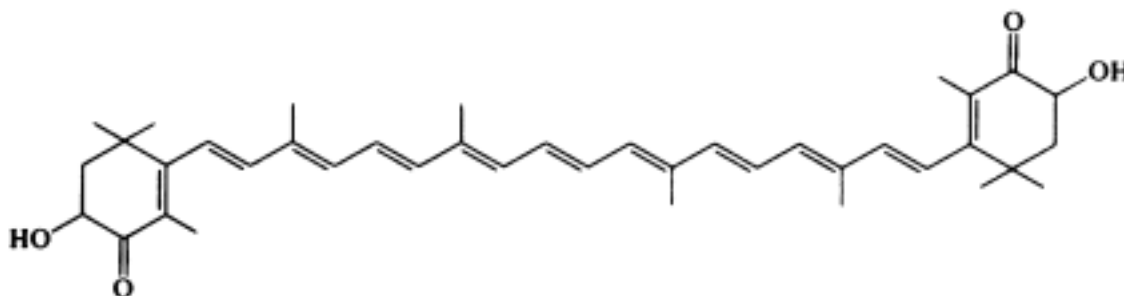


HPLC and spectrophotometric analysis of carotenoids from *Haematococcus* algae powder

Background: The astaxanthin molecule has two asymmetric carbons located at the 3 and 3' positions of the benzenoid rings on either end of the molecule. Different enantiomers of the molecule result from the exact way that these hydroxyl groups (-OH) are attached to the carbon atoms at these centers of asymmetry (Figure 1). If the hydroxyl group is attached so that it projects above the plane of the molecule it is said to be in the R configuration and when the hydroxyl group is attached to project below the plane of the molecule it is said to be in the S configuration. Thus the three possible enantiomers are designated R,R', S,S' and R,S' (meso).

Figure 1

Astaxanthin



Free astaxanthin is the form in which the 3 and 3' hydroxyl groups are not bonded, an astaxanthin monoester has one fatty acid bonded to a 3 position, and an astaxanthin diester has a fatty acid bonded to each of the 3 positions. Free astaxanthin and its mono- and diesters from *Haematococcus* have optically pure (3S,3'S)-chirality (Renstrom *et al.*, 1981). The monoesters and diesters are relatively non-polar, whereas free carotenoids are relatively polar. The solvents used in this extraction and assay takes these factors into account, thus variations to this procedure may affect the outcome.

The carotenoid fraction of *Haematococcus* algae contains about 70% monoesters of astaxanthin, 10% diesters of astaxanthin, 5% free astaxanthin, with the remainder consisting of β -carotene, canthaxanthin, and lutein. Accurate quantification of esterified astaxanthin by HPLC is difficult, however, various systems have been developed and validated for the analysis of free astaxanthin. Thus, esterified astaxanthin must first be hydrolyzed by either a chemical or enzymatic procedure to yield all free astaxanthin. The enzymatic hydrolysis method is

preferable as it is simple, complete and does not oxidize the astaxanthin molecule when performed carefully. The enzymatic hydrolysis of carotenoid esters is a slight modification of the procedure developed by Jacobs P.B., R.D. LeBoeuf, S.A. McCommas, and J. D. Tauber, 1982. Thus, the HPLC assay quantifies free astaxanthin by separation and absorbency using a standard curve. Astaxanthin from salmon and trout muscle does not need to be hydrolyzed as it is enzymatically cleaved by a non-specific lipase upon uptake from the gut and is in the free form.

This HPLC method was first published by Vecchi M., V. Muduna, and E. Glinz, 1987 and subsequently validated by Hoffmann-La Roche. The method is published in the Roche Bulletin “Determination of Stabilized Astaxanthin in Carophyll Pink, Premixes, and Fish Feeds” by Dr. S. Weber, Mr. W. Hardi and Dr. J. Schierle as well as by Schuep and Schierle, 1995. The HPLC method for astaxanthin determination is the most commonly used procedure by feed companies and analytical laboratories in Norway and Chile.

The method has also been accepted by the Canadian Food Inspection Agency analytical laboratories for registration of *Haematococcus* algae meal in salmonid feeds (Registration Number 990535) and the US Food and Drug Administration (21 CFR 73.185). Preferably, a Luna 3 um analytical silica column and guard should be used for this procedure (Phenomenex part #00F-4162-E0). The Luna column provides significantly better repeatability and ruggedness. The spectrophotometric quantification of astaxanthin has been determined empirically and yields a close estimation, but is not as accurate as HPLC. Spectrophotometric methods are not reliable with mixed carotenoid samples.

NatuRose Technical Bulletin #017 describes the procedure for extraction and purification of carotenoids from oils and Bulletin #007 from finished feeds. NatuRose Technical Bulletin #003 describes a thin-layer chromatography procedure for NatuRose.

PART 1: Extraction of carotenoids from *Haematococcus* algae meal.

Materials Required

Analytical balance

Refrigerated centrifuge (1200 RCF at 3500 RPM is sufficient)

Acetone (minimum technical grade) containing 500 mg/L BHT (Sigma B 1253)

Dimethyl sulphoxide (DMSO)

Spectrophotometer (scanning)

25 ml volumetric flask with lid

10 ml centrifuge tubes with lid

Vortexer

Glass, sand or zirconium beads (through 20 mesh)

Pipettes

Water bath at 50 C

Procedure

Note: All manipulations should be performed in low light and temperatures, as carotenoids are very sensitive to light, oxygen and heat. It is recommended that the assay be performed in a darkened room while keeping the temperatures at 5 C if possible.

Extraction Procedure

Perform duplicates for each sample.

- 1) Weigh approximately 25 mg of dried *Haematococcus* algae powder into the 10 ml centrifuge tube. Record the weight
- 2) Add 3 grams of glass beads to the tubes.
- 3) Add 5 ml of DMSO to the centrifuge tubes, place in pre-heated water bath at 45-50 C for 15 minutes. Vortex for several times during the incubation.
- 4) Centrifuge at 3800-4200 rpm for 3 minutes to pellet the cell material.
- 5) Pipette the supernatant and collect in the 25 ml volumetric flask.
- 6) Add 5 mls of the acetone to the centrifuge tube, vortex vigorously for 30 seconds. Centrifuge tubes at 3500 rpm for 3 minutes to pellet the cell material. Transfer supernatant to the volumetric flasks.
- 7) If supernatant still has color, repeat step 6 until centrifuged acetone layer is less than 0.05 absorbency. Four extractions with acetone are usually ample.
- 8) After all the supernatant is collected in the volumetric flask, bring the volume to 25 ml with acetone.
- 9) Cap the flask and invert gently to mix. Pipette an aliquot (5-7 mls) into a clean tube and centrifuge for 5 minutes at 3500 rpm to remove any particulate matter which may have carried over in the transfers.
- 10) Read the maximum absorbency (approx. 471-477 nm) against an acetone blank on the spectrophotometer.
- 11) If the absorbency is greater than 1.25 it is necessary to dilute the sample with acetone and adjust the calculation for the dilution (absorbency readings are linear between 0.25 and 1.25). A 1:5 dilution (2 parts astaxanthin extract to 8 parts of acetone) will typically adjust the final absorbency within the proper range.
- 12) Proceed to Part 2 for hydrolysis of the carotenoid esters.

Calculations:

Total carotenoid quantification

$$\text{Carotenoids (mg) extracted} = \frac{\text{Abs max}}{250} \times 25 \text{ ml acetone} \times \text{Dilution}$$

Approximate astaxanthin percentage:

$$\text{Percent Astaxanthin} = \frac{\text{Carotenoids (mg) extracted}}{\text{sample wt (mg)}} \times 80$$

PART 2: Hydrolysis of carotenoids from *Haematococcus algae* meal.

Materials:

Scanning spectrophotometer

Haematococcus algae meal acetone extract

Cholesterol esterase from *Pseudomonas fluorescens* (Sigma C-9281, 10,000 units per gram).

Petroleum ether

0.05 M Tris-HCl buffer pH 7.0 (store refrigerated)

Sodium sulfate decahydrate (Na₂SO₄-10H₂O)

37 degree Celsius water bath

Pipettes

4 glass test tubes with caps

Nitrogen manifold

Fume hood

Vortex

Cholesterol esterase preparation

NOTE: Cholesterol esterase must be kept frozen at -20C until made into these working stock vials. The enzyme activity may be lost if not transported and stored properly.

1) It is best to make a single stock solution and freeze 2 ml aliquots for later use. Prepare a 3.4 units/ml stock solution of enzyme in 0.05 M Tris-HCl pH 7.0 buffer. If the 100 unit vial is purchased, add entire contents of vial to 29.4 mls of the Tris buffer. If the 500 unit vial is purchased, add the entire contents of the vial to 147 mls of water. Rinse inside of vial well to remove all enzyme, mix well solution well.

2) Aliquot solution into 1 or 2 ml vials and store unused stock solutions frozen at -20 C.

Procedure

Note: All manipulations should be performed in low light and temperatures, as carotenoids are very sensitive to light, oxygen and heat. It is recommended that the assay be performed in a darkened room while keeping the temperatures at 5°C if possible.

- 1) Adjust the *Haematococcus* algae acetone extract so that the absorbency (474-476 nm) is between 0.75-1.25.
- 2) Transfer 3 ml of the extract to a test tube and add 2 ml of 0.05 M Tris HCl buffer.
- 3) Equilibrate the tube in 37 C degree water bath for 2 minutes.
- 4) Add 300 ul of enzyme stock solution to the test tube and cap. React in 37 C degree water bath for 45 minutes with gentle mixing
- 5) Add 1.0 gram of sodium sulfate decahydrate and 4 ml of petroleum ether to tube and vortex for 30 seconds.
- 6) Centrifuge the tube for 3 minutes at 3500 RPM. This will separate the layers and cause the carotenoids to migrate from the aqueous phase into the petroleum ether.
- 7) Remove the petroleum ether layer containing the carotenoid mixture with a pipette to a clean tube.
- 8) Add 3-4 ml of petroleum ether to tube. Vortex than centrifuge the tube.
- 9) Pipette the petroleum ether layer to the tube tube with the carotenoids mixture.
- 10) Dry down under nitrogen gas.
- 11) Re-dissolve into 3 ml of mobile phase for HPLC analysis (82/18 hexane:acetone).

PART 3: HPLC analysis of carotenoids from *Haematococcus* algae meal.

Materials:

NatuRose acetone extract prepared according Part 2.
Astaxanthin standard (Sigma Chemical Company #A 9335)
Acetone (minimum technical grade) containing 500 mg/L BHT
HPLC instrument capable of integration and detection at 470-476 and peripherals
1% orthophosphoric acid in methanol
HPLC grade running solvent : 82/18 hexane: acetone, v/v (not containing BHT)
Chloroform
Hexane
Five Volumetric flasks (100 ml)
Sonicator
Spectrophotometer

Note: All manipulations should be performed in low light and temperatures, as carotenoids are very sensitive to light, oxygen and heat. It is recommended that the assay be performed in a darkened room while keeping the temperatures at 5 C if possible.

Preparation of standard solution and standard curve for astaxanthin

- 1) Dissolve approximately 3 mg of astaxanthin standard in a 100 ml volumetric flask with 10 ml of chloroform. Sonicate in a water bath to aid solubility. The solution is then made up to the 100 ml volume with hexane. This is the Stock Solution.
- 2) Transfer 5, 10, 15 and 20 mls of the Stock Solution to four 100 ml volumetric flasks containing 4 ml of chloroform. Make up to 100 ml volume with hexane. Sonicate in a water bath to aid solubility. These will result in an approximately 1.5, 3.0, 4.5 and 6.0 ug per ml solution, respectively. These are the Standard Solutions.
- 3) Immediately after preparation of the Standard Solutions, measure the absorbance (A) of each by spectrophotometer at the maximum (approx. 470nm).
- 4) The astaxanthin concentration is then calculated according to the following formula:

$$\text{Astaxanthin (ug/ml or mg/L)} = \frac{\text{Absorbance} \times 10,000}{2100}$$

2100 = E (1%/ 1 cm) = Standard absorbance of a 1% astaxanthin solution (weight/volume) in a 1-cm cuvette at 474 nm in hexane.

- 6) Aliquots of the freshly prepared Standard Solutions are then injected into the HPLC system for producing the stand curve of astaxanthin. (See below)

Reference: Astaxanthin; determination of stabilized, added astaxanthin in fish feeds and pre-mixes. In, Carotenoids Volume 1A:Isolation and Analysis. 1995. Birkhauser and Verlag basel.. W. Schuep and J. Schierle.

HPLC Column Preparation

This procedure is necessary to condition a newly purchased column. Thereafter, it is only required at about 6 month intervals, or if peaks start tailing off, broadening, or the backpressure increases significantly. The Luna column should be dedicated for this application only.

- 1) Run the 1% orthophosphoric acid through the guard and column at 0.5 ml/min for 1-2 hours to coat the silica matrix.

2) Equilibrate with running solvent at 0.5-1 ml/min with running solvent for 3-4 hours. Peaks shape and resolution generally improves after running a few samples.

HPLC operation with standards and samples

1) Conditions for running standards and samples are as follows:

Flow rate: 1.2 ml/min

Temperature: Ambient

Running solvent: hexane/acetone isocratic (82:18 v/v)

Injection volume (loop size): 20 ul

Column: Preferably, a Luna 3 um analytical silica column can be used (Phenomenex part # 00F-4162-E0). Alternatively, a LiChrosorb 5 um silica 60 HPLC column (250mm x 4.0mm) with similar guard column material. Run time: 10-15 minutes

Detection limit: 0.1 ppm in feeds

Retention times (varies slightly with running solvents and other conditions):

Beta-carotene:	1.4 min
Canthaxanthin:	2.8 min
Di-cis astaxanthin	5.5 min
E-astaxanthin:	5.8 min
9Z-astaxanthin	6.3 min
13Z-astaxanthin:	6.7 min
Lutein:	8.6 min

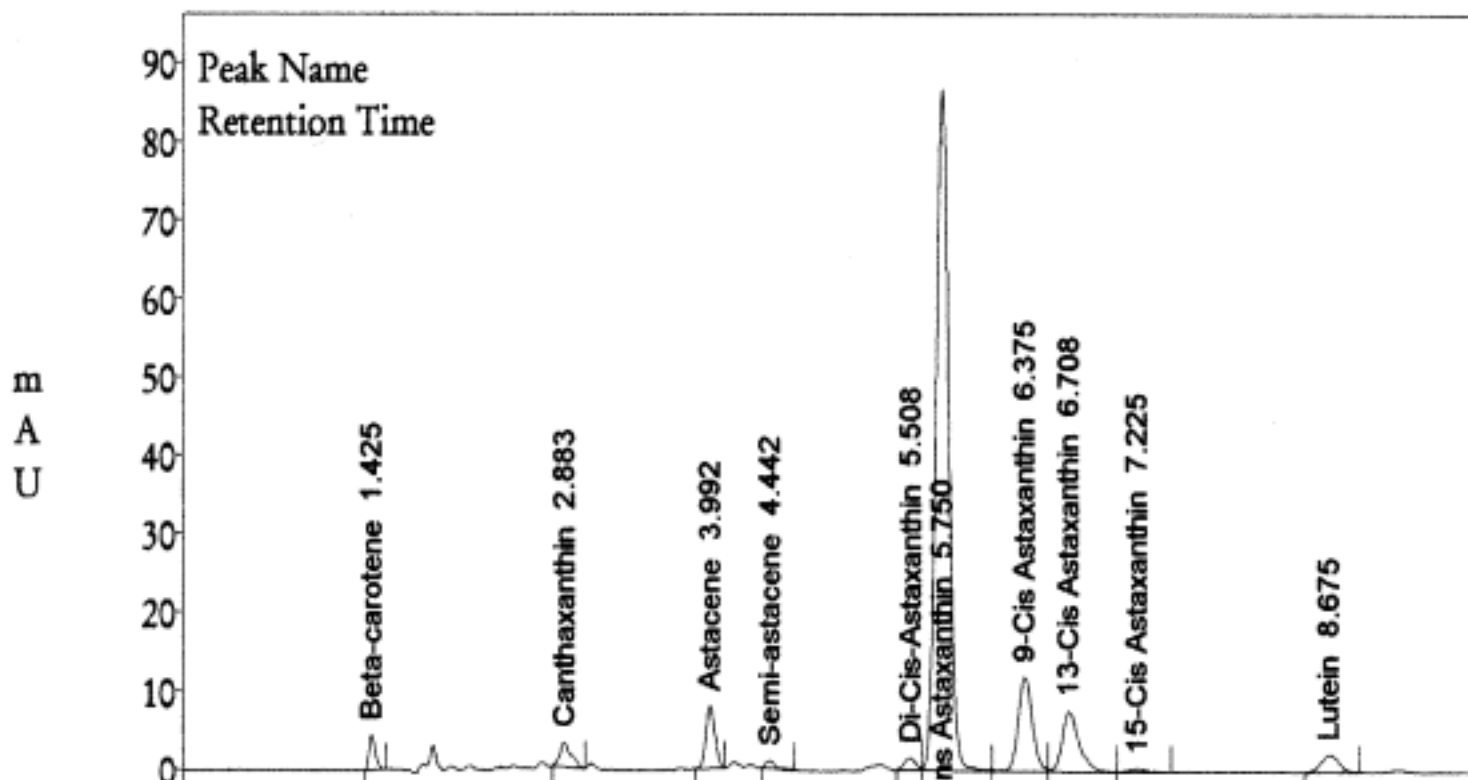
2) Immediately after preparation, 20 ul aliquots of the standard solutions are injected into the HPLC system. Peak shapes and resolution generally improve after running a number of samples through the column. The peak areas of the E/Z isomers of astaxanthin are determined separately and averages are calculated for each isomer. From the resulting means and from the spectrophotometric measured astaxanthin concentration, a response factor (RF) for E-astaxanthin is calculated according to:

$$\text{RF} = \frac{\text{P(trans)} + 1.2 \text{P(9-cis)} + 1.6 \text{P(13-cis)} + \text{P(di-cis)}}{\text{C}}$$

Where P(trans), 1.2 P(9-cis), 1.6 P(13-cis), and di-cis are the determined peak areas for all-trans, 9-cis, 13-cis, and di-cis astaxanthin respectively, and C is the concentration of the standard astaxanthin solution as determined spectrophotometrically. The multipliers 1.2 and 1.6 are introduced because the specific responses of 9-cis and 13-cis are 1.2 and 1.6 times lower than that of the all trans-isomer. A concentration curve can then be constructed, or entered into the software of the system for automatic calculations. The various E and Z-isomers of astaxanthin are separately determined and then summed, yielding the total astaxanthin content of the sample. The standard curve will correlate peak area of total astaxanthin vs. actual astaxanthin concentration in the test samples in ug/ml (mg/L). This can usually be done through the software of the particular HPLC system.

3) Sample aliquots of 20 ul from samples can now be quantified from step 11 of Part 2, based on the standard curve.

Typical chromatogram of carotenoids from NatuRose or BioAstin powder



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