terms of growth. This suggests the addition of Corbinol to diets 2 and 4 had no effect on the palatability of the diet or the active ingredient (2-palmitoylaminoproprionic acid) had no adverse effects on fish health. Feeding response and fish growth are both good indicators of any problems the fish may be experiencing, as fish will not consume unpalatable diets resulting in an inferior growth performance. Likewise, fish that are exposed to factors inducing stress either abiotic (such as high temperatures or pollution) or biotic (namely disease) would demonstrate a lack of feeding response, again resulting in poor or even retarded growth. Nevertheless, the inclusion rate of Corbinol (1000 mg kg⁻¹) was 0.1 % of the diet, but 2-palmitoylaminoproprionic acid the active ingredient of Corbinol only constitutes 10 % of its mass. The remaining 90 % is talc which acts as a bulking agent making Corbinol a stable free flowing miscible white powder, with good handling characteristics for use on an industrial scale.

Two independent assessment approaches were used to monitor the progress of the fish in terms of pigmentation. The traditional Roche colour reference (SalmoFan™) was routinely employed as the subjective indicator of colour perception, as used by the salmonid industry as a simple tool to assess quality. This was also supported by quantitatively determining
the astaxanthin content in tissue extracts from freshly excised fillet samples, using high performance liquid chromatography, at each stage of the trial. The results reported for these parameters display trends, which are indicative of a rapid development in pigmentation of the flesh and external integument of the fish for both pigmented diets. The Roche colour score (SalmoFan™) data at week zero for the 20 randomly sampled fish were not of significance since the white muscle remained unpigmented at this stage. This is rationalised by the fact that week zero was the effective start of the trial evaluation when fish were conditioned to the experimental diets. Thus, fish evaluated at this stage (week 0) represented the base level of pigmentation for comparison of each treatment on termination of the experiment at week twelve. No visual colouration could be distinguished for any of the fillets observed at weeks four, eight and twelve from those fish fed either of the unpigmented diets (1: negative control/basal diet and 2: 1000 mg kg⁻¹ Carbinol). As discussed in chapter 4, the SalmoFan™ cannot ascribe a colour score for fillets that remain unpigmented or only contain very low concentrations of astaxanthin. Thus, the scores for diets 1 and 2 were not significantly different (P>0.05) at any stage of the experiment. However, a visual colouration was evident at week four for those fish receiving the pigmented diets (3 and 4). This became more apparent after weeks eight and twelve. At week four, the fish fed diet 3 containing 40 mg kg⁻¹ Carophyll Pink™ (positive control) and those fed diet 4 containing 40 mg kg⁻¹ Carophyll Pink™ and 1000 mg kg⁻¹ Carbinol gained 4 and 3 colour score units (CSU) resulting in 23 and 22 CSU’s respectively, but were not significantly (P>0.05) different. At week 8 the pigmentation of the fillets became more enhanced for both pigmented dietary treatments, resulting in diet 3 increasing by 4 CSU’s to 27 and diet 4 increasing by 3 CSU’s to 25. However, the mean scores for each dietary treatment were not significantly different (P>0.05), this probably due to the large variation in the colour scores awarded by the sensory panel. The Roche colour score system is subjective, and can be affected by illumination and presentation of the fillets (Foss et al., 1987). Distinguishing perceived colour may be impeded by the presence of
muscle fat deposits (Kestin, pers comm.). Likewise, the perceived colour may be enhanced by the presence of blood due to impaired bleeding (Storebakken and No 1992; Foss et al., 1987). At week twelve (termination of the experiment) the colour scores again increased for both pigmented diets. Diet 3 (40 mg kg\(^{-1}\) Carophyll Pink™) increased by 1 CSU to 28 whereas diet 4 increased by 3 CSU’s to 28. Again, the scores for each of the pigmented dietary treatment were not significantly different (P>0.05). However, the colour scores for both pigmented diets (3 and 4) were significantly (P<0.001) higher than those gained for the unpigmented diets (1 and 2) at weeks four, eight and twelve.

The astaxanthin concentration of the epaxial white muscle generally supported the visual assessment of those fish fed the unpigmented diets (1 and 2) and those receiving the pigmented diets (3 and 4) at all sampling sessions (weeks zero, four, eight and twelve). At week 0 no astaxanthin was detected in the flesh of any of the fish sampled, literally providing blank fish to pigment. The fish fed the basal diet (1, negative control) remained unpigmented throughout the twelve-week experimental period. Whereas the unpigmented diet containing 2-palmitoylaminoproprionic acid (Corbinol 1000 mg kg\(^{-1}\)) demonstrated retention of very low concentrations of a carotenoid in their epaxial white muscle after four weeks of receiving Corbinol. The carotenoid was determined to be zeaxanthin (Bowen, pers comm.). Yet, the concentration of zeaxanthin measured at weeks eight and twelve were not significantly different (P>0.05) as those measured at week four. This is probably due to the large variation in the concentration of zeaxanthin retained in the flesh of the individual fish receiving diet 2 (Corbinol 1000 mg kg\(^{-1}\)). The presence of zeaxanthin in the epaxial white muscle of those fish receiving the unpigmented diet (2) containing Corbinol 1000 mg kg\(^{-1}\) is rationalised by the fact that both diet 2 and the basal diet (1, negative control) when analysed revealed a mean total carotenoid content of approximately 0.5 mg kg \(^{-1}\) which remained stable throughout the twelve-week experimental period. The carotenoid content of this diet may be accounted for by the results of experiment 3.1 that determined several of the dietary ingredients, namely extruded wheat and vitamin premix
contained low levels of the carotenoid zeaxanthin (Whyte, pers comm.). This finding seems to suggest that 2-palmitoylaminoprorionic acid does have an effect on increasing the bioavailability of zeaxanthin in rainbow trout. At week four the fish receiving the pigmented diets (3 and 4) demonstrated retention of astaxanthin in their flesh. The concentration of astaxanthin in the flesh of fish fed either diets 3 or 4 were significantly (P<0.001) higher than diet 2, but not significantly different (P>0.05) from each other. At week eight the concentration of astaxanthin in the flesh of those fish fed the pigmented diets increased by approximately 29 and 4 % for diets 3 and 4 respectively. The difference in epaxial white muscle astaxanthin concentration between those fish fed diet 3 (positive control) containing 40 mg kg⁻¹ synthetic astaxanthin (Carophyll Pink™) and those receiving the diet 4 containing 40 mg kg⁻¹ Carophyll Pink™ and 1000 mg kg⁻¹ Corbinol were not significantly different (P>0.05), but were significantly (P<0.001) higher than those fed diet 2. The concentration of astaxanthin in flesh of those fish receiving the pigmented diets increased by approximately 13 and 36 % for diets 3 and 4 respectively, at week twelve, again showing no significant differences (P>0.05), but both being significantly (P<0.001) higher than the fish fed diet 2.

These findings propose Corbinol had no detectable effect on increasing the bioavailability of astaxanthin in rainbow trout, even though under normal conditions rainbow trout absorb zeaxanthin far more poorly than astaxanthin (Schiedt, 1988). The differing effect Corbinol had on zeaxanthin compared to astaxanthin is probably due to the difference in their chemical structures. Astaxanthin has an extra pair of carbonyl groups, one located at C4 and the other at C4', each being situated in one of the β-ionone rings. Thus, being devoid of the two carbonyl groups makes zeaxanthin smaller, less polar and more hydrophobic than astaxanthin. The increased absorption of zeaxanthin as compared to astaxanthin may be further rationalised by the effect Corbinol has on lipid membranes. The confirmation of phospholipids and the weak attractions formed between the heads of neighbouring molecules results in a loose hexagonal packing and the formation of natural pores (Garnett...
and Jones, 1993). The characteristic hole-size profile of different cell types varies greatly, which is predominantly due to the quantity and type of glycolipids and intra-membrane and peripheral proteins present (Garnett and Jones, 1993). Corbinol permeablises cell membranes by altering the simple hexagonal packing and manipulating its natural vacancies, creating pores of varying sizes that allow the passage of desirable molecules (Garnett and Jones, 1993). The use of Corbinol in the present experiment was to create pores within the epithelial cells of rainbow trout's gastrointestinal tract to increase the rate of astaxanthin absorption in the gut. It seems that a combination of the size and hydrophobicity of zeaxanthin, enables it to pass through the pores created by Corbinol in the target cells of gastrointestinal tract, whereas the slightly larger astaxanthin was not able to pass through the pores. Yet, astaxanthin is terminally hydroxylated with a hydrophobic central region and its length corresponds to the thickness of the membrane's phospholipid bilayer (Britton, 1991). These facts together with the results gained from the present investigation trial suggests astaxanthin is most likely being retained in the pores created by Corbinol. These chemical characteristics of astaxanthin dictate its location within cells, either non-covalently bonded to specific proteins such as actomyosin or within cell membranes where it has two roles (Armstrong, 1999). The primary role of cell membrane located astaxanthin is to function as an antioxidant, as it inhibits oxidative injury (lipid peroxidation) of the cell membrane (Kurashige et al., 1990). The second role of astaxanthin located within cell membranes is structural, as it spans the membrane acting as trans-membrane rivets stabilising both the inner and outer leaflets and regulating membrane fluidity by increasing its rigidity (Milon et al., 1986). In the context of salmonid pigmentation, Schiedt et al. (1989) observed the phenomenon of a luminescent colour in the flesh of Atlantic salmon (Salmo salar) that had very low concentrations of astaxanthin in their epaxial white muscle tissue. Schiedt et al. (1989) suggested that this phenomenon was probably due to the retention of astaxanthin within cell membranes. Nevertheless, the
absorption of zeaxanthin is not as beneficial to rainbow trout as it is a metabolite of astaxanthin (Schiedt, 1988).

The net apparent retention (NAR) of astaxanthin was calculated for all the diets fed to rainbow trout. The NAR values for diets 2, 3 and 4 reflected the astaxanthin concentration measured in the epaxial white muscle of those fish fed the respective diets, where diet 2 > diet 3 > diet 4 > diet 1. However, the concentration of zeaxanthin in the epaxial white muscle of those fish fed diet 2 was significantly (P<0.001) lower than the astaxanthin concentration measured in the muscle of those fed either diet 3 or diet 4. The lower NAR of astaxanthin for diets 3 and 4 is vindicated by the fact that diets 3 and 4 contained approximately 70 times more pigment than the diets 1 and 2. Those fish receiving the basal diet (1 negative control) had no detectable astaxanthin in their flesh, so NAR was not applicable. Whereas diet 2 containing 1000 mg kg⁻¹ Corbinol despite being unpigmented actually managed to pigment the epaxial white muscle in the respective fish. The NAR value for the unpigmented diet 2 containing Corbinol was approximately 21 % being significantly (P<0.01) higher than pigmented diets (3 and 4). The NAR values for diets 3 and 4 were not significantly different (P>0.05). The NAR value calculated for diet 2, is the highest value observed in any of the trials undertaken in Chapters 4-6 and is much higher than the typical 15 % NAR for astaxanthin reported in the literature (Torrissen, 1995).

Hardy et al. (1990) identified several factors that influence the retention of dietary astaxanthin in salmonids, these include losses during diet preparation and storage, the wastage of diet during feeding, gut absorption, metabolism and excretion. The 40 % increased NAR for diet 2 as compared with Torrissen (1995) is rationalised by the suggestion discussed in chapter 4 that dietary pigment concentration appears in the denominator of the NAR equation, making dietary carotenoid concentration a critical factor in gaining high NAR values. Only comparing NAR values further suggests that the active ingredient in Corbinol (2-palmitoylaminoprorionic acid) does increase the bioavailability of carotenoids in rainbow trout. Although, the NAR value for the pigmented
diet (4) containing Corbinol was approximately 10% lower than the NAR calculated for the pigment positive control diet (3).

Dietary carotenoid content of the feeds was calculated on total carotenoid content of Carophyll Pink™ (8% free astaxanthin). The two pigmented diets (3 and 4) targeted 40 mg kg⁻¹ astaxanthin, but on analysis both diets only yielded approximately 35 mg kg⁻¹, even though they remained stable throughout the twelve week storage period, approximately incurring a 12% loss from their target concentrations. Garnett (*pers comm.*) determined Corbinol to be temperature stable and did not expect any degradation when exposed to the harsh conditions of an extruder, so Corbinol was added into the macro ingredients prior to extrusion. Combining this with the primary aim of the trial (determine the efficacy of Corbinol as a suitable enhancer to increase muscle pigmentation of rainbow trout) it seemed prudent to also add the astaxanthin to the diet prior to extrusion enabling the pigment and Corbinol to be mixed *in situ*. Even though the results of trial 3.2 demonstrated approximately 40% degradation of Carophyll Pink™ astaxanthin occurred during extrusion. Such losses were predicted, so post extrusion the astaxanthin concentration of diets 3 and 4 were determined and any losses compensated for by adding extra astaxanthin as a top-coat, and so the target concentration of astaxanthin was achieved (Hjelmisvet, *pers comm*). The losses may have been incurred during transportation where the ambient temperature was not controlled.

In conclusion, Corbinol provided very similar flesh colouration as compared to the Roche Carophyll Pink product at the final assessment (week 12). The degree of perceived pigmentation as evaluated by the SalmoFan™ was adequate and favourable resulting in a deep pink colour for the fillets. There were no obvious differences between the groups apparent to the trained observer. Additionally, direct measurement of the astaxanthin in the flesh by chromatographic techniques (HPLC) confirmed that the rainbow trout were able to absorb and assimilate the astaxanthin in presence of Corbinol to a similar degree as the fish receiving the positive control diet (40 mg kg⁻¹ Carophyll Pink™). Finally there were no
apparent problems with respect to the health of the rainbow trout at the end of the trial and no indication of any internal lesions or abnormalities associated with supplementation of the diets with the Corbinol product. It therefore may be concluded that Corbinol is not an effective enhancer for the deposition of free astaxanthin (Carophyll Pink™) under the conditions of the present investigation. However, Corbinol does seem to have an effect on the uptake of other carotenoids, as those fish receiving diet 2 (1000 mg kg⁻¹ Corbinol) retained zeaxanthin in their flesh even though the diet was not supplement with astaxanthin. So further investigations should be undertaken, for example the effect of Corbinol on salmonids receiving canthaxanthin (Carophyll Red™), zeaxanthin and of course Haematococcus pluvialis. Corbinol could then be recommended as a possible enhancer for the pigmentation of trout and salmon destined for the table market.
Chapter 7

General discussion
Chapter 7: General Discussion

Biotechnological considerations (algae production)

Many aspects of using the micro-alga *Haematococcus pluvialis* as a natural alternative to synthetic astaxanthin (namely Carophyll Pink™), for pigmenting the flesh of commercially produced salmonid species, have been investigated during this study. Nevertheless, prior to becoming a viable product that can be produced economically and satisfy market demand, much work still has to be undertaken, primarily with respect to production of the *H. pluvialis* cells.

The mass production of micro-algae as primary producers (via photosynthesis) was first developed in Germany during the early 1940's, where diatoms propagated under nitrogen deficient conditions accumulated relatively large quantities of lipid (Chen, 1996). Since then, new species such as *Dunaliella salina*, *Spirulina platensis* and of course *H. pluvialis* have been exploited for their ability to produce β-carotene, phycocyanin and astaxanthin, respectively (Borowitzka, 1992). However, the fundamental methods for industrial scale production of these photosynthetic cells have largely remained unchanged. This is because the predominate issue in the cultivation of photoautotrophic algae such as *H. pluvialis* on a commercial scale, is the sustained trapping of solar radiation at an adequate intensity to allow growth all year at a fairly constant rate (Richmond, 1992).

Usually, the simplest and cheapest method to cultivate photoautotrophic algae is within open reactors or ponds which operate under the conditions dictated by the external environment (Chen, 1996). Even though the culture is able to directly harness solar radiation, utilise atmospheric carbon dioxide and are economical to construct, this type of system does have its disadvantages. These include low cell density, high evaporation rates, contamination of the culture, no control of the culture temperature or photon density and difficulty in estimating the duration of the culture (Richmond, 1992; Margalith, 1999). The majority of these problems are dependent on the location of the culture, which obviously
has to have a high number of days of visible sun-shine in order to maximise photosynthesis, growth and product formation (Borowitzka, 1999). Consequently, only a few species of algae are commercially produced using this method to date. Yet, with respect to the commercialisation of any product, the aim of any industrial enterprise for the firm involved is to maximise profit margins, which usually means employing the most cost effective production technology. Hence, the majority of biotechnology firms producing photoautotrophic algae on a commercial scale have opted to grow algae that biosynthesise one or more high value products, in open reactor systems. Sufficient progress has been made to improve pond design, such as the use of inclined raceways and the introduction of paddles into ponds, both of which increase yield (Tredici and Materassi, 1992). These design features primarily cause turbulent flow within the pond which increases mass transfer of nutrients such as carbon dioxide, maintains the algal cells in suspension so reducing shading of the cells and allows a higher cell density to be maintained (Tredici and Materassi, 1992). This is the option taken by the Cyanotech Corporation (Kona, Hawaii, USA) who are the only firm commercially producing an algal product (NatuRose™) for the aquaculture industry. The constraints and disadvantages arising from pond production of *H. pluvialis* are more far reaching than those discussed here and obviously require further research. Even if the cultivation of *H. pluvialis* in open pond systems had been optimised, its physical properties as a product and its efficacy for pigmenting salmonid flesh are other matters for concern that have been evaluated in the experiments and feeding trials undertaken during this study.

**Stability of astaxanthin in feeds**

The concentration of astaxanthin in the feed pellets, when produced and bagged at the factory and then at the time they are dispensed to the fish, are not necessarily the same concentration presented to the fish on ingestion. Due to its reactive nature, astaxanthin is readily degraded, being sensitive to oxidation, photodegradation, acids and bases (Latscha,
1990). The loss of astaxanthin from feed pellets primarily occurs due to water erosion and degradation resulting from exposure to solar radiation. Both types of loss follow first order kinetics (as demonstrated in chapter 3) regardless of the astaxanthin being in the free form (as found in synthetic Carophyll Pink™) or the esterified forms (mono or di, as found in the natural *H. pluvialis* cells). Nevertheless, in both the water erosion and light degradation experiments, the astaxanthin residing in the NatuRose™ (*H. pluvialis*) cells proved to be significantly more stable than the synthetic Carophyll Pink™ product. This is simply due to the *H. pluvialis* cell wall providing protection against light and water erosion. The loss of free astaxanthin into water will probably occur regardless of the presence of a cell wall, due to natural partitioning between the two phases (oil and water). As demonstrated in chapter 3, at least a 3% loss of astaxanthin is expected to occur due to partitioning, and will probably be higher, due to the turbulent flow the pellets encounter under commercial conditions. However, this loss may be restricted by the addition of an emulsifying agent such as lecithin, which could help maintain the integrity of the astaxanthin solution / cod liver oil emulsion on the pellets surface when they are immersed into an aqueous phase. Taking into consideration the loss of astaxanthin due to pellet erosion by water and the affects of light degradation along with oxidation during storage, it seems as if an appreciable amount of the astaxanthin applied to the pellet will never actually enter the fish. Further work on stability (using the CPS suntest system as described in chapter 3) could be carried out to combine degradation factors such as light and water. Certain organics dissolved in natural water courses, such as humic substances, have been shown to affect the rate of photodegradation of crude oil (Zepp *et al.*, 1985). Humic substances could act as either photo-sensitisers, increasing the rate of photo-oxidation of astaxanthin or conversely, they may be light absorbing compounds, which decrease light intensity and the subsequent rate of photodegradation (Mill *et al.*, 1981; Ali 1994). Undoubtedly, the rate of degradation would increase in proportion to that of the water temperature (Thominette and Verdu, 1984). Thus, the effect of temperature could also be tested,
bearing in mind the fact that other species can utilise astaxanthin, but their optimum temperatures vary greatly from that of salmonids (15°C). Examples include the Arctic charr (*Salvelinus alpinus*) and red sea bream (*Chrysophrys major*) which have optimum temperatures of 5 and 23°C, respectively (Metusalach *et al*., 1996). Other environmental factors to be considered are the dissolved oxygen content of the water and the presence of other oxidants such as peroxides, both of which may increase the photo-oxidation rate of hydrocarbons (Miller, 1983). Fukuda *et al*. (1988) demonstrated water salinity to affect the rate of photodegradation, also Miller and Zepp (1979) noted water turbidity to decrease the rate of photodegradation through light attenuation and partitioning of particulate matter (Ali, 1994). Even competition by other processes, such as biodegradation due to bacteria, may have an affect on the rate of photodegradation (Ali, 1994). Hence, further experiments using the CPS Suntest system could be undertaken to assess the effects of the aforementioned biotic and abiotic factors.

*Feeding trials with rainbow trout*

The general trend from the results of the feeding trials strongly suggest the natural pigment source (*Haematococcus pluvialis*) is less efficient than a synthetic counterpart (Carophyll Pink™) for pigmenting the flesh of salmonids. Thus, a higher dietary inclusion rate of *H. pluvialis* is required to achieve the same effect when compared to synthetic astaxanthin. The simplest option would be to increase the target dietary astaxanthin content, by increasing the quantity of *H. pluvialis* added to the diet. However, ‘arbitrarily’ increasing the *H. pluvialis* content of a diet may have negative effects on the fish, as reported by Sanderson and Jolly (1994) and, Davies (*pers comm.*). In both experiments rainbow trout were fed high oil content commercial diets, containing the red yeast *Phaffia rhodozyma*, which had 10% (Sanderson and Jolly, 1994) and 14% (Davies, *pers comm.*) inclusion rates (by weight). The fish in both experiments demonstrated retarded growth that was probably due to the high RNA and DNA content of the *Phaffia* cells (Davies, *pers comm.*). In both
experiment, the *Phaffia* content of the diet was so high that it became a feed ingredient, whereas the quantities of *H. pluvialis* used in the present feeding trials was so low that it can be classed as an additive. Also, the dose response of astaxanthin follows a hyperbolic relationship, where dietary astaxanthin concentrations greater than 100 mg kg⁻¹ for sexually maturing fish (as used in the present feeding trials) lies on the plateau (Storebakken and No, 1992). Likewise, for mature salmonids the plateau is reached at approximately 50 mg kg⁻¹ (Olsen and Mortensen, 1997). Therefore, increasing the dietary content above 100 mg kg⁻¹ would not produce an increase in the astaxanthin concentration of the flesh (Storebakken and No, 1992). Another option could be to calculate the amount of *H. pluvialis*, required using the total astaxanthin content of the cells. Again this would increase the quantity of *H. pluvialis* needed, as total astaxanthin content of the cells is approximately 85% of the total carotenoid content (Lorenz, 1999).

The lower efficacy of *H. pluvialis* as compared to the synthetic astaxanthin in trials 4.1 (using cold pressed) and 4.2 (using extruded diets) led to several hypotheses. The primary hypothesis proposed that the bioavailability of astaxanthin was reduced in *H. pluvialis* cells due to the impediment of the cell wall. The primary hypothesis was fundamentally supported by the results gained in the trial that utilised cold pressed feeds and demonstrated that feeding trout *H. pluvialis* with intact cell walls resulted in the flesh remaining unpigmented. Hence, an alternative to elevating the astaxanthin content of the diets could be increasing the bioavailability of astaxanthin by producing more surface cracking and cavities in the cell covering or by removing the cell covering completely. Different methods, such as the use of a French press, a ball mill or an x-press could be tested to identify which method could be introduced into the down streaming processing of the *H. pluvialis*, to induce cracks or holes into the cell wall. The degree of cracking and the number of holes could be estimated using scanning electron microscopy in an attempt to assess the most efficient method. The second hypothesis was that the bioavailability of astaxanthin was effectively reduced in *H. pluvialis* cells, due to astaxanthin only
constituting 85% of the total carotenoid content of which the majority (90%) is esterified to fatty acids. Hence, trial 5.1 was carried out to test the first hypothesis and trial 5.2 to test the second hypothesis. The results from trial 5.1 showed that the presence of the disrupted \textit{H. pluvialis} cell wall had no affect on the bioavailability of the astaxanthin retained within the cells, as the carotenoids within the cells were assimilated to the same degree as the total carotenoid extract. This strongly suggests that the degree of cell wall disruption in the NatuRose™ product is more than adequate to allow pigmentation. The results of trial 5.2 demonstrated that the bioavailability of mono-esterified astaxanthin is significantly higher than that of di-esterified astaxanthin. Yet, neither was significantly different to the total carotenoid extract or the synthetic astaxanthin. These results suggest that the degree of esterification is a limiting factor, but as the content of di-esters is relatively low then it has no significant effect on the overall bioavailability of the total carotenoid content of \textit{H. pluvialis} presented to fish in a cellular form.

The overall low bioavailability and high cost of adding astaxanthin from both natural (\textit{Haematococcus pluvialis}) and synthetic (Carophyll Pink™) sources to salmonid diets led to the search for novel feed additives that had the potential to increase astaxanthin absorption and bioavailability. Hence, trial 6.1 was designed to study the efficacy of 2-palmitoylaminoproprionic acid (Corbinol) \textit{in vivo} using rainbow trout. 2-palmitoylaminoproprionic acid was chosen as it had previously been demonstrated \textit{in vitro} to increase the passage of astaxanthin through cell membranes (Garnett, unpublished data). However, the results from trial 6.1 showed 2-palmitoylaminoproprionic acid had no effect on the net apparent retention (NAR) of astaxanthin in the flesh of rainbow trout when tested \textit{in vivo}. Yet, in this trial only one inclusion rate of 2-palmitoylaminoproprionic acid (Corbinol) was tested. A further trial could be carried out using various inclusion rates of 2-palmitoylaminoproprionic acid to find whether increased inclusion levels increase the bioavailability of astaxanthin in the gut and its concentration in the epaxial white muscle. However, increasing the inclusion level of 2-palmitoylaminoproprionic may have adverse
affects on fish health, so the trial should also study the general pathology of all fish sampled. A similar trial could also examine the affects of 2-palmitoylaminoprorionic acid on *H. pluvialis* derived astaxanthin.

Nevertheless, the use of novel additives to increase the bioavailability of astaxanthin still has potential. One such additive that may have the desired effect is the vitamin E derivative, d-Alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS). Astaxanthin is classed as a terpenoid and TPGS has been designed to aid the uptake of lipid soluble drugs, therefore, marrying these facts seems to suggest that TPGS would probably aid the uptake of astaxanthin. In fact, TPGS has been demonstrated to increase the uptake of the strongly lipophilic drug cyclosporine in children after liver transplantation (Sokol *et al.*, 1991). Likewise, TPGS has also been illustrated to increase cyclosporine bioavailability in healthy volunteers (Chang *et al.*, 1996), and increase the uptake of vitamin D in children with chronic cholestasis (Argoa *et al.*, 1992). Sokol *et al.* (1991) suggests TPGS functions as a micellar solubilising agent that results in the increased bioavailability of lipophilic molecules. Simply, TPGS has the ability to render lipid soluble molecules to become water soluble, potentially increasing bioavailability of astaxanthin by utilising a second route of absorption. Applying this rationale to astaxanthin may well increase its bioavailability in salmonid species. Hence, an experiment could be designed to test the efficacy of TPGS when added to diets pigmented with astaxanthin at an appropriate industrial inclusion rate. Both Carophyll Pink™ and NatuRose™ may then be tested against a negative (unpigmented) control and positive (pigmented) control diets for both astaxanthin sources. Another proposal would be to test the efficacy of TPGS at different inclusion rates within the diets for each of the astaxanthin sources under similar conditions of feeding.

The statistical model and the linear regressions used for analysing the results of the feeding trials was limited to using just weight as the independent variable. Time was considered and may have been more applicable in those trials where the fish were fed for up to twelve weeks on the experimental diets and sampled every four weeks (this could enable a serial
assessment of colour formation in the fillets to be made and closely followed). However, the constraints of these experiments, in terms of the size of the tanks used and the numbers of fish they could sustain was not large enough to allow samples to be taken more frequently. Hence, it was deemed that not enough sampling points were obtained from the experiment to use time as an independent variable. The trial initiated to test the efficacy of Corbinol (2-palmitoylaminopropionic acid) demonstrates why time was not viable as an independent variable. The design of this investigation only enabled the use of fish weight as the independent variable and this was used to rationalise why no regression model could be designed to identify a difference between the diets, with respect to Corbinol and Roche Colour Score (RCS). This is also true for astaxanthin deposition within the flesh. However, in this investigation it was shown that the product moment correlation coefficients demonstrated a negative correlation between the variable fish weight and both responses [RCS and astaxanthin concentration for the pigmented diets containing synthetic astaxanthin (Carophyll Pink™) after twelve weeks of feeding].

Considering only those results from the trials that used commercially produced extruded diets; it seems that those fish receiving the control diets pigmented with synthetic astaxanthin (Carophyll Pink™) obtained significantly different net apparent retention values. Taking the different dietary inclusion levels of the pigment used in the various trials into consideration, it seems that those fish in trials where the astaxanthin was dissolved in cod liver and added to the basal diet as a top-coat, had higher net apparent retention values for astaxanthin.

Therefore, the type of oil used in the diet could be a factor affecting pigmentation of salmonid species. Further experiments could be carried out to ascertain whether the type of oil into which synthetic astaxanthin is dissolved and H. pluvialis cells are suspended and used to top-coat the basal diet, has any influence on either the growth of fish or the bioavailability of astaxanthin from various sources. The use of fish oils (such as cod liver oil) may increase pigmentation purely because these oils are more palatable, so the fish
readily consume more diet and have increased pigmentation. Also, fish oils may be more effective than vegetable oils or oils from terrestrial species, as rainbow trout may be able to assimilate them more readily, probably being due to the fatty acid profile of fish oils being similar to that of the rainbow trout. An observation made during the filleting of fish in all the experiments may hold the key as to why some trout obtained higher NAR values than others. During excision of the viscera, it was noted that in most trials the fish had relatively intact feed pellets in their gut right up to the pyloric region where astaxanthin is thought to be absorbed (White et al., unpublished). In those trials where astaxanthin was added prior to extrusion, the majority of the pigment would have been located within the pellet matrix. Conversely, in trials where the astaxanthin was added post extrusion, the majority of the pigment would have been located on the surface of the pellets making it more readily available to the fish. Even the net apparent retention values gained for those fish receiving a cold pressed laboratory diet containing synthetic astaxanthin was appreciably higher than those receiving extruded diets to which the astaxanthin was added prior to extrusion. This is probably due to the fish receiving cold pressed diets digesting them more readily than a commercially produced extruded diet. Cold pressed pellets are much softer than extruded pellets and are probably more porous, so most likely became saturated with water and started to structurally degrade whilst in the water column. In comparison, extruded pellets are designed to have low porosity, so take longer to become saturated with water and structurally degrade, remaining intact in the fish gut much longer.

Diet storage, stability and protection

The storage stability of astaxanthin in top-coated diets seems to be lower than that added to diets prior to extrusion, despite the fact that the application of the pigments as a top-coat (post extrusion) enabled target levels to be easily achieved. Also, top-coated diets demonstrated far greater production stability than those diets where the astaxanthin was added prior to extrusion. However, the addition of ethoxyquin to the oil/astaxanthin
solutions prior to top-coating seemed to increase the stability of astaxanthin when applied to an extruded basal diet, as compared to those without. Ethoxyquin acts as an antioxidant, reducing the rate of astaxanthin oxidation, possibly by being less stable than astaxanthin, and so ethoxyquin is probably oxidised in preference to astaxanthin. It seems 100 mg kg\(^{-1}\) inclusion rate of ethoxyquin in the experimental diets was sufficient to protect the pigments (namely astaxanthin) throughout the duration of the trial. In this respect, it would be of interest to assess the efficacy of ethoxyquin as an additive to increase the bioavailability of astaxanthin from both synthetic and natural (H. pluvialis) sources. The experiment could include different inclusion levels of ethoxyquin to identify an optimal working concentration. As with any additive, the pathology of the fish should be assessed to identify any abnormalities caused by its use.

Feeding rate, presentation and pigment efficacy
As discussed in chapters 4, 5 and 6 the residual time of the feed pellets within the gut probably affects the net apparent retention of astaxanthin, as feeding the fish to satiation will reduce the time feed pellets reside in the gut as compared to chart feeding regimes. Perhaps a simple way to increase the net apparent retention of astaxanthin is to alter their feeding patterns. A typical study would investigate the net apparent retention of astaxanthin by varying feeding rate and meal size, whereby diets made using the same basal ingredients, extruded and top-coated with a designated astaxanthin content (50 mg kg\(^{-1}\)), would be fed to different groups of fish. The daily ration (calculated to percentage weight of the group biomass) would be fed using several different regimes. This could be achieved by feeding the total ration in one meal, equally in two meals every twelve hours, equally in three meals every eight hours or using a demand feeder such as the Aquasmart™ system which allows the fish to obtain food when they desire. Perhaps dividing the ration into several meals would not only increase the resident time of the pellets in the gut, but could also maintain the concentration of astaxanthin in the plasma at its optimal level. As
White et al. (unpublished) recently demonstrated, it takes 24 hours for the astaxanthin concentration to peak in rainbow trout plasma, post prandially. The maintenance of astaxanthin in the plasma at an elevated level may increase the net apparent retention of astaxanthin in the muscle by ensuring astaxanthin is constantly available for deposition. Taking into consideration that it takes 24 hours for the concentration of astaxanthin to peak in the plasma and the high cost of pigmented diets, it may be more economical to feed a pigmented diet every other meal or every other day. Wathne et al. (1998) demonstrated that Atlantic salmon (Salmosalar) fed pigmented diets in alternate meals as compared to continually feeding pigmented diets, had no effect on muscle pigmentation after 168 days of feeding. The results from this initial trial are positive with respect to being more economical in terms of feeding costs, which are estimated to be an 8% saving, using 20% as the cost of adding astaxanthin to a commercial diet. However, the logistics of using an alternating feeding regime may be difficult to execute in a commercial environment, especially if the fish are fed using demand feeders.

Application to other species
As mentioned other farmed fish species utilise astaxanthin for pigmenting either their skin, which include the red sea bream (Chrysophrys major) and the yellow tail (Serilagaiingu) or the flesh of Arctic char (Salvelinus alpinus) and salmonid species. Hence, all these species could in theory use H. pluvialis as a source of astaxanthin to enhance their pigmentation, as desired for their respective markets. In Japan, red sea bream and yellow tail are highly prized, where skin colouration is used by the consumer as a guide to the quality of the fish presented at market. In the extreme, skin colouration can adversely affect the fish’s market value, particularly the red sea bream, which in Japanese culture is synonymous with good fortune (Ibrahim et al., 1984). Feeding trials have recently been completed using H. pluvialis as source of astaxanthin to pigment red sea bream and yellow tail. The trial using yellow tail demonstrated H. pluvialis to be a superior pigment source
than Carophyll Pink™ (synthetic astaxanthin). Lorenz (unpublished data) demonstrated the concentration of astaxanthin in the skin of yellow tails receiving *H. pluvialis* was significantly higher than those receiving Carophyll Pink™. These results were reinforced by the results of a visual comparison carried out by an expert panel (Lorenz, unpublished data). This may be due to the fact that the astaxanthin in both *H. pluvialis* and the yellow tails skin is in the esterified form and perhaps unlike rainbow trout, yellow tails are able to absorb esterified astaxanthin from the gut without the need for de-esterification. In a similar trial using red sea bream, no significant differences in the astaxanthin content of the skin were identified between those fish fed a diet containing *H. pluvialis* and those receiving a diet pigmented with Carophyll Pink™ (Bowen, pers comm.). This trial was repeated using gilthead sea bream (*Sparus aurata*) which is prized in many Mediterranean nations. Again no significant differences were obtained between those fish fed a diet containing *H. pluvialis* and those receiving a diet pigmented with Carophyll Pink™ (Bowen, pers comm.). However, on analysis, the only carotenoid identified in the skin was leutin which was also present in the skin of those fish fed an unpigmented control diet (Bowen, pers comm.). These results may be rationalised by the fact that the diet had a high content of leutin due to the presence of corn gluten. Arctic charr as a cold water species has great potential in terms of aquaculture, but has a highly variable ability to retain astaxanthin within its epaxial muscle (Hatlen et al., 1995). This may be due to Arctic charr being able to readily bind other carotenoids in their flesh, namely idoxanthin that provide a different hue to the flesh (Aas et al., 1997). To date no work has been published on the efficacy of *H. pluvialis* as source of astaxanthin to pigment the flesh of Arctic charr. Hence, a simple feed trial could be initiated to study the ability of *H. pluvialis* to pigment Arctic charr. However, due to the ability of Arctic charr to utilise different carotenoids for flesh pigmentation coupled with the variable carotenoid content of *H. pluvialis* the, resulting colour of the flesh may be too variable for commercial exploitation.
Other potentially exploitable sources of astaxanthin for aquaculture

An array of other organisms have been examined as potential astaxanthin sources to pigment cultured fish, including animals and fungi. Within the animal kingdom, only invertebrates belonging to the phylum crustacea have been used to pigment salmonids, primarily due to the waste (head and carapace) of certain crustacea containing appreciable quantities of astaxanthin (Castillo et al., 1982). Torrissen et al. (1982) demonstrated that rainbow trout fed diets containing the waste from shrimp (Pandulus borealis) processing plants were able to assimilate the astaxanthin. However, this source proved to be unstable and would require intensive processing to produce a viable product. Antarctic krill (Euphasia superba) has been widely demonstrated to be a potential source of astaxanthin for salmonids (Arai, et al., 1987; Storebakken, 1988; Mori et al., 1990) and even the oil extracted from the crayfish carapace has been advocated as being a potential source of astaxanthin. Gouveia et al. (1996) demonstrated another microalgae Chlorella vulgaris to be a viable alternative to pigment rainbow trout flesh. Yet, like H. pluvialis, Chlorella vulgaris contains an appreciable quantity of esterified astaxanthin that also seems to limit its bioavailability. Hence, an alternative to H. pluvialis could be Chlorococcum wimerri that has approximately 95% of its astaxanthin in the free form (Lee, pers comm.). A great deal of interest has also been shown in fungi, namely the yeast Phaffia rhodozyma, which has also been widely advocated as a pigment source for salmonids (Johnson et al., 1980; Gentles pers coom.; Sanderson and Jolly; 1994). Even though the main problem with Phaffia rhodozyma is its low astaxanthin content, its use has been demonstrated by Nakano and Tosa (1995) as having a positive affect on fish health and it has also been shown to have the ability to reduce oxidative stress in rainbow trout (Nakano et al., 1999).

Application of natural sources of astaxanthin to aquafeeds

If any natural alternative to synthetic astaxanthin is deemed commercially viable, then its use and design as a food additive must be carefully deliberated, with considerations falling
into two categories, physical parameters and cost. In terms of physical parameters, the product design must take into account four main characteristics, namely bioavailability, stability, miscibility and handling (Roche, 1998). The formulation of high energy feeds that provide optimal nutrient profiles and increased growth rates, means fish farmers are able to use less feed to obtain the desired biomass for market. Hence, the bioavailability of the active ingredient within additives has become a critical factor, generally being regarded as the most important physical parameter, as the proportion of astaxanthin absorbed has to be maximised to provide the desired flesh colour. Miscibility of a product refers to how well it mixes with the other raw ingredients, being dependent on particle size and particle number (Roche, 1999b). To obtain good miscibility the particles should be uniform in size and shape, preferably spherical, as they move in a rolling motion and mix more readily than randomly shaped particles that tend to tumble (Gulbrandsen, pers comm.). An example of such a product is Carophyll Pink™, which is reputed by its manufacturers (Hoffmann-La Roche, Basel, Switzerland) to have excellent miscibility when added in solid form to the raw ingredients prior to extrusion (Roche, 1999c). This is primarily due to its uniform consistency of spherical beadlets that have a mean diameter of 0.3 mm and a particle number of 0.1 million g⁻¹, which also provide high flowability (Roche, 1999c). These handling properties are important, as high flowability means the product freely flows and so requires little handling and is therefore ideal for large-scale commercial use.

The present study employed scanning electron microscopy to observe the topography of desiccated *H. pluvialis* cells from two sources, *i.e.* commercial NatuRose™ and the experimental cells produced by Liverpool John Moores University. The electron micrographs showed the cells from both sources to also be spherical in shape and quite uniform in size and so have a relatively high particle number. Even though these attributes are desirable, the relatively small particle size (45 μm) of *H. pluvialis* cells results in the product having poor handling properties. This product creates copious amounts of dust when being poured and readily adheres to a wide range of surfaces within the working
environment, including machinery and the handler. These problems could be costly, in terms of losing a relatively large quantity of the product to the environment that is non-redeemable, and the possibility of installing of extra equipment required to make the use of the product safe to the handler. Thus, as its use may cause environmental pollution, an environmental impact study may be required prior to its use on an industrial scale. As discussed, astaxanthin is readily degraded, so any product containing such a sensitive compound has to provide protection against the aggressive processes which it will be exposed to with the employment of modern feed production techniques, mainly the harsh environment encountered within the extruder. The Carophyll Pink™ beadlets are composed of astaxanthin emulsified with several antioxidants (vitamin C and ethoxyquin) bound in a starch-gelatine matrix and top-coated with starch (Roche, 1999b). The formulation of the Carophyll Pink™ product affords the encapsulated astaxanthin thermal and oxidative stability, concomitantly increasing shelf life.

Market potential and future perspectives
All of the previously stated factors are inter-linked, particularly bioavailability, which can be highly influenced by the final formulation of the product. Thus, a careful balance must be achieved to gain a working product that optimises each parameter with respect to the constraints imparted by the others. This is no easy task, that is further complicated by economics, which must be considered on two levels, production costs and market costs (Livesey, 1998; Griffiths and Wall, 1999; Sloman, 2000). Production costs have to take into account a myriad of parameters (many of which have already been discussed), that are collectively regarded by many as the most critical factor. This is because total production costs directly affect profit margins, which are subsequently affected by market value (cost to the consumer) (Griffiths and Wall, 1999). However, the introduction of a new astaxanthin product into the market such as one developed from Haematococcus pluvialis is rather intricate, as the producers of astaxanthin work in a oligopoly market, i.e. a market
that only has a few manufacturers/sellers (Sloman, 2000). In this particular case, at present only four firms commercially produce astaxanthin products for use in the aquaculture industry. Hoffmann-La Roche and BASF (Germany) both manufacture synthetic astaxanthin products, that approximately possess 70 and 25 % of the global market share, respectively (Baker, pers comm.). The remaining 5 % of the market is unequally divided between the Cyanotech Co-operation (Kona, Hawaii, USA) and the Archer Daniel Midland Company (USA), both of which produce astaxanthin from natural sources, in the form of dried algal (H. pluvialis) cells and dried yeast (Phaffia rhodazyma) cells, respectively (Baker, pers comm.). A market operating under an oligopoly state, means each firm (oligopolist) is mutually interdependent on each other, whereby each firm will be affected by the actions of its rivals (Livesey, 1998). Such that if one firm changes the specification of the oligopoly product, then its rivals will respond by changing either the specification or advertising of their product. Hence, firms in such a market system cannot afford to ignore the actions or reactions of their competitors (Sloman, 2000).

When determining the price of a product entering into an oligopoly market, the producer has to opt for one of two opposing policies, competition or collusion. Competition would entail pricing the new product considerably lower than that of its competitors, which has an element of risk and could initiate a price war (Griffiths and Wall, 1999). The extent to which firms will reduce the market cost of their products and the duration of the price war is usually dependent on the size of the firm that initiated the war. Nevertheless, in the short term a price war is always beneficial to the consumer. If the instigator is large enough to sustain low profits or even long term losses on their new product then eventually the market price will equilibrate at a lower price acceptable to the majority of the firms involved (Griffiths and Wall, 1999). However, if the new firm is relatively small then a price war could put them out of business, as they will probably be unable to sustain low profitability or long term losses. Thus, a firm entering a new product into an oligopoly market will probably opt to collude with its competitors. Direct collusion where several
firms form a cartel to fix the price of a product, under British and American law is illegal (Sloman, 2000). However, the new firm will probably tacit collude with its competitors, whereby it will price its new product similar to that of the dominant firm, resulting in dominant firm price leadership (Sloman, 2000). This type of collusion is beneficial to all the oligopogists as it sustains the market cost and helps maximise profits, it also has the added advantage of being legal (Sloman, 2000). Taking into consideration all the aspects of an oligopoly market, it seems prudent for any firm entering the astaxanthin market producing *H. pluvialis* to price their product according to that of the dominant firm, which at present is set at approximately £ 112 kg\(^{-1}\) (Baker, *pers comm.*). Direct collusion may be occurring in the astaxanthin market, as the producers of synthetic astaxanthin (Roche and BASF) were each fined several hundred million dollars by a United States court for breaking anti trust legislation by collusion (Ziggers, 2000). Both firms were found guilty of collusion by elevating and fixing the price of synthetic vitamins for the animal feed market (Ziggers, 2000).

Nevertheless, other firms such as Bioprocess (Keflavic, Iceland) and Astacarotene (Stockholm, Sweden) intend to commercially produce *H. pluvialis* using computer controlled fermentation vessels that utilise artificial light sources. Such systems will enable far higher yields than open ponds, as they are able to optimise the culture conditions allowing shorter production times. This, combined with the identification of the major stress factors that induce the production of secondary carotenoids in *H. pluvialis*, suggests large scale production of astaxanthin could be viable. However, large scale production of astaxanthin is hampered by the fact that the conditions required to form secondary carotenoids in *H. pluvialis* are antagonistic to growth, which makes the production of astaxanthin via fermentation more complex. This is because maximising the culture conditions requires a two step process, where the first step has to optimise biomass production and the second step has to optimise astaxanthin production. Obviously, optimisation conditions should be tested using a pilot scale fermentation vessel, but *H.
*pluvialis* is autotrophic, using photosynthesis to produce glucose, so requires light for growth not only to maximise cell yield but also to maximise astaxanthin production. The requirement of light further complicates the design of the fermentation vessel and causes particular problems for scale up, as the photon flux density required constantly varies throughout the media and light penetration is restricted (Harker *et al.*, 1995). Even so, the initial cost of the equipment and the operational costs would probably outweigh the profitability brought with enhanced production rates. The major problem with using this type of fermentation technology is the size (volume) to which commercial vessels can be built, to enable production to meet the demand of the aquaculture industry, whilst being profitable. With respect to the aforementioned firms that intend to utilise this technology to commercially cultivate *H. pluvialis*, it seems they could only make their firms commercially viable if they aim their products at the human health market. This is because *H. pluvialis* would be classed and marketed as a herbal supplement, which commands a higher price, because astaxanthin is an antioxidant whose use could reduce the risk of certain types of cancer (as it reduces oxidative stress) (Gonzalez, *et al.*, 1993). Tablets containing *H. pluvialis* derived astaxanthin can be used and marketed as a preventative medicine, as achieved by La Haye Incorporated Laboratories (Redmond, Washington, USA), a company that sells *H. pluvialis* derived product called astaZANTHIN™ in this very market. The human health market for *H. pluvialis* is a much smaller market than for aquaculture, so supply could easily meet demand. As it is not an additive for another product, it is without the problem of secondary demand (Gregory, *pers comm.*). According to a market analysis by Gregory (*pers comm.*), the price commanded by the producer of *H. pluvialis* for the health food market is approximately one thousand times greater than that for the aquaculture industry. The high premium obtained in the health food market makes the heavy investment made by companies utilising fermentation technology, to produce a safe sterile *H. pluvialis* product and an appreciable profit margin.

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The basic rationale for this project is based on three simple facts, the first being salmonids have an elitist image dependant upon their distinguishing pink coloured flesh which is due to the presence of astaxanthin (Torrissen et al., 1995). It is therefore of great economic importance that salmonids either wild or farmed, have the preferred pigmentation commanded by the consumer (Torrissen et al., 1989). The second, that salmonids like other animals are unable to synthesise astaxanthin \textit{de novo} (Anderson, 2000). The third, that manufactured feed formulations destined for salmonids must contain astaxanthin, which is costly to synthesise on a commercial scale, resulting in astaxanthin being an expensive product to add to salmonid diets. Hence, the overall aim of this study was to develop and exploit a natural alternative to synthetic astaxanthin that can be easily and cheaply produced.

\textit{Summary}

A general trend seems to emerge when taking the results of the four feeding trials into account that incorporated \textit{H. pluvialis} or its products into diets. Overall, the efficacy of \textit{H. pluvialis} as a source of astaxanthin to pigment rainbow trout epaxial muscle seems to be lower than that of synthetic astaxanthin (Carophyll Pink™). Nevertheless, \textit{H. pluvialis} derived astaxanthin showed a similar dose response to the synthetic form, confirming that it was assimilated from the diet. Also, when intact \textit{H. pluvialis} cells were added to the diets, they had no affect on growth performance. In fact, on termination of all four feeding trials, no indication of any internal lesions or abnormalities associated with supplementation of the diets with any of the algal products were observed, suggesting \textit{H. pluvialis} poses no apparent risk to the health of rainbow trout. This is confirmed by the fact that in the latter half of 2000, the NatuRose™ (\textit{Haematococcus pluvialis}) product manufactured by the Cyanotech Corporation was granted approval by the US federal drug agency (FDA) for use in salmon diets to pigment salmon flesh (Dotzel, 2000a). Likewise, on the same day, the Ecotone™ (\textit{Phaffia rhodozyma}) product manufactured by the Archer
Daniel Midland Company was also granted FDA approval for use in salmon diets (Dotzel, 2000b). Analysis of the *Haematococcus pluvialis* product demonstrated it to be far superior in terms of water and light stability. Likewise, the stability of *H. pluvialis* incorporated into diets, also proved to be a more stable source of astaxanthin than its synthetic counterpart. Taking all the data into consideration from the present studies, it seems that the efficacy of *Haematococcus* as a product to pigment salmonid flesh has considerable potential. It may therefore be concluded that *Haematococcus pluvialis* could in fact prove to have commercial potential and be a viable and effective option for pigmenting farmed salmonids, which also has the added characteristic of being a natural product.

Ultimately, consumer preference and demand for naturally reared and organically farmed fish will dictate future trends, as pressure in the United Kingdom and more recently in Europe necessitates a renewed focus on novel ingredients and additives that are effective and safe to use in the aquafeed industry.
References


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