

Technical report

Analysis of Total Astaxanthin in algae meal prepared from Haematococcus pluvialis.

(TR.1002.001)

- ***The method used at Aquasearch production facility in Kona, Hawaii, to determine the total astaxanthin content of Haematococcus pluvialis algae meal is described and discussed.***
- ***This method is able to determine total astaxanthin in Haematococcus pluvialis algae meal within a 5% error margin.***

INTRODUCTION

Astaxanthin is a red carotenoid pigment (xanthophyll) that *Haematococcus pluvialis* cells accumulate in response to stress conditions. The method described herebelow is used on a routine basis at Aquasearch production facility in Kona to control astaxanthin content in Aquaxan HD algae meal. It consists of two steps: an astaxanthin extraction phase in DMSO (dimethyl sulfoxide) and a measure of astaxanthin light absorption by spectrophotometry at 489 nm.

Total astaxanthin measurements reported on Aquasearch's certificates of analysis and guaranteed on product specifications are based on this method.

METHODOLOGY

Supplies and Equipment

- Precision waterbath
- Disposable plastic pipettes
- Glass cuvette
- Spectrophotometer
- Production lab computer
- Vortex mixer
- Weight scale balance
- Aluminium boats
- 15 ml sample tubes
- Pipettor with DMSO
- 1.0 ml pipette

For further details, contact:

Aquasearch Inc.,
73-4460 Queen Kaahumanu Highway,
Suite 110, Kailua-Kona, HI 96740, USA
Tel: 808-326 9301, Fax: 808-326 9401

000150

Technical report

- Lab tape
- Sharpie marker
- Latex gloves
- Astaxanthin analysis data sheet
- Amber coloured jars
- Disposable clear tubes with caps

Precision

Samples analysed from early May '98 through the first week in August '98 indicate an average %error of 2.94 (n = 65) using this method.

Procedure

A. Pre-labelling

- 1) Label 2 15ml tubes for each sample to be analysed (eg. 1A, 1B, 2A, 2B, etc).
- 2) Label 2 aluminium boats for each sample to be analysed (eg. 1A, 1B, 2A, 2B, etc).
- 3) Label 2 amber coloured jars for each sample to be analysed (eg. 1A, 1B, 2A, 2B, etc).

B. Extraction

- 1) Turn on weight balance, place uncapped 15ml tube in a small cup and zero out the readout (zero). Take sample and weigh 0.02 gr - 0.03 gr into 15ml tube. You can use a small plastic disposable pipette to make weighing easier. Record wt. on data sheet in "WEIGHT OF SAMPLE gr" column. Cap tube.
- 2) Repeat step # 1 for all samples.
- 3) Add 1ml DIH₂O. Add 9ml DMSO. Vortex well. Place in 70 °F waterbath for 30 minutes.
- 4) Remove from waterbath, wipe tubes dry with paper towel and centrifuge 3-5min.
- 5) Remove tubes from centrifuge, and pour supernatant into numbered amber jars. Supernatant should be poured with the highest angled portion of the pellet abreast of the amber jar. This is extract process #1. Record in a "EXTRACTIONS" column a checkmark for each extraction completed.
- 6) Repeat step #5 for all tubes.
- 7) Add 10ml DMSO to each tube & cap tube after DMSO has been added. Vortex well. Place in waterbath for 30 minutes and repeat this process until the 4th extraction is completed or unless advised that all of the pigment has been removed (sometimes as much as 5 extractions).
- 8) Amber coloured jars should be stored in refrigerator until you're ready for the next extraction.
- 9) Prepare disposable clear plastic tubes by labelling each tube the same as the 15ml tubes. Add 10ml DMSO to each tube. These disposable tubes are the dilution tubes. Next remove 1ml of the sample from the amber jar. Add the 1ml of your sample to the disposable clear tube containing 10ml DMSO.
- 10) Ensure that the sample you remove corresponds to the appropriate numbered dilution tube. Mix well by inverting 3 times.

C. Spectrophotometric readings

- 1) Turn on spectrophotometer at least 15 min. before making measurements.
- 2) Ensure your ABS WAVELENGTH is 489nm (see Appendix A). Blank the spectrophotometer with glass cuvette filled with DMSO.

Technical report

- 3) Fill glass cuvette halfway with next sample & discard. Fill cuvette again with sample, place in spectrophotometer and record your measurement (ABS_{489}). Repeat with remaining samples.
- 4) On data sheet, prepare a column "EXTRACT VOL ML": to determine this number, refer to the no. of times you did an extraction for the sample you are measuring. Multiply by 10 and this will be your extract vol. (eg. Sample 1A had 3 extraction checkmarks. $3 \times 10 = 30$. Thus 30 ml is your extract vol.

D. Calculation of astaxanthin content

To calculate the total astaxanthin content of the extracted *Haematococcus* meal use the following formula:

$$\%astax = \frac{ExtractVol(ml) * Abs_{489} * 1.1}{WeightOfSample(gr) * 190.8}$$

DISCUSSION

Astaxanthin is soluble in organic solvents such as alcohol, acetone or DMSO (dimethyl sulfoxide)¹. DMSO has been found to be one of the best solvents for pigment extraction from green algae² and our own in-house research has confirmed that DMSO is the most appropriate solvent for extraction of *Haematococcus* algae meal and cysts.

After extraction, the absorbance of the DMSO extract is measured at 489 nm in a spectrophotometer. The absorbance is normalised to the volume of solvent and mass of meal extracted. This method is a satisfactory evaluation of total astaxanthin since HPLC analyses conducted at the University of Hawaii³ have shown that astaxanthin represents 95.5% of total carotenoids in *Haematococcus pluvialis* flakes prepared from harvested reddened cells, and 85.4% of total pigments (with chlorophylls representing 10.6% of total pigments, and lutein 3.2%). Other studies have confirmed similar pigment levels in *Haematococcus pluvialis* reddened algae^{4,5,6}. The absorption measurement is carried out at 489 nm because the absorption maximum of the red meal extract shifts to 489nm as the cells naturally redden and accumulate astaxanthin as illustrated in appendix A.

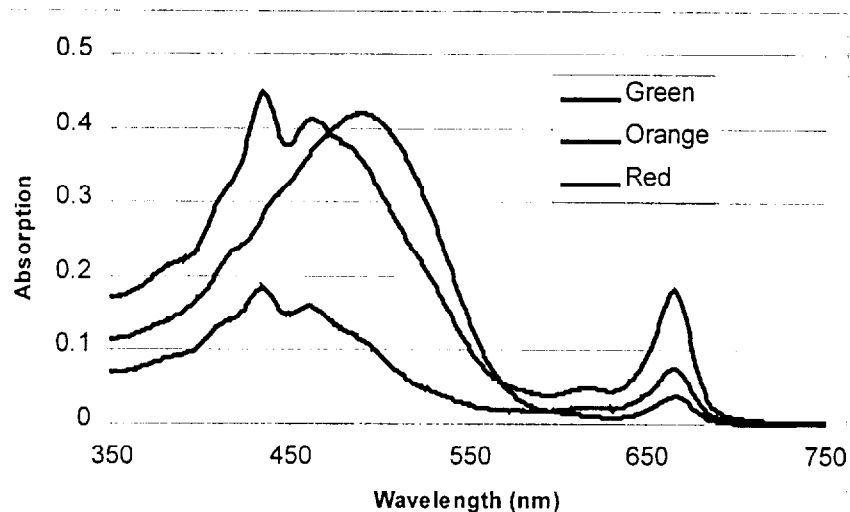
References

1. Cordero B., Otero A., Patiño M., Arredondo, Fabregas, J. 1996. Biotech. Lett. 18: 213-218.
2. Wright S.W., Jeffrey S.W., and Mantoura R.F.C. 1997. In "Phytoplankton Pigments in Oceanography", Jeffrey S.W. and Wright S.W. (Eds), pp: 261-282.
3. Latasa P., 1995. Report to Aquasearch Inc.
4. Fan L., A. Vonshak, R. Gabbay, J. Hirshberg, Z. Cohen, and S. Boussiba. 1995. Plant Cell Physiol. 36: 1519-1524.
5. Yuan J.-P., X.-D. Gong, and F. Chen. 1996. Biotech. Tech. 10: 655-660.
6. Kobayashi M., T. Kakizono, S. Nagai. 1991. J. Ferment. Bioeng. 71: 335-339.

Technical report

APPENDIX A: ABSORPTION PEAKS OF DMSO EXTRACTS PREPARED FROM HAEMATOCOCCUS ALGAE AT VARIOUS STAGES OF THE REDDENING CYCLE.

The astaxanthin absorption measurement after extraction in DMSO is carried out at 489 nm because the absorption maximum of the red meal extract shifts to 489nm as the cells naturally redden and accumulate astaxanthin. The figure below shows three different absorption spectra of extracts obtained from *Haematococcus* cells in (a) the green stage, (b) after initiating to redden (orange), and (c) when fully red, corresponding to a shift from chlorophylls to astaxanthin as the dominant pigment in the cells. Note the shift in absorption maximum towards 489 nm.



Technical report

Derivation of astaxanthin light absorption coefficients in different solvents.

(TR.1004.001)

Measurements conducted at Aquasearch's Kona Research and Development facility indicate that:

- ***The light extinction coefficient of astaxanthin in different solvents varies significantly.***
- ***The wavelength of maximum light absorption of astaxanthin also varies in different solvents.***

BACKGROUND

We expect that Quality Control (QC) laboratories at our customers' facilities will routinely analyze our *Haematococcus* algal meal products for astaxanthin content. In general, total astaxanthin is estimated from light absorption measurements of extracts. The extracts are usually prepared by grinding the algal meal in an organic solvent. The extract is then clarified by either filtration or centrifugation. The light absorption of the extract provides an estimate of pigment content. To accurately estimate the content of astaxanthin in a solvent it is critical to know what the absorption coefficient of astaxanthin is in that specific solvent and at what wavelength (usually the wavelength of maximum absorption as determined in a scanning spectrophotometer).

We have made a series of measurements of astaxanthin absorption in five different solvents to determine both the wavelength of maximum absorption and the extinction coefficient of astaxanthin in those solvents. This data can be used by our customers to analyse the astaxanthin content of algal meal in the solvent of their choice.

EXPERIMENTAL CONDITIONS

Pure, free, astaxanthin was obtained from the Sigma Chemical Company (lot number 87H5002). According to the manufacturer, the molar extinction coefficient of this product in chloroform at a wavelength of 489 nm is $E_{\text{cm}}^{1\text{cm}} = 101$.

A working solution of pure, free, astaxanthin was made up in dimethyl sulfoxide (DMSO). Five test tubes were filled with 10 ml of each of the following solvents: DMSO, acetone, methanol (MeOH), dimethylformamide (DMF) and chloroform (CHCl_3). Each of the tubes was added either 50, 100, 200, 400, or 1010 μL of the astaxanthin solution.

Technical report

The light absorption of each solution was then measured between 425 and 550 nm in a Shimadzu model UV1201 scanning spectrophotometer with a 1 cm cuvette. The absorption spectra were used to estimate the wavelength of maximum absorption of astaxanthin and its extinction coefficient in each solvent.

RESULTS:

Light Absorption by Free Astaxanthin in Organic Solvents

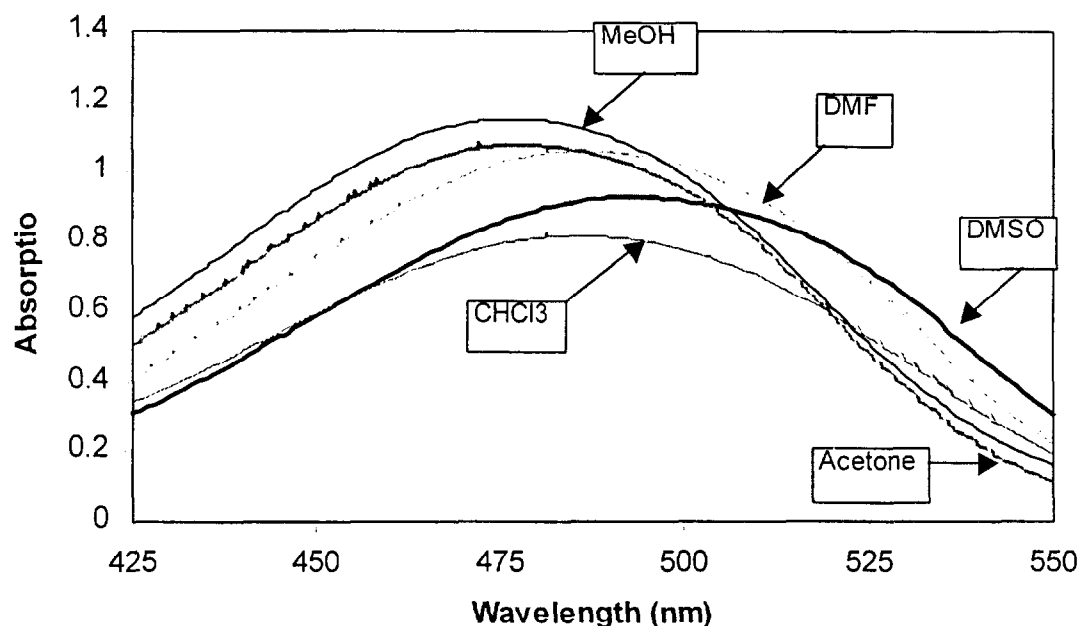


Figure 1 shows the light absorption spectra of the same amount of astaxanthin in 5 different solvents. Note that both the wavelength of maximum absorption and extinction coefficient are different for each solvent.

Our results of the extinction coefficient calculations are summarised in the table below.

Solvent	Wavelength of maximum absorption	Molar Extinction Coefficient	Mass Extinction Coefficient
	nm	$\text{l mol}^{-1} \text{cm}^{-1}$	$100 \text{ ml g}^{-1} \text{cm}^{-1}$
DMSO	492	118.2	1980.7
Acetone	477	130.0	2177.4
MeOH	477	137.7	2306.6
DMF	486	123.2	2064.7
CHCl ₃	486	101	1692.2

Technical report

Comparison of HPLC and spectrophotometric analyses of astaxanthin content in *Haematococcus pluvialis* algal meal

(TR.1005.001)

Analyses conducted at Aquasearch's Kona Research and Development facility indicate that:

- ***Spectrophotometric analysis of combined sequential DMSO extracts of *Haematococcus pluvialis* algal meal is a reliable estimate of total astaxanthin content.***
- ***Astaxanthin content as determined by reversed-phase high-performance liquid chromatography (HPLC) of combined sequential acetone extracts correlates closely with values determined by spectrophotometry.***
- ***HPLC analyses provide additional information, i. e., ratios of free astaxanthin to mono- and di-esterified astaxanthin.***

BACKGROUND

Aquasearch's laboratory routinely uses a spectrophotometric method to analyse our *Haematococcus pluvialis* algal meal product for total astaxanthin content (see Technical Report TR.1002.001). The spectrophotometric assay offers several advantages: it is accurate, reproducible, and technically simple. In addition, the required instrumentation (a UV-visible spectrophotometer) is a relatively common piece of equipment, and thus this method of analysis should be reproducible in the quality control laboratories of our customers.

However, a spectrophotometric measurement gives an estimate only of total astaxanthin. Astaxanthin possesses two hydroxyl moieties, either or both of which may be esterified (chemically bonded) to a fatty acid, resulting in a mono- or diester form, respectively. It may sometimes be of interest to determine the form in which the astaxanthin occurs, i. e., free (unconjugated) or esterified. Natural astaxanthin from *Haematococcus pluvialis* occurs predominantly as monoesters (about 80-85% of total astaxanthin), with smaller amounts of diesters (about 10-15%) and of free astaxanthin (about 2-4%). Changes in these ratios may reflect different physiological states of the alga, or in variations in processing of the algal meal.

Technical report

We have therefore developed an alternative method for measuring both total astaxanthin and its distribution between the free and esterified forms. This method is based on reversed-phase high-performance liquid chromatography (HPLC) with diode-array detection, using external standards for calibration. Samples are extracted into acetone as an HPLC-compatible, alternative solvent to DMSO. Separation of both pure astaxanthin standards and of mixtures (e. g., extracts of *Haematococcus pluvialis*) is achieved with a C18 column, using methanol-water mixtures as the mobile phase. This is illustrated by the example below, which demonstrates that for high-quality *H. pluvialis* algal meal (where astaxanthin is the major pigment present), spectrophotometric and HPLC determination of astaxanthin content are in good agreement.

EXPERIMENTAL CONDITIONS

GENERAL

All solvents used are HPLC-grade or better and are thoroughly degassed (preferably with argon) immediately before use. Solutions of astaxanthin are light- as well as air-sensitive and should be protected from light (e. g., covering containers with aluminum foil, working under subdued light) as much as is practical. Astaxanthin appears reasonably stable at room temperature if protected from oxygen and light.

PREPARATION OF STANDARDS

Detector response from a diode-array detector is not directly comparable to the response from a single-wavelength (visible light) spectrophotometer, since the diode-array response is generally obtained from more than one diode (that is, more than a single wavelength). Also, since the flow cell is exposed to light of all wavelengths simultaneously, there is the potential for fluorescent contamination of the detected signal. Thus, the routine use of standards is mandatory for accurate quantification of HPLC peaks.

Synthetic, racemic astaxanthin (catalogue number A9335, lot number O78H1178, purity by HPLC 99.3%) was purchased from Sigma Chemical Co. for use as an external standard. As an alternative standard, astaxanthin diacetate was prepared. Astaxanthin diacetate offers the advantage of increased stability to oxidation, and displays chromatographic behaviour similar to that of free astaxanthin. The visible spectral properties of astaxanthin diacetate are identical to that of free astaxanthin.

To prepare astaxanthin diacetate, 10 mg of astaxanthin (from a newly opened bottle) was peracetylated with 5.0 mL acetic anhydride in 5.0 mL HPLC-grade pyridine (freshly dried over BaO). The reaction was carried out over 22 h at room temperature, in the dark, under N₂ positive pressure. The reaction was quenched on ice by addition of 10 mL 0.1 M HCl (aq.). The clear, colourless aqueous epiphase was discarded, and the organic phase washed four times with 20 mL 0.1 M HCl (aq.) and twice with 20 mL dd H₂O. The organic phase was dried over Na₂SO₄ and the solvent evaporated. The residue was stored under N₂, in the dark at -20 °C. HPLC analysis (see below) showed the reaction to be quantitative, with a single component eluting at 11.1 minutes and no trace of starting material (free astaxanthin) observed. The diode-array spectrum of this single component consisted of a single peak with broad absorption maximum of approximately 476 - 478 nm in the HPLC mobile phase, identical to that of authentic free astaxanthin.

Typically, a concentrated (3 - 5 mg/mL) solution of synthetic astaxanthin diacetate (or of free astaxanthin) is made in dichloromethane. Aliquots of this concentrate are added to methanol or acetone to produce diluted "standard" solutions for use as spectrophotometric and HPLC standards. At least five standard solutions should be prepared, with optical densities spanning the range of 0.1 to 1.0 (478 nm). Absorbance measurements at 478 nm are taken in a 1 cm cuvette immediately

Technical report

after the standard solutions are made. Aliquots of the standard solutions are transferred to HPLC autosampler vials, covered with argon, and the vials sealed and loaded into the precooled (10 °C) autosampler carousel.

PREPARATION OF SAMPLES FOR HPLC ANALYSIS

Samples destined for HPLC analysis must meet a number of criteria. Samples must be dissolved in a solvent compatible with the chromatographic system in use (preferably in the mobile phase or similar solvent). They should not contain particulates that may increase back-pressure or otherwise block the HPLC flow path. Particulates may be removed by filtration (0.2 µm or finer) or by centrifugation. Additionally, they may require preliminary purification (e. g., by passing through a solid-phase extraction cartridge or other quick chromatographic system) if contaminated with large amounts of lipids or other substances that interfere with HPLC or degrade column performance.

CHROMATOGRAPHY

Chromatographic conditions are as follows. The column is a Platinum EPS C18 column, in the Alltech proprietary "Rocket" format, 7 mm x 53 mm (Alltech Associates). This is a non-encapped, low (5%) carbon-load, 3 Å particle size packing material with appropriate porosity (100 Å) for carotenoid separation. An appropriate replaceable guard column (e. g., Alltech Associates' "AllGuard" guard cartridge in Platinum EPS C18) should always be used with this column and replaced when necessary to maintain optimal peak resolution as well as minimise back-pressure. Injections are made with an autosampler fitted with a refrigerated carousel and a 100 µL sample loop; typically 20 µL (partial-loop) injections are made for optimal peak resolution. Flow rate of mobile phase is 2.0 mL/min. Detection is with a diode-array detector, typically scanning 300 – 600 nm at 1 or 2 Hz/nm. Integration is done using a minimum bandwidth with detection centered on 478 nm.

To distinguish between free astaxanthin and its diacetate, samples are isocratically eluted with 82.5% methanol, 17.5% water. Under these conditions, free astaxanthin elutes at 9.3 minutes and its diacetate at 11.1 minutes. The diode-array spectra of free astaxanthin and its diacetate are identical.

For routine analytical runs, standards are isocratically eluted with 90% methanol, 10% water. Both free astaxanthin and its diacetate elute at ~2.5 minutes under these conditions. The run time for standards is usually 5 minutes. Generally a minimum of five standards are run at least in triplicate. It is not necessary to change mobile phase composition between consecutive standard runs. For a long series of samples (total run time >12 hours), sets of standards are chromatographed between several unknown samples.

The astaxanthin peak is integrated (under these conditions from baseline to baseline) and quantified as milli absorption units (mAU). The amount (in micrograms) of astaxanthin injected can be calculated from the spectrophotometrically-measured absorbance ($A_{478\text{nm}}$) and the (micro)molar extinction coefficient $\epsilon = 0.1251/\mu\text{M}$ (Britton, 1995):

$$[(A_{478\text{nm}} \div 0.1251/\mu\text{M}) \times 0.000020 \text{ L} \times 596.8 \mu\text{g}/\mu\text{mol}] = \mu\text{g injected (in 20 } \mu\text{L)}$$

A convenient expression of the diode-array response factor for astaxanthin is absorption units per µg (AU/µg). This value does vary to some extent (it is affected by lamp condition, for example) but should be consistent between replicate samples of different concentrations and within a 24-hour run. Lack of consistency between the response factor values for standards may indicate degradation of the standard or other problems. In our hands, typical values of this response factor for a given set of analyses (total run time <24 hours) have been ~2500 to 3400 AU/µg with a percent standard deviation from the mean of <3.5%.

Technical report

Unknown mixtures of astaxanthin or its esters are eluted as follows:

0 min to 30 min: 10% water, 90% methanol
30 min to 32 min: linear gradient to 100% methanol
32 min to 45 min: 100% methanol

Under these conditions, free astaxanthin elutes at about 2.5 minutes, the monoesters elute as several peaks between 7 and 18 minutes, and the diesters elute as several peaks between 33 and 36 minutes. Astaxanthin peaks are identified by their diode-array spectra and integrated. All-*E* (all-*trans*) astaxanthin and its esters have a diode-array spectrum consisting of a single peak with broad absorption maximum of approximately 476 - 478 nm in the mobile phase used. There may be minor "shadow" peaks following the all-*trans* peaks with spectra that have a hypsochromic shift to approximately 465 nm and development of a shoulder at about 375 nm (the "*cis* peak"); this is probably due to the presence of mono-*cis* isomer(s). This method does not distinguish between the three possible astaxanthin stereoisomers (all *S*, all *R*, and *meso*). The response factor obtained from the standards is used for calculating the amount of astaxanthin in the unknowns.

AN EXAMPLE

Six samples (about 100 to 200 mg each) of *Haematococcus pluvialis* dry meal (lot number 990610MIX) were weighed into tared 15-mL polypropylene conical centrifuge tubes. Each sample was sequentially extracted using one of the following three methods (2 tubes per extraction method):

- A. Add 0.5 mL DMSO to tube; heat 2 minutes in 70 °C water bath; remove from heat; add 4.5 mL acetone; vortex 1 minute; centrifuge 5 minutes in clinical centrifuge; decant supernatant into amber glass jar. Repeat 4 more times for a combined final volume of 25 mL.
- B. Add 0.5 mL DMSO to tube; vortex 1 minute; add 4.5 mL acetone; vortex 1 minute; centrifuge 5 minutes in clinical centrifuge; decant supernatant into amber glass jar. Repeat 4 more times for a combined final volume of 25 mL.
- C. Add 5.0 mL acetone; vortex 1 minute; centrifuge 5 minutes in clinical centrifuge; decant supernatant into amber glass jar. Repeat 4 more times for a combined final volume of 25 mL.

All of the above sequential extraction methods were sufficient to remove most pigment from the algal meal, leaving a dark pink residual pellet after centrifugation. The fourth and fifth extractions removed only small additional amounts of pigment as judged by the increasingly pale color of the solvent.

Immediately after extractions were complete, an aliquot of each extract was removed and diluted as required into acetone for spectrophotometric analyses. The remaining extracts were stored refrigerated under a blanket of argon gas, prior to HPLC analysis.

Standard solutions of astaxanthin diacetate were prepared and chromatographed as described above. The diode-array response factor for astaxanthin diacetate obtained from integration of standard runs (five concentrations, triplicate analyses) was 3317 ± 113 AU/ μ g astaxanthin diacetate. This was used for calculation of the astaxanthin content of the *Haematococcus pluvialis* extracts. For these extracts, areas under the curve for peaks identified as free or esterified all-*E* (all-*trans*) astaxanthin were integrated.

Technical report

Results of these analyses are given in the following table.

Sample number (extraction method)	Sample dry weight (mg)	Astaxanthin content (mg), by spectrophotometry (478 nm)	Astaxanthin content as percent dry weight, by spectrophotometry (478 nm)	Mean (standard deviation), <i>n</i> = 6	Astaxanthin content (mg), by HPLC	Astaxanthin content as percent dry weight, by HPLC	Mean (standard deviation), <i>n</i> = 6
1 (A)	98.1	2.40	2.44	2.36 (0.06)	2.40	2.45	2.40 (0.05)
2 (A)	117.3	4.17	2.35		4.21	2.37	
3 (B)	124.3	2.93	2.36		2.99	2.40	
4 (B)	188.6	4.52	2.39		4.41	2.34	
5 (C)	106.1	2.48	2.34		2.48	2.34	
6 (C)	195.4	4.42	2.26		4.82	2.47	

The distribution of astaxanthin as the free xanthophyll, monoester, and diester forms were determined from the HPLC analyses. The results are given in the following table.

Sample number (extraction method)	Percent astaxanthin as free xanthophyll	Mean (standard deviation), <i>n</i> = 6	Percent astaxanthin as monoesters	Mean (standard deviation), <i>n</i> = 6	Percent astaxanthin as diesters	Mean (standard deviation), <i>n</i> = 6
1 (A)	2.8	3.1 (0.4)	85.6	84.5 (1.7)	11.6	12.5 (1.3)
2 (A)	3.2		83.4		13.4	
3 (B)	3.5		84.4		12.1	
4 (B)	3.5		81.9		14.6	
5 (C)	3.0		84.8		12.2	
6 (C)	2.4		86.7		10.9	

As there is no official (e. g., AOAC) method for determination of astaxanthin content in *Haematococcus pluvialis* algal meal, a sample of the same lot (990610MIX) of *H. pluvialis* algal meal was sent for independent, parallel analysis to a laboratory (Dr. Bjørn Bjerkeng, Akvaforsk, Norway) well-experienced in determining astaxanthin content in fish feed or fish flesh. A sample of the meal was weighed out, treated with 0.1 g Glucanex® (Novo Nordisk) in 5 mL distilled water for 2 h at 30 °C, and a 500 L aliquot extracted in 40 mL 25% methanol, 75% chloroform. The extract was homogenised and centrifuged, and an aliquot of the chloroform layer dried and redissolved in HPLC mobile phase for analysis. The Akvaforsk HPLC analysis used a silica column pretreated with phosphoric acid, a mobile phase consisting of 14% acetone in *n*-hexane, a flow rate of 1.2 mL/min, and detection at 470 nm (Vecchi et al. 1987). This analysis reported an astaxanthin content (as percent dry weight) of 2.18% (Akvaforsk final analysis report, project S827, 1 July 1999). This value is approximately 10% lower than the values obtained by us either by spectrophotometry (2.36%) or by HPLC (2.40%), for samples from the same lot of algal meal. This may be explained by the fact that at Akvaforsk the enzyme-treated meal was extracted only once into organic solvent, whereas in our laboratory the samples were extracted five times. The extraction method used by Akvaforsk is similar to that used at Hoffmann-La Roche for extracting free (synthetic) astaxanthin in the gelatin matrix used for fish feed (Weber 1988; Bühler-Steinbrunn and Manz, 1988), while the sequential extraction method used in our laboratory is similar to methods described for extraction of carotenoids from macro- and microalgae (Haugan et al. 1995; Yuan & Chen 1998). We have observed that a single extraction is not capable of extracting all astaxanthin from the algal meal, probably due to the high concentrations of astaxanthin and lipids in the alga and further complicated by the non-homogeneity of the matrix (algal cellular structures). The distribution of astaxanthin among the free, monoester, and diester forms (3.6%, 81.8%, and 14.6%, respectively) reported by Akvaforsk (Akvaforsk final analysis report, project S827, 1 July 1999), is similar to that determined by our laboratory's HPLC method (3.1%, 84.5%, and 12.5% respectively).

Technical report

Results of the analyses show that, for these samples, the correlation between our spectrophotometric and HPLC analytical methods is high; this is as expected as in these samples astaxanthin is the main xanthophyll and main carotenoid present and there is little chlorophyll. For both our spectrophotometric and HPLC methods, variation in results is less than 5%, and thus compares favourably with the variability ranges reported by workers at Hoffmann-La Roche for HPLC analysis of astaxanthin in fish feeds and premixes ($\pm 10\%$, Weber 1988) and for spectrophotometric analysis of astaxanthin isolated by open-column chromatography from fish feeds ($\pm 16\%$, Bühler-Steinbrunn and Manz, 1988).

In all cases the estimate of astaxanthin content is dependent on the extractability of the xanthophyll into organic solvent, and thus the obtained values of astaxanthin content are best stated as "extractable astaxanthin" rather than "total astaxanthin". Since even at the highest measurable levels of astaxanthin content there was some visible red pigment that was not extractable left in the pellet, the measured levels must actually slightly underestimate the total astaxanthin content.

For *Haematococcus pluvialis* algal meal samples with low ($<1\%$ by spectrophotometer) astaxanthin content, the HPLC analysis gives a lower estimate of astaxanthin content than does the spectrophotometer (data not shown). The spectrophotometric method does not distinguish between astaxanthin and other materials that may absorb in the same region (e. g., other carotenoids or products of chlorophyll degradation), and hence HPLC is a more accurate method of determining astaxanthin content in these instances.

REFERENCES

- Britton, G. UV/Visible spectroscopy. In: "Carotenoids. Volume 1B: Spectroscopy." G. Britton, S. Liaaen-Jensen, and H. Pfander (editors), p. 57, Birkhäuser Verlag, Basel, 1995.
- Bühler-Steinbrunn, I. I., & Manz, U. Determination of stabilized astaxanthin in fish feeds by open-column chromatography on silica gel. In: "Analytical Methods for Vitamins and Carotenoids in Feed." H. E. Keller (editor), pp.62-64, F. Hoffman-La Roche & Co., AG, Basel.
- Haugan, J. A., Aakermann, T., & Liaaen-Jensen, S. Worked examples of isolation and analysis. Example 2: macroalgae and microalgae. In: "Carotenoids. Volume 1A: Isolation and Analysis." G. Britton, S. Liaaen-Jensen, and H. Pfander (editors), pp.215-226, Birkhäuser Verlag, Basel, 1995.
- Vecchi, M., Glinz, E., Meduna, V., & Scheidt, K. (1987) HPLC separation and determination of astacene, semiastacene, astaxanthin, and other ketocarotenoids. *J. High Resolution Chromatogr. Chromatogr. Commun.*, 10:348-351.
- Weber, S. Determination of stabilized, added astaxanthin in fish feeds and premixes with HPLC. In: "Analytical Methods for Vitamins and Carotenoids in Feed." H. E. Keller (editor), pp.59-61, F. Hoffman-La Roche & Co., AG, Basel.
- Yuan, J.-P. & Chen, F. (1998) Chromatographic separation and purification of *trans*-astaxanthin from the extracts of *Haematococcus pluvialis*. *J. Agric. Food Chem.*, 46:3371-3375.