

Analysis of Natural Astaxanthin Derived from *Haematococcus* Microalgae in Astaxanthin Oleoresin, Astaxanthin Gelcaps, Astaxanthin Beadlets, and *Haematococcus* Biomass (3/15/2013)

For questions or comments please contact Gerald R. Cysewski, Ph.D., Chief Science Officer, Cyanotech Corporation, email: gcysewski@cyanotech.com ; Phone: (808) 334-9420

1.0 Introduction

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 as shown in Figure 1 below.

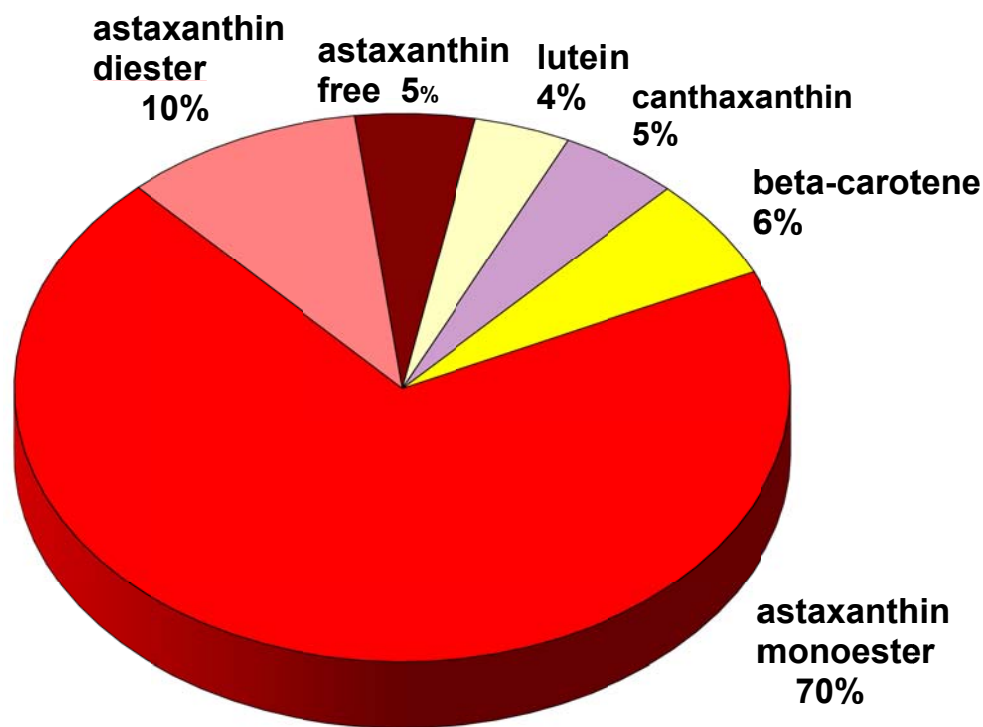


Figure 1. Carotenoid Complex in *Haematococcus*

Accurate quantification of esterified astaxanthin is difficult because the astaxanthin is esterified to a number of different fatty acids. 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. To verify the accurate extraction and analysis of *Haematococcus* algae products, an internal standard (trans- β -apo-8'-carotenal) is added and analyzed.

Spectrophotometric Quantification

Spectrophotometric quantification of astaxanthin yields a close approximation of astaxanthin, but is not as accurate as HPLC. Spectrophotometric quantification is influenced by other mixed carotenoids and any degradation products of astaxanthin present in the sample.

HPLC Analysis

Astaxanthin analysis by High Performance Liquid Chromatography (HPLC) provides the most accurate quantification of astaxanthin as well as mixed carotenoids and degradation products of astaxanthin present in the sample. However, it is essential that all esterified astaxanthin be completely hydrolyzed prior to HPLC analysis.

Safety

Proper safety precautions should be followed. Protective gear should include but is not limited to safety glasses and solvent-resistant gloves. Extractions should be performed in a fume hood that is in proper working condition. Measures should be taken to minimize solvent exposure through inhalation, ingestion, and skin contact.

General Considerations

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 20° C if possible. Carotenoids can absorb into and onto plastic and only glassware should be used.

2.0 Reagents

- Tris HCl (EMD P/N 9310)
- 1.0 N NaOH solution for pH adjustment (Fisher P/N S318-1)
- Methanol (VWR P/N JT9093-3) with 0.05% BHT (Sigma P/N B1253)
- DI or Distilled water
- pH buffer calibration solution
- Acetone (minimum technical grade) containing 500 mg/L BHT (Sigma B 1253)
- Trans- β -apo-8'-carotenal (Sigma P/N:10810) as internal standard
- Astaxanthin standard (Chromadex P/N ASB-00001695-005)
- Cholesterol Esterase (1 vial of 1000 units enzyme per vial, VWR P/N IC 10543991)
- Petroleum Ether (such as VWR P/N 4980-08)
- Anhydrous sodium sulfate (such as VWR P/N SX0761-1)
- Hexanes, HPLC Grade (such as VWR P/N HX 0290-1)
- Acetone, HPLC Grade (such as VWR P/N 9002-03)
- Dichloromethane (DCM) as dissolution solvent (such as VWR P/N BDH1113)
- Dimethylsulfoxide (DMSO, such as VWR P/N BDH1115-4LP)

3.0 Equipment

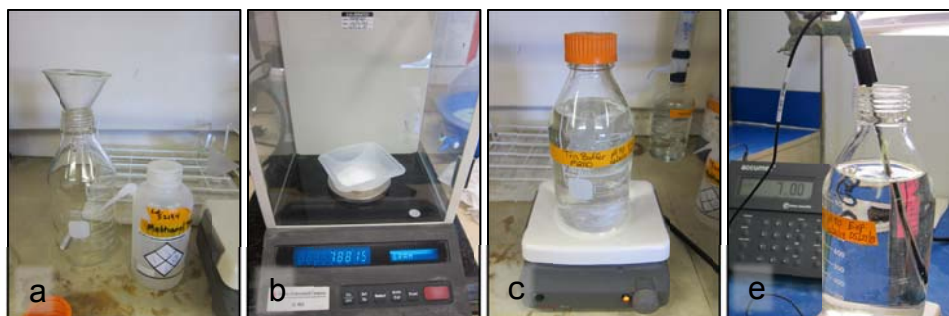
- Glass bottles with stir bar, 125 mL, 250 mL and 1000 mL
- Magnetic stir plate
- Graduated cylinders, 500 mL and 1000 mL
- glass beakers, 250 mL and 500 mL
- pH meter
- Funnel

- Analytical balance (0.1 mg accuracy)
- Centrifuge capable of 4200 rpm
- Volumetric flasks with stopper (3 mL, 10 mL, 50 mL, or 100 mL)
- 10 mL centrifuge tubes with caps
- 12 mL test tubes with lids
- 20 mL glass sample vials
- Small spatula
- Vortex mixer
- Volumetric pipettes (1 mL, 2 mL)
- Pipette, 5 mL adjustable
- Pasteur pipettes with bulbs
- MeOH squirt bottle
- 5 mL amber vials with tops
- Gas-tight glass syringes, 50 μ L, 100 μ L
- Spectrophotometer and controlling computer
- Water bath set at 35-37° C
- Nitrogen manifold
- Fume hood
- Aluminum foil
- Parafilm
- HPLC column: Luna 3 μ Silica(2), 100Å 150 x 4.60 mm, Phenomenex (P/N 00F-4162-E0) with suitable guard column (Phenomenex P/N AJO 4348 & KJO 4282)
- HPLC system with UV/VIS detector

4.0 Preparation of 0.05M Tris Buffer, pH 7.0

Tris buffer is added to the enzymatic digest of astaxanthin in the quantitative analysis of BioAstin products for astaxanthin and other carotenoids. Tris buffer helps provide a neutral pH for the enzyme cholesterol esterase to function and is prepared at a concentration of 0.05 molar (M) with the pH adjusted to 7.0.

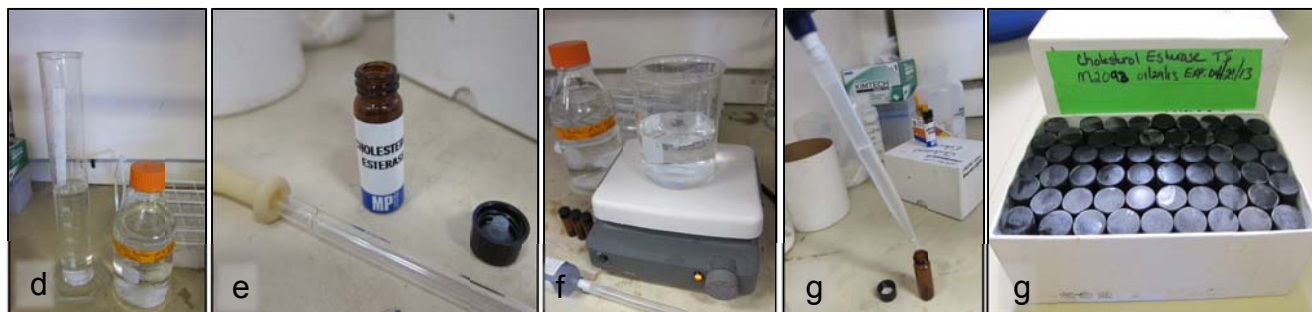
- a) Rinse the following glassware first with MeOH then secondly with DI water: glass bottle with stir bar, graduated cylinder, and funnel.
- b) Weigh out 7.88 g of Tris HCl and transfer the white solid quantitatively to the 1000 mL glass bottle containing a stir bar.
- c) Add 1000 mL DI water to the glass bottle and stir on a stir plate until all solid material has been dissolved.
- d) Calibrate pH meter to pH 7.00 \pm 0.01
- e) Adjust the pH of the solution to 7.00 using 1.0 N NaOH solution.
- f) Store under refrigeration at 4° C for up to 3 months. When unsatisfactory enzymatic digestion of astaxanthin is observed, check integrity and pH of solution, when necessary prepare a fresh solution, discarding the old one appropriately.



5.0 Cholesterol Esterase Preparation

Cholesterol esterase is commercially available as a solid. In order to be able to use the enzyme for the HPLC analysis of astaxanthin, it has to be solubilized in a buffer that stabilizes the enzyme during storage but does not affect its activity during the hydrolysis (de-esterification) step of the analysis. For ease of use, pre-measured aliquots of the enzyme solution are prepared and kept at -20°C . A cholesterol esterase solution is prepared so as to contain 3.33 enzyme units per mL.

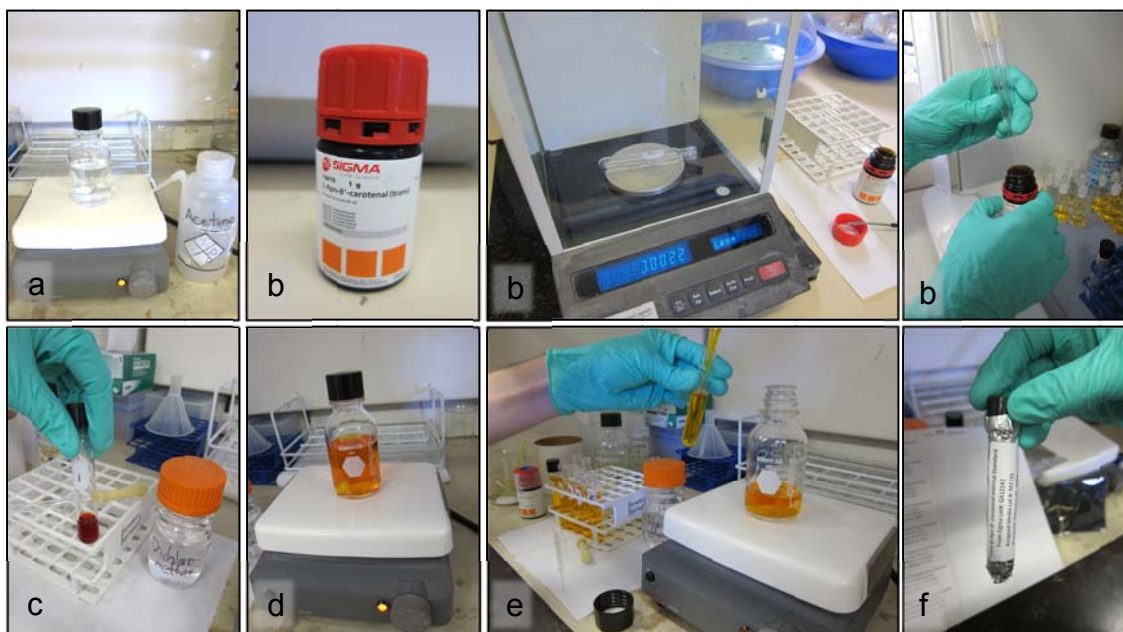
- Rinse 500 mL beaker, stir bar, and 500 mL graduated cylinder three times with MeOH using squirt bottle.
- Rinse items in step 1 six times with DI. water.
- Rinse 500 mL beaker with a small amount of Tris buffer.
- Measure 300 mL of Tris buffer into the 500 mL graduated cylinder.
- Since the amount of enzyme in each vial is quite small, open carefully and place the cap upside down on the counter (small amounts of enzyme may be adhering to the inside of the cap), and pipette 3.0 mL Tris buffer from graduated cylinder into the vial and 0.5 mL Tris buffer into the caps. Pour the Tris buffer from the rinses of the vial into the 500 mL beaker and remove the Tris buffer from caps using the pipette, transferring it to the beaker as well. Repeat 3 times.
- Gently stir till dissolved (~15 min).
- Using the 5 mL pipette set at 3.6 mL, fill as many 5 mL amber vials as possible with the enzyme solution. Seal the vials with caps, and store in freezer at $< -20^{\circ}\text{C}$ for up to three months until used. Label with the date prepared, assigned media lot number.



6.0 Preparation of Trans- β -Apo-8'-Carotenal Internal Standard Solution

The preparation of standard solutions and subsequent calibration of the HPLC are integral parts of the quantitative analysis of microalgae products by HPLC.

- a) Add 100 mL of acetone to a 125 mL glass bottle containing a small stir bar and place on stir plate with moderate stirring.
- b) Obtain the trans- β -apo-8'-carotenal standard compound. Into a clean and dry test tube, weigh out 2.2 mg of the trans- β -apo-8'-carotenal standard compound. Gently blow out the air from the bottle with N₂, quickly cap.
- c) To dissolve, add 3 mL (about 2 Pasteur pipette volumes) of dichloromethane (DCM) to the test tube containing the standard compound. This is the Compound Solution SI. Make sure that all of the compound is properly dissolved. Note: Organic solvents (such as DCM and diethyl ether) have been shown to catalyze the isomerization of carotenoids. Thus it is important to minimize the time the standard compounds are 'in contact' with these solvents.
- d) To the 125 mL bottle containing acetone add all of the Compound Solution SI and mix well. The concentration of the trans- β -apo-8'-carotenal internal standard solution is approximately 22 μ g/mL.
- e) Into separate clean and dry test tubes, pipette as many 10 mL aliquots of the standard solution as possible. Cap the test tubes tightly.
- f) Wrap each of the test tubes containing the trans- β -apo-8'-carotenal internal standard solution with aluminum foil and label them with the: type of standard, lot # of standard compound used, date prepared, and media preparation number. Store under refrigeration at < 4° C for up to one month until used.

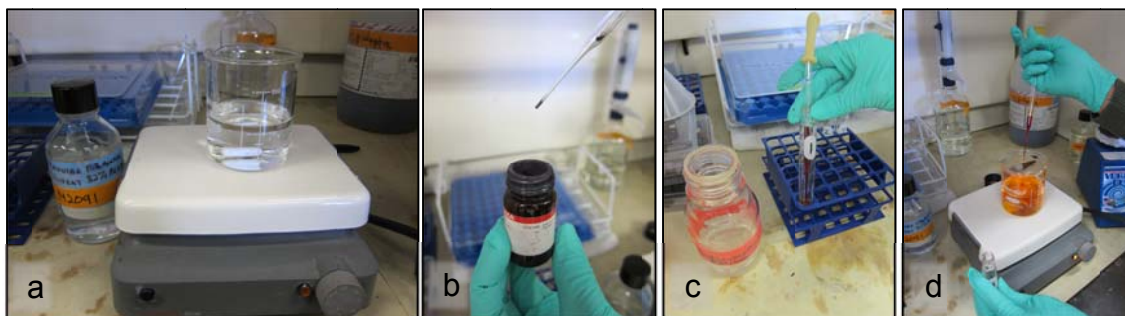


7.0 Preparation of Trans-Astaxanthin Daily Calibration Check Standard

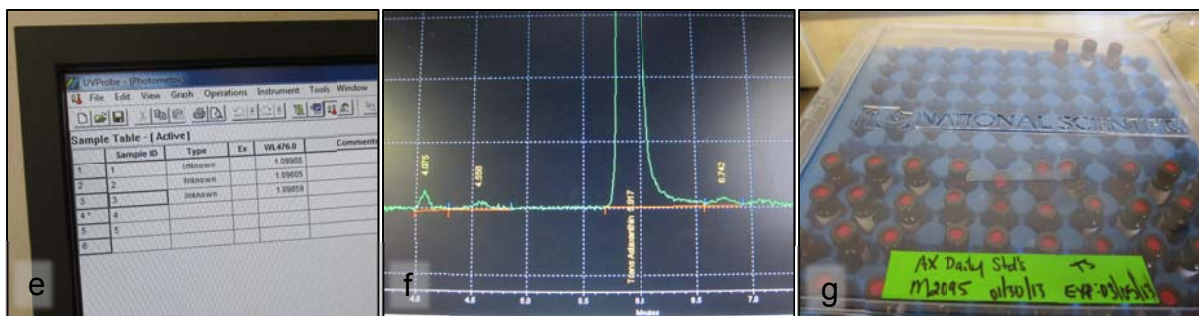
The preparation of standard solutions and subsequent calibration of the HPLC are integral parts of the quantitative analysis of microalgae products by HPLC.

- a) Add approximately 120 mL of HPLC Running Solvent (82% Hexane: 18% Acetone) to the 250 mL glass bottle containing a small stir bar and place on stir plate with moderate stirring.
- b) Obtain the trans-astaxanthin standard compound, and with a glass pipette (no bulb), transfer 1 mg or less of the compound to a test tube (dip pipette into compound bottle, this should supply ample amount for the preparation). Gently blow out the air from the bottle with N₂, quickly cap.

- c) To dissolve, add 1.5 mL (about 1 Pasteur pipette volume) of dichloromethane (DCM) to the test tube containing the standard compound. Make sure that all of the compound is properly dissolved. This is the Compound Solution AX. Note: Organic solvents (such as DCM and diethyl ether) have been shown to catalyze the isomerization of carotenoids. Thus it is important to minimize the time the standard compounds are 'in contact' with these solvents.



- d) For the following steps the use of the spectrophotometer is required. Use HPLC Running Solvent (82% Hexane:18% Acetone) as a blank when determining the absorbance of the standard solutions.
- e) To the 250 mL bottle containing Running Solvent (82% Hexane:18% Acetone) add enough of the Compound Solution AX to yield an absorbance of approximately 1.0 at the wavelength of 474 – 476 nm. Once an absorbance of approximately 1.0 is achieved, take at least three measurements and record the corresponding absorbances and calculate and record their average value including the lot # of the compound found on the bottle label.
- f) Now that the standard solution is prepared, into separate clean and dry test tubes, previously wrapped with aluminum foil, pipette as many 10 mL aliquots of the standard solution as possible. Cap the test tubes tightly. Inject and analyze the prepared standard solution on the HPLC System following procedure **10.0 HPLC Analysis** below for the determination of the standard compound's spectrophotometric purity.
- Spectrophotometric Purity: After the standard solution has been run on the HPLC, analyze it to show the peak areas of all detectable peaks and determine the spectrophotometric purity of the standard compound according to **7.1 Calculations** below, entering the pertinent information in the respective HPLC Notebooks. Make sure that only *detectable* peaks are included by reviewing the chromatogram used to quantitate the standard compound and making appropriate changes to the integration of the chromatogram.
 - With the spectrophotometric purity determined, calculate and record the concentration of the Trans-Astaxanthin Daily Calibration Check Standard as shown in **7.1 Calculations** below.
 - Inject and analyze at least five replicates of the Trans-Astaxanthin Daily Calibration Check Standard and report the average HPLC concentration of all replicates.
- g) Label each of the test tubes containing the Trans-Astaxanthin Daily Calibration Check Standard with: type of standard, lot # of standard compound used, concentration of standard, date prepared, and media preparation #. Place test tubes in freezer for storage at < -20° C for storage up to one month until used.



7.1 Calculations

a) Average Absorbance =
$$\frac{\text{absorbance1} + \text{absorbance2} + \text{absorbance3}}{3}$$

b) Spectrophotometric Purity =
$$\frac{\text{HPLC Peak Area of Standard Compound}}{\text{HPLC Peak Areas of All Detectable Peaks}}$$

c) Concentration of Standard (in mg/mL) =
$$\frac{\text{average absorbance of standard solution} \times \text{purity}}{217}$$

where *purity* is

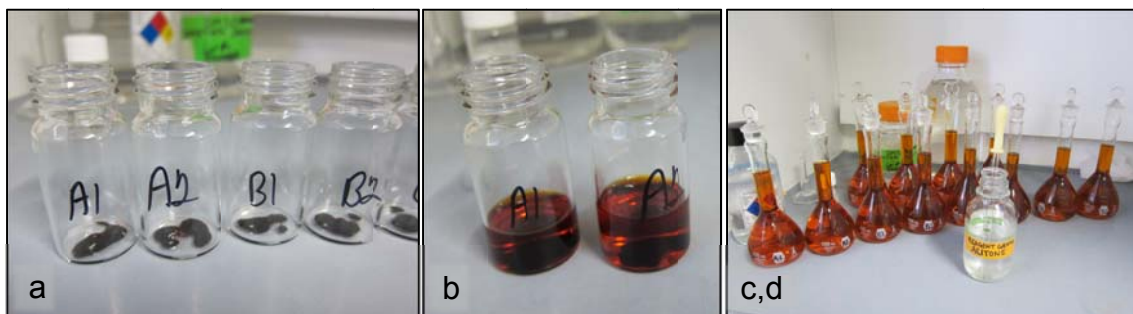
- the claimed purity of the compound as per the Certificate of Analysis, expressed as a decimal (for example: purity as per CoA = 98.6% → 0.986) or
- the spectrophotometric purity as calculated in step above.
- 217 is the extinction coefficient for Trans-Astaxanthin at a wave length of 474 nm in Running Solvent (82% Hexane:18% Acetone) .

Note: Use the spectrophotometric purity when a standard compound's Certificate of Analysis does not show an expiration date nor a stated purity, or the expiration date of a standard compound is expired and the standard compound shows a purity >90%.

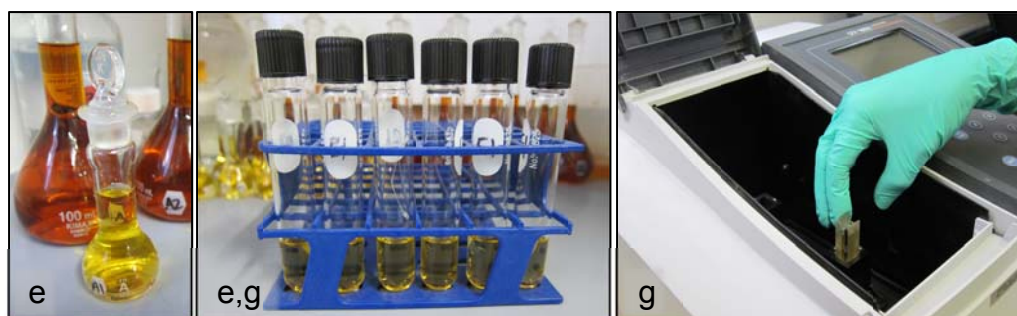
8.01 Extraction of Carotenoids from Astaxanthin Oleoresin

Note: Perform duplicate extractions for each sample

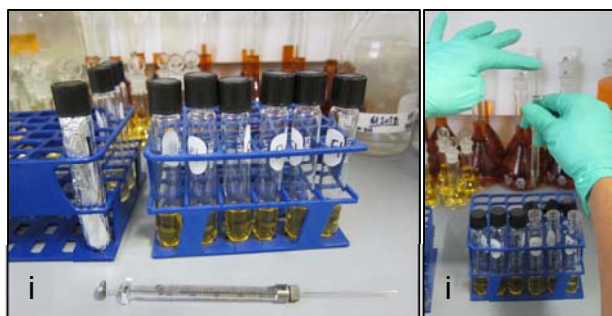
- Using the small spatula, stir the oleoresin sufficiently, then weigh approximately 25 mg of astaxanthin oleoresin into a clean and dry 20 mL glass sample vial. Record the weight to the nearest 0.1 mg
- Pipette acetone into the vial to wash the resin off the sides and dissolve the oleoresin.
- Transfer the acetone solution into a 100 mL volumetric flask using a Pasteur pipette. Wash the tube with acetone until all oleoresin is removed and the acetone is colorless, transferring the rinsates to the 100 mL volumetric flask.
- After all the rinse acetone is collected in the volumetric flask, let the solution equilibrate to room temperature (20° C), then bring the flask to volume with acetone and mix well.



- e) Prepare a 1/5 dilution by pipetting accurately 2 mL of first dilution into a clean and dry 10 mL volumetric flask. Bring the flask to volume with acetone, stopper and mix flask well. This is the second dilution. If a 1/5 dilution is used in this step the total dilution **D** is 500.
- f) (Typically a 1/5 dilution is adequate. Occasionally dilutions of 1/2 (5 mL sample in 10 mL total volume) are required for less concentrated samples or dilutions of 1/10 (1 mL sample in 10 mL total volume) are required for more concentrated samples.)
- g) Read and record the maximum absorbency on a Spectrophotometer (approx. 472 - 478 nm) of the second dilution against an acetone blank. (Absorbency readings are linear between 0.50 and 1.50). Verify that the absorbance of the duplicate samples are within 3% of each other before proceeding. If the duplicate samples are not within 3% of each other, repeat steps e) through f) above. If still not within 3%, either prepare a third sample that should fall within 3% of one of the two initial samples, or redo duplicates. Note it down in appropriate notebook.
- h) Once it is confirmed that the duplicates are within 3%, select ONE of the duplicates for the following, keeping note of which one is used. Pipette accurately 3 mL of the second dilution to a separate clean, dry test tube. Note: Make sure to note which duplicate is used at this time.



- i) To the test tube containing 3 mL of the second dilution add 50 μ L of internal standard (trans- β -apo-8'-carotenal) using the 50 μ L glass syringe. Make sure the syringe is rinsed after use with acetone.

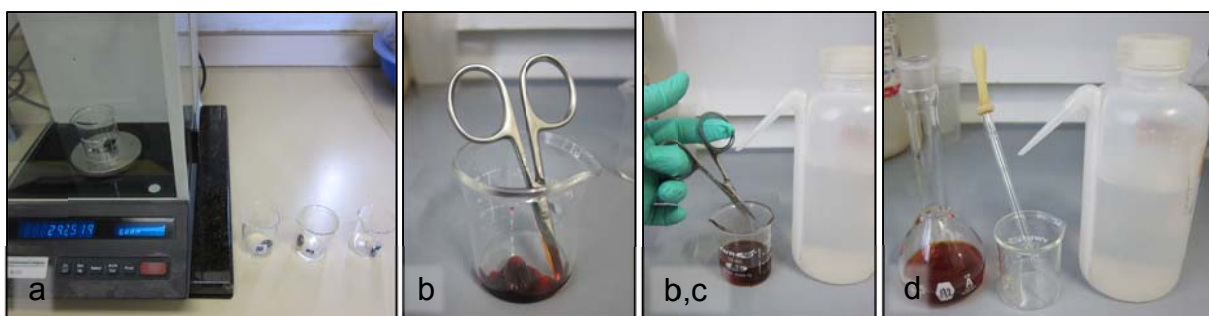


- j) Proceed to **9.0 Hydrolysis of Carotenoids from *Haematococcus* Algae Products**.

8.02 Extraction of Carotenoids from Astaxanthin Gelcap

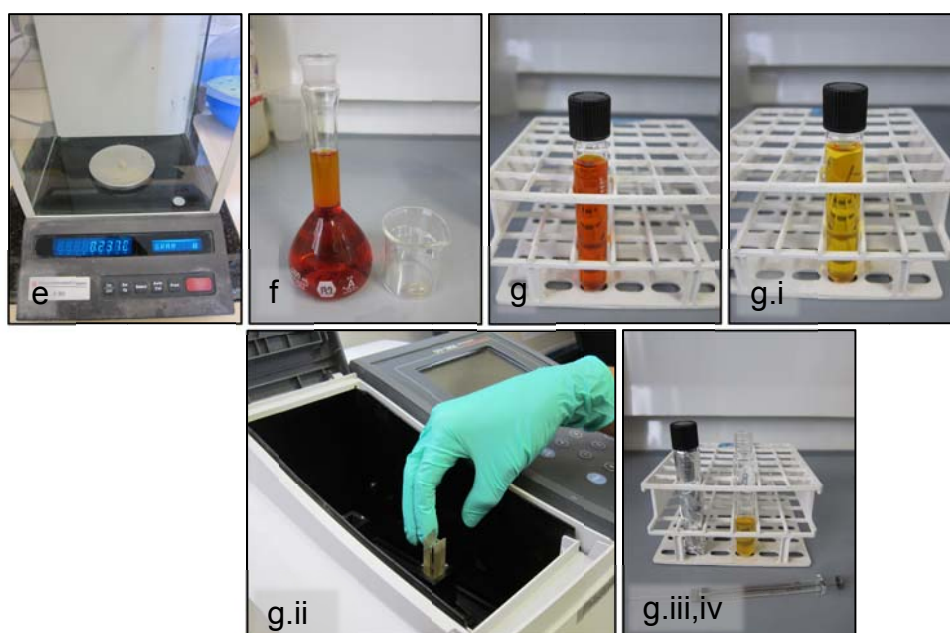
Note: Perform quadruplicate extractions for each sample

- Weigh out accurately four gelcaps into individual, tared 50 mL glass beakers and record the weight to the nearest 0.1 mg.
- Using small scissors carefully cut open the gelcap. Then fill the beaker with 20 mL acetone to avoid splashing of gelcap content. Note: Some gelcaps' content is partially solid or semi-solid and a sonication step may be necessary to facilitate the removal of all the content. If sonication is indicated, using small scissors carefully cut open the gelcap in a 20 mL screw cap vial. Add enough acetone to cover the gelcap. Close the vial tightly and place in the sonicator bath for 15 min.
- Rinse the scissors with acetone into the beaker.
- Transfer the rinsate quantitatively to a 100 mL volumetric flask using a Pasteur pipette. Rinse the beaker and gelcap with acetone until the rinsate is colorless. Add acetone to a final volume of ca. 80 mL. Note: Some gelcaps' content is partially solid or semi-solid and a sonication step may be necessary to facilitate complete dispersion/dissolution in the acetone solution. If sonication is indicated, place the stoppered volumetric flask in the sonicator bath and sonicate for 20 minutes or until the gelcap content is completely dispersed.



- Set the beaker with the empty gelcap aside to dry for at least 30 minutes. Accurately weigh the dried, empty gelcap and record the weight.
- After all the supernatant is collected in the volumetric flask and the gelcap content is completely dispersed, let the solution equilibrate to room temperature (20° C), then bring the flask to volume with acetone and mix well.
- Transfer an aliquot (8-10 mL) into a clean, dry test tube and centrifuge for 3 minutes at 3800-4200 rpm to remove any particulate matter which may have carried over in the transfers. This is the first dilution.

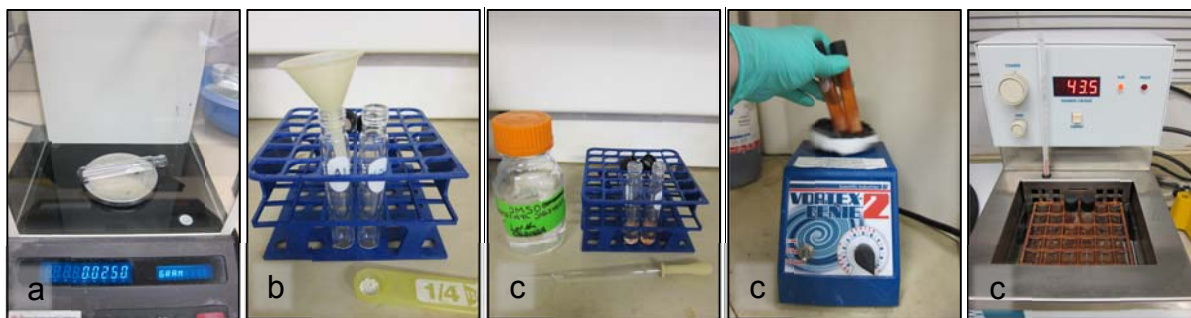
- i. Pipette 1 mL of extract into a clean, dry 10 mL volumetric flask and bring to volume with acetone to prepare a 1/10 dilution. Stopper the flask and mix well. This is the second dilution.
- ii. Optional: Read and record the absorbency at 474 nm of the second dilution against an acetone blank on the Spectrophotometer. (Absorbency readings are linear between 0.50 and 1.50).
- iii. Pipette accurately 3 mL of the second dilution into a separate clean, dry test tube.
- iv. To the test tube containing 3 mL of the second dilution add 50 μ L of internal standard (trans- β -apo-8'-carotenal) using the 50 μ L glass syringe. Make sure the syringe is rinsed after use with acetone.
- v. Proceed to **9.0 Hydrolysis of Carotenoids from *Haematococcus* Algae Products.**



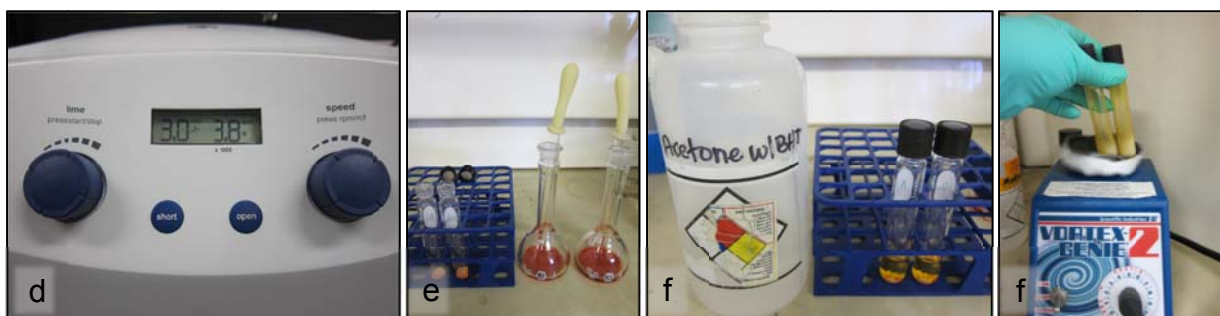
8.03 Extraction of Carotenoids from Astaxanthin Beadlets or Dried *Haematococcus* Biomass

Note: Perform duplicate extractions for each sample

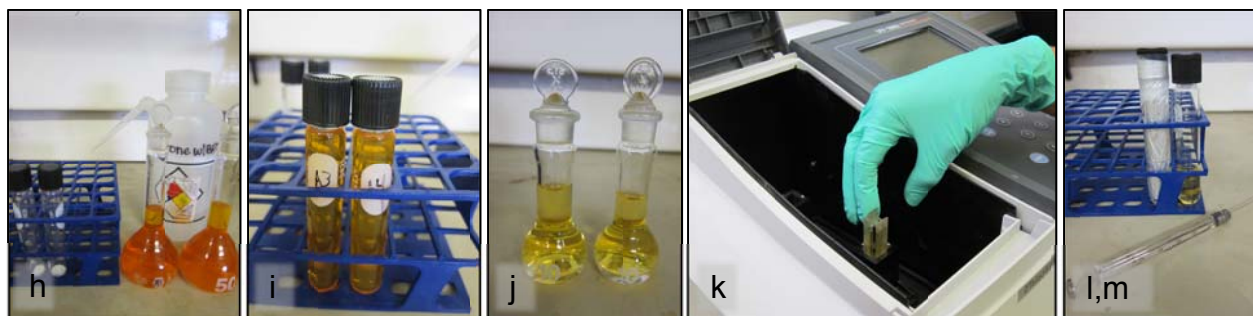
- a) Weigh approximately 25 mg of astaxanthin beadlets or dried *Haematococcus* biomass into a clean and dry 10 mL centrifuge tube. Record the weight to the nearest 0.1 mg. Observe the appearance of the sample and note any unusual physical characteristics such as clumps, burned or discolored material.
- b) Add 0.5 grams of glass beads to the tube.
- c) Add 2-3 mL of DMSO to the centrifuge tube, cap tightly and mix; then place the tube in the pre-heated water bath at 43-46°C for 15 minutes. Mix the sample a few times during this step.



- d) Remove the tube from the water bath and centrifuge at 3800-4200 rpm for 3 minutes to pellet the cell material.
- e) Transfer the supernatant to a 25 mL or 50 mL volumetric flask using a Pasteur pipette.
- f) Add 2-3 mL of acetone to the centrifuge tube, mix vigorously for 30 seconds. Centrifuge tube at 3800-4200 rpm for 3 minutes to pellet the solid material. Transfer supernatant to the volumetric flask.
- g) Repeat steps d-f until the supernatant is colorless, transferring the supernatant to the volumetric flask. Four to five extractions with acetone are usually enough.



- h) After all the supernatant is collected in the volumetric flask, let the solution equilibrate to room temperature (20° C), then bring the flask to volume with acetone and mix well.
- i) Transfer an aliquot (8-10 mL) into a clean, dry test tube and centrifuge for 3 minutes at 3800-4200 rpm to remove any particulate matter which may have carried over in the transfers. This is the first dilution.
- j) Prepare a 1/5 dilution by pipetting accurately 2 mL of extract into a clean and dry 10 mL volumetric flask. Bring the flask to volume with acetone, stopper and mix flask well. This is the second dilution.
 - a. Typically a 1/5 dilution is adequate. Occasionally dilutions of 1/2 (5 mL sample in 10 mL total volume) are required for less concentrated samples or dilutions of 1/10 (1 mL sample in 10 mL total volume) are required for more concentrated samples.
- k) Read and record the absorbency at 474 nm of the second dilution against an acetone blank on the Spectrophotometer. (Absorbency readings are linear between 0.50 and 1.50). Using the calculations below, verify that the duplicate samples are within 3% of each other before proceeding. If the duplicate samples are not within 3% of each other, repeat steps 8.0Cj through 8.0Ck. If still not within 3%, either prepare a third sample that should fall within 3% of one of the two initial samples, or redo duplicates.



- l) Once it is confirmed that the duplicates are within 3%, select ONE of the duplicates for the following: Pipette accurately 3 mL of the second dilution to a separate clean, dry test tube. Note: Make sure to note which duplicate is used at this time.
- m) To the test tube containing 3 mL of the second dilution add 50 μ L of internal standard (trans- β -apo-8'-carotenal) using the 50 μ L glass syringe. Make sure the syringe is rinsed after use with acetone.
- n) Proceed to **9.0 Hydrolysis of Carotenoid from *Haematococcus* Algae Products**.

8.1 Calculations

- Total Carotenoid Quantification

- Carotenoids (mg) extracted = $\frac{\text{Abs max} \times \text{volume of acetone} \times \text{dilution}}{210}$,
where 210 is the extinction coefficient of astaxanthin in acetone

- Approximate Astaxanthin Percentage

$$\text{Percent Astaxanthin} = \frac{\text{Carotenoids (mg) extracted} \times 85\%}{\text{sample wt (mg)}}$$

where 85% is the percentage of astaxanthin of the total carotenoid content

- Relative percent difference (RPD) of duplicates

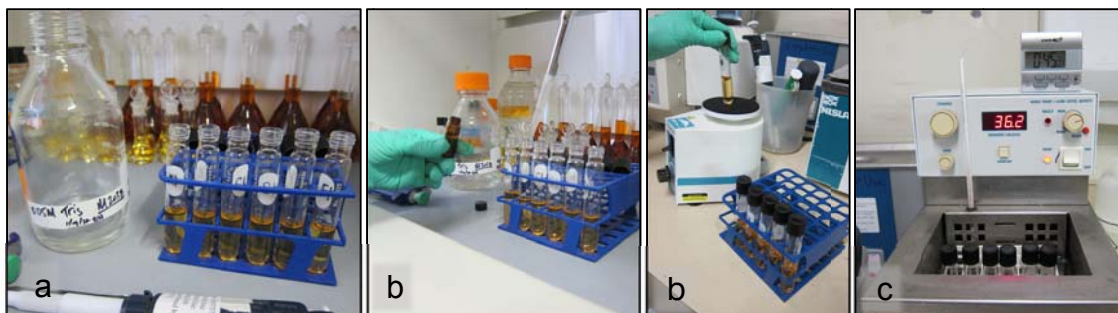
$$\text{RPD} = \frac{|R1 - R2|}{R} \times 100 \text{ ,}$$

where $|R1 - R2|$ = absolute difference between the duplicates

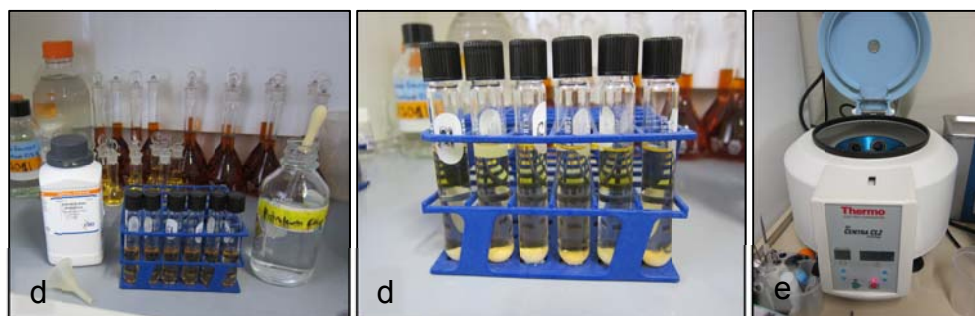
9.0 Hydrolysis of Carotenoids from *Haematococcus* Algae Products

Haematococcus algae products comprise astaxanthin that has been esterified with fatty acids. These fatty acids need to be removed completely, since the HPLC method employed for the analysis of astaxanthin only quantifies 'free' (non-esterified) astaxanthin. This reaction is catalyzed by the enzyme cholesterol esterase and is employed here.

- a) To the test tube containing the 3 mL of the second dilution with added internal standard (trans- β -apo-8'-carotenal), add 2 mL of 0.05M Tris buffer, pH7.0. Cap the test tube and mix well.
- b) To this test tube, add 600 μ L of cholesterol esterase stock solution (600 μ L of cholesterol esterase stock solution contains 2 units of enzyme). Cap the test tube and mix well. (BioAstin gelcap products should receive 900 μ L of cholesterol esterase stock solution (900 μ L of cholesterol esterase stock solution contains 3 units of enzyme). Note: Make sure the solution is completely thawed before dispensing.
- c) Place the test tube in the water bath set at 35-37° C for 45 minutes, mixing the solution frequently.



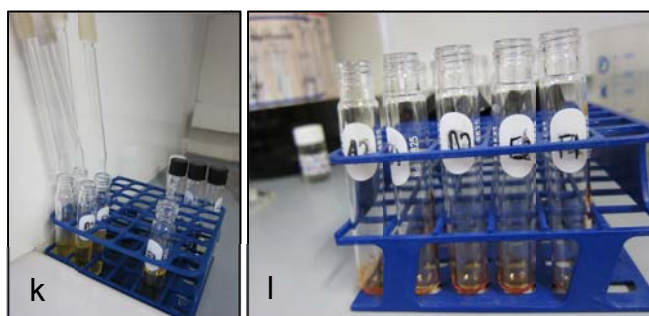
- d) Remove the test tube from the water bath and add 0.5 g of sodium sulfate and 2 mL petroleum ether. Cap the test tube and mix vigorously.
- e) Centrifuge the tube at 3500 to 4200 rpm for <30 seconds.



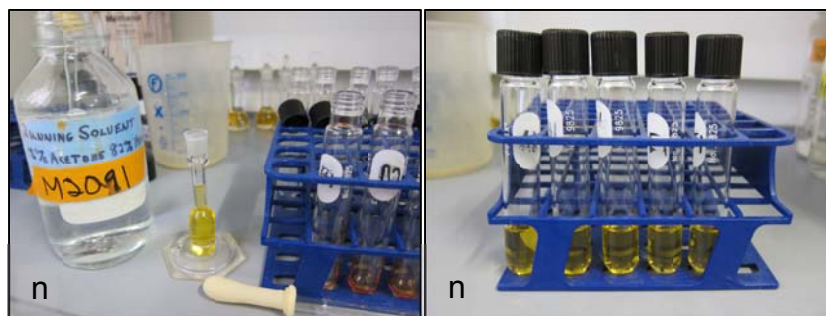
- f) Remove the test tube from the centrifuge and remove the upper, colored (petroleum ether) layer, transferring it to a separate clean, dry test tube. Make sure that no water is transferred in this step.
- g) Again add 2 mL petroleum ether to the test tube. Cap the test tube and mix vigorously.
- h) Centrifuge at 3500 to 4200 rpm for <30 seconds.
- i) Remove the test tube from the centrifuge and remove the upper, colored (petroleum ether) layer, transferring it to the test tube containing the first petroleum ether solution. Make sure that no water is transferred in this step.
- j) Repeat steps g) through i) until the upper (petroleum ether) layer is colorless. Three to four separate additions are usually necessary.



- k) Turn on the nitrogen gas flow to the nitrogen manifold. Place one of the Pasteur pipettes of the nitrogen manifold into the test tube containing the combined petroleum ether solutions. Make sure the pipette does not reach into the solution or splashing may occur. Adjust the nitrogen gas flow by opening or closing the control valve to facilitate the evaporation of the petroleum ether from the test tube.
- l) Once the petroleum ether is completely evaporated, remove the nitrogen manifold from the test tube and turn off the nitrogen gas flow.



- m) Inspect the test tube containing the dried petroleum ether residue for water that may have been carried over in the petroleum ether extraction.
- a. Note: It is important that NO water is present in the sample prior to the HPLC analysis. If water is present in the test tube, add <math><0.5\text{ g}</math> of anhydrous sodium sulfate and 1.5 mL petroleum ether to the test tube and mix gently. The anhydrous sodium sulfate will react with the water, forming 'clumps' of hydrated sodium sulfate and leaving the petroleum ether water free. Transfer the petroleum ether to a clean, dry test tube and rinse the sodium sulfate with petroleum ether until colorless, transferring and combining the petroleum ether solutions. Evaporate the petroleum ether solution as outlined in steps k - m)
- n) If no water is present, transfer the extract quantitatively into a 3 mL volumetric flask using HPLC Running Solvent (82%Hexane:18%Acetone). Bring the 3 mL flask to volume with 'running solvent' and mix well. Transfer the solution to a clean and dry test tube for HPLC analysis. The sample is now ready for HPLC analysis.



10.0 HPLC Analysis

Detector: UV/Vis detector at 474 nm and 458 nm

Column: Luna 3 μ Silica(2), 100Å 150 x 4.60 mm, Phenomenex (P/N 00F-4162-E0) with suitable guard column (Phenomenex P/N AJO 4348 & KJO 4282)

Column temperature: Ambient (20 - 25°C)

Flow rate: 1.2 mL/minute

Injection volume: 1.2 μ L

Mobile phase running solvent: 82% Hexane:18% Acetone, Isocratic

Retention times for Identification

Compound	Retention time (minutes)
Beta-Carotene	1.4
Trans- β -Apo-8'-Carotenal (internal Standard)	1.9
Canthaxanthin	2.9
Astacene	4.0
Semi-Astacene	4.4
Di-Cis Astaxanthin #1	5.2
Di-Cis Astaxanthin #2	5.4
Trans Astaxanthin	5.6
9-Cis Astaxanthin	6.3
13-Cis Astaxanthin	6.6
15-Cis Astaxanthin	7.1
Lutein	8.6

(See Appendix A for representative HPLC chromatogram)

With HPLC system running and stable under conditions listed above:

1. First run triplicate diluted trans- β -apo-8'-carotenal standards prepared under section **6.0 e iii** with detection at 458 nm. Review chromatograms and record peak area for trans- β -apo-8'-carotenal at a retention time of 1.9 minutes. Peak area of replicates should be within 3% of each other. Calculate average peak area. This is P_{IS} . (Peak area internal standard)
2. Run triplicate trans-astaxanthin standards prepared under section **7.0-f** with detection at 474 nm. Review chromatograms and record peak area for trans-astaxanthin at a retention time of 5.6 minutes. Peak area of replicates should be within 3% of each other. Calculate average peak area. This is P_{STA} . (Peak area trans-astaxanthin standard)

- a. Calculate Spectrophotometric Purity as shown in section 7.1 b and make any required adjustment to the Concentration of Standard as shown in section 7.1 c. This is C_{STA} . (Concentration standard trans-astaxanthin)
3. Run hydrolyzed astaxanthin sample prepared under section 9.0 n with detection at 458nm and 474 nm.
 - a. Record peak area of trans- β -apo-8'-carotenal peak determined at 458 nm. This is P_{ISS} . (Peak area internal standard sample)
 1. Percent recovery of the internal standard, PR_{IS} , is used as a measure to insure carotenoids are recovered during **9.0 Hydrolysis of Carotenoids from *Haematococcus* Algae Products**. PR_{IS} , in the astaxanthin sample is calculated as:

$$PR_{IS} = (P_{ISS} \times 100) \div P_{IS} \quad \text{Eq (1)}$$

- b. PR_{IS} must be 97 ± 5 . If PR_{IS} is outside this limit, repeat procedure **9.0 Hydrolysis of Carotenoids from *Haematococcus* Algae Products**.
4. Run hydrolyzed astaxanthin sample prepared under section 9.0 n with detection at 474 nm. Record the peak areas of di-cis astaxanthin #1, P_{DCA1} , di-cis astaxanthin #2, P_{DCA2} , trans astaxanthin, P_{TA} , 9-cis astaxanthin, P_{9CA} , 13-cis astaxanthin, P_{13CA} , and 15-cis astaxanthin, P_{15CA} .

- a. Calculate the peak area ratios of astaxanthin, PAR_{AX} as:

$$PAR_{AX} = (P_{DCA1} + P_{DCA2} + P_{TA} + 1.133 \times P_{9CA} + 1.60 \times P_{13CA} + P_{15CA}) \div P_{STA} \quad \text{Eq (2)}$$

Where:

1.133 is the response factor for 9-cis astaxanthin

1.60 is the response factor for 13-cis astaxanthin

P_{STA} is the peak area of the trans astaxanthin standard from 2. above.

- b. Calculate the per cent astaxanthin in the sample, AXP , as:

$$AXP = (PAR_{AX} \times C_{STA} \times D \times 100) \div W \quad \text{Eq (3)}$$

Where:

C_{STA} is the trans astaxanthin concentration of the standard solution from 2.a above.

D is the total sample dilution in section 8. f above.

W is the weight of sample used in section 8. a above.

11.0 References

- Jacobs P.B., R.D. LeBoeuf, S.A. McCommas, and J. D. Tauber. 1982. The cleavage of carotenoid esters by cholesterol esterase. *Comp. Biochem. Physiol.* 72B: 157-160.
- Quackenbush, F.W., *Journal of Liquid Chromatography*, 10:643-653, (1987)
- *ORA Laboratory Procedure*, Food and Drug Administration. Document No.: ORA-LAB.5.9, Version 1.1, 10-01-03.

- Renstrom B. and S. Liaaen-Jensen. 1981. *Fatty Acid Composition of some Esterified Carotenols*. Comp. Biochem. Physiol. B., Comp. Biochem. 69: 625-627.
- Vecchi M., V. Muduna, and E. Glinz. 1987. *HPLC Separation and Determination of Astacene, Semiastacene, and other Keto-carotenoids*. J. High Res. Chrom. And Chrom. Commun. 10:348-351.
- Weber S., W. Hardi, and J. Schierle. *Determination of Stabilized Astaxanthin in Carophyll Pink Premixes*. Hoffmann-La Roche Ltd. publication, CH-Basle.

12.0 Example

An analysis of BioAstin SCE5, 5% natural astaxanthin oleoresin extracted from Haematococcus, was conducted following the procedures outlined above. The details of the analysis are:

- The average absorbance of three samples of trans astaxanthin standard as prepared in procedure **7.0 f** read at 474 nm was 0.757
- The weight of oleoresin used in procedure **8.0** was **W** = 33.8 mg
- The first dilution in procedure **8.0 e** was 100 and the second dilution in procedure **8.0 f** was 10. This results in a total dilution of **D** = 1000.

The standard solutions and sample solutions were run on an HPLC as describe in procedure **10.0 HPLC Analysis** and produced the chromatograms presented in **Appendix A**.

From chromatogram 1, the peak area internal standard is:

$$P_{IS} = 113,954$$

From chromatogram 2, the peak area internal standard sample is:

$$P_{ISS} = 112,751$$

Percent recovery of the internal standard, **PR_{IS}**, is calculated from **Eq (1)** in section **10 3.a**.

$$PR_{IS} = (112,751 \times 100) \div 113,954 = 98.9$$

Which meet the criteria that **PR_{IS}** = 97 ± 5

From chromatogram 3, the peak area for the trans-astaxanthin standard is:

$$P_{STA} = 772,789$$

One other peak was detected with an area of 272. The purity is then:

$$772,789 \div (772,789 + 272) = 1.000 \text{ (essentially 100\% pure)}$$

close to 100% purity. The average absorbance of three samples of trans astaxanthin standard as prepared in procedure **7.0 f** read at 474 nm was 0.757. The concentration of the trans astaxanthin standard is calculated from **7.1 c**:

$$C_{STA} = 0.757 \div (217 \times 1.0) = 0.00349 \text{ mg/mL} = 3.49 \text{ }\mu\text{g/l}$$

Where:

1.0 is purity of 100%.

From chromatogram 4 the peak area of the various isomers of astaxanthin in the sample solution are:

$$P_{DCA1} = 4,507$$

$$P_{DCA2} = 4,691$$

$$P_{TA} = 300,667$$

$$P_{9CA} = 47,002$$

$$P_{13CA} = 24,080$$

$$P_{15CA} = 3,904$$

The peak area ratios of astaxanthin, PAR_{AX} , is calculated from **Eq (2)** in section **10 4.a**.

$$PAR_{AX} = (4,507 + 4,691 + 300,667 + 1.133 \times 47,002 + 1.6 \times 24,080 + 3,904) \div 772,789$$

$$PAR_{AX} = 0.5289$$

The per cent astaxanthin in the sample is calculated from **Eq (3)** in section **10 4.b**.

$$AXP = 0.5289 \times 0.00349 \times 1,000 \times 100 \div 33.8$$

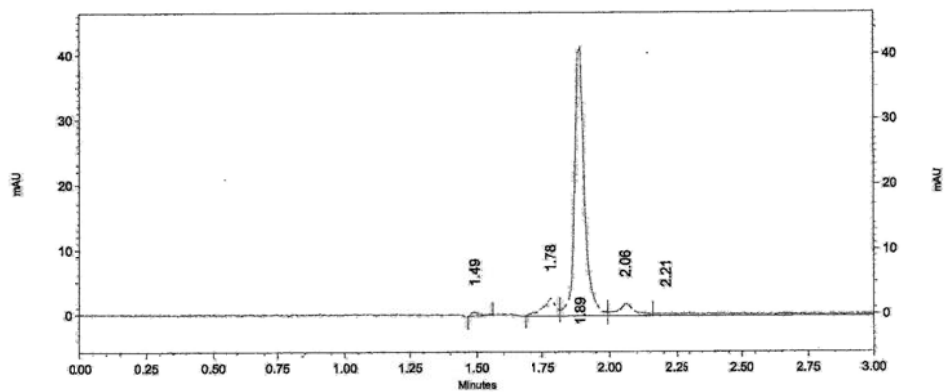
$$AXP = 5.46 \% \text{ (w/w)}$$

Appendix 1 HPLC Chromatograms

HPLC Analysis Report

Sample: Apo internal Standard 458nm Detector
Sample ID:
File Name: C:\32Karat\Projects\Default\Data\2012\Nov\121130\M1999-apo1
Method Name: C:\32Karat\Projects\Default\Methods\Axmethods\Current Methods\Apoverity-2.met
Acquired: 11/30/2012 1:14:17 PM

Det 168-458nm Results	
Name	Area
apo-carotenal (spike)	113954



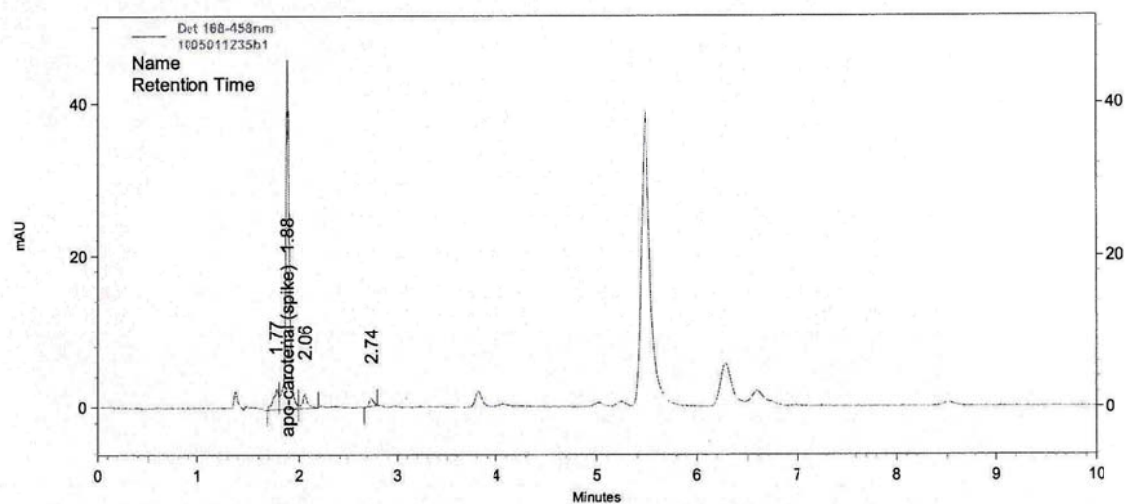
ls

HPLC Analysis Report

Sample: 1005011235 5% SCE 458 Detector APO Method
Sample ID: 1005011235b1
Method Name: C:\32Karat\Projects\Default\Methods\Axmethods\Current Methods\Apomethod-2.met
Acquired: 11/30/2012 1:39:27 PM

Det 168-458nm Results

Name	Retention Time	Area
apo-carotenal (spike)	1.88	112571

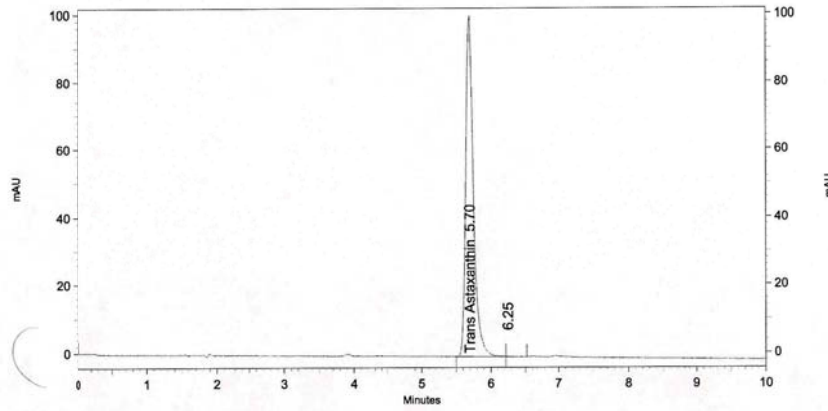


internal
der

HPLC Analysis Report

File Name: Ax Daily Std 474 Detector Concentration: 3.49ug/ml
File Path: C:\32Karat\Projects\Default\Data\2013\Jan\130107\M2073
Method Name: C:\32Karat\Projects\Default\Methods\Axmethods\Current Methods\AxmethodQC-2-130104.met
Acquired: 1/7/2013 1:29:02 PM

Det 168-474nm Results			
Name	Retention Time	Area	
Trans Astaxanthin	5.70	772789	
	6.25	272	
Totals		773061	



as n

HPLC Analysis Report

Sample: 1005011235 SCE5 474 Detector
Sample ID:
File Name: C:\32Karat\Projects\Default\Data\2012\Nov\121130\1005011235
Method Name: C:\32Karat\Projects\Default\Methods\Axmethods\Current
 Methods\AxmethodHPLC-2-13(104).met
Acquired: 11/30/2012 1:39:27 PM

Det 168-474nm Results		
Name	Retention Time	Area
Beta Carotene	1.38	4656
apo-carotenal (spike)	1.88	107694
Canthaxanthin	2.74	6996
Astacene	3.81	12176
Semi Astacene	4.10	6895
Di-Cis Astaxanthin#1	5.03	4507
Di-Cis Astaxanthin#2	5.22	4691
Trans Astaxanthin	5.48	300667
9-Cis Astaxanthin	6.28	47002
13-Cis Astaxanthin	6.60	24080
15-Cis Astaxanthin	7.01	3904
Lutein	8.51	8452

