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| CYANOTECH | TITLE: HPLC Analysis of Carotenoids from <i>Haematococcus</i> Algae Products | IMPLEMENTATION DATE: 04/04/2008 |
|           | FORM# AL-01  | REV# 3                          |
|           |  | EFFECTIVE DATE: 04/04/2008      |
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1.0 PURPOSE

The carotenoid fraction of *Haematococcus* algae contains about 70% monoesters of astaxanthin, 10% diesters of astaxanthin, 5% free astaxanthin, with the remainder consisting of  $\beta$ -carotene, canthaxanthin, and lutein. Accurate quantification of esterified astaxanthin 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.

2.0 POLICY

The Quality Unit conducts lab operations and analyses according to the FDA's Good Manufacturing Practices for Dietary Supplements.

3.0 SCOPE

QC Department and all Technicians who operate the HPLC system. This analysis is to be used for samples from production and samples from finished goods for customers. It is for NatuRose<sup>®</sup> and powders destined for further processing, as well as for beadlets and astaxanthin oleoresins, including gelcaps.

4.0 RESPONSIBILITY

QC Department. It is the responsibility of all QC personnel to conduct HPLC analyses according to this procedure. It is the responsibility of the QC Lab Manager to ensure that this procedure is followed.

5.0 REFERENCES ; AI-02

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.

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.

6.0 EQUIPMENT/MATERIALS

HPLC computer, Beckman HPLC system, also see procedure section for details per product type.

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7.0 PROCEDURE:

**7.1A Extraction of carotenoids from *Haematococcus* algae powder, flake, or beadlets**

*\*Note: for flake and powders note Ax Beads/white specs, A= 0-5, B=6-10, C=11+, when viewed in large petri dish.*

**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

**Extraction Procedure (Perform duplicates for each sample).**

- 1) Weigh approximately 25 mg of dried *Haematococcus* algae powder, flake, or beadlets 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 3 minutes at 4200 rpm to remove any particulate matter which may have carried over in the transfers.
- 10) Dilute the sample with acetone. Typically a 1in5 dilution is adequate. Occasionally dilutions of 1in4 are required for weak samples or dilutions of 1in7 are required to strong samples.
- 11) Read and record the maximum absorbency (approx. 471-477 nm) against an acetone blank on the spectrophotometer. (Absorbency readings are linear between 0.25 and 1.50). Using the following calculations, verify that the duplicate samples are within 3% of each other before proceeding.
- 12) Proceed to Part 2 for hydrolysis of the carotenoid esters.

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### 7.1B Extraction of carotenoids from Astaxanthin Oleoresin

#### 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)  
 Spectrophotometer (scanning)  
 100 ml volumetric flask with lid  
 10 ml centrifuge tubes with lid  
 Vortexer  
 Pipettes

#### Extraction Procedure *(Perform duplicates for each sample).*

- 1) Weigh approximately 25 mg of astaxanthin oleoresin into the 10 ml centrifuge tube. Record the weight
- 2) Pipette acetone into the tube to wash the resin off the sides and suspend the oleoresin in acetone.
- 3) Transfer into the 100ml volumetric flask using a Pasteur pipette. Wash the tube with acetone until all oleoresin is removed and transfer to 100ml volumetric flask.
- 4) Bring the 100ml volumetric flask up to volume, cap and mix properly.
- 5) Accurately pipette 8ml of acetone into a clean test tube and add 2ml of the extract from the 100ml volumetric flask for a 1in5 dilution.**
- 6) Read and record the maximum absorbency (approx. 471-477 nm) against an acetone blank on the spectrophotometer. (Absorbency readings are linear between 0.25 and 1.50). Using the following calculations, verify that the duplicate samples are within 3% of each other before proceeding.
- 12) Proceed to Part 2 for hydrolysis of the carotenoid esters

### 7.1C Extraction of carotenoids from Astaxanthin Gelcaps

#### 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)  
 Spectrophotometer (scanning)  
 100 ml volumetric flask with lid  
 10 ml centrifuge tubes with lid  
 Vortexer  
 Pipettes  
 50ml glass beakers  
 small pair of scissors

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**Extraction Procedure** *(Perform quadruplicates for each sample).*

- 1) Weigh out accurately one gelcap into a tared 50ml glass beaker.
- 2) Using the small scissors, carefully cut open the gelcap. Then fill the beaker with 20ml acetone to avoid splashing of gelcap content.
- 3) Rinse the scissors with acetone into the beaker. Transfer the rinsate quantitatively to a 100ml volumetric flask using a Pasteur pipette. Rinse the beaker and gelcap with acetone until the rinsate is colorless. Set the beaker with empty gelcap aside to dry.
- 4) Accurately weigh the dried, empty gelcap.
- 5) Bring 100ml volumetric flask to volume with acetone, cap and mix.
- 6) Pipette 1ml of extract into a clean glass tube and add 9ml of acetone to prepare a 1in10 dilution.
- 7) Read and record the maximum absorbency (approx. 471-477 nm) against an acetone blank on the spectrophotometer. (Absorbency readings are linear between 0.25 and 1.50).
- 8) Proceed to Part 2 for hydrolysis of the carotenoid esters

**Calculations:**

**Total carotenoid quantification**

$$\text{Carotenoids (mg) extracted} = \frac{\text{Abs max}}{210} \times \text{volume of acetone} \times \text{dilution}$$

**Approximate astaxanthin percentage:**

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

**Gelcaps only: Total content of gelcap (mg)**

$$\text{Gelcap weight (mg)} - \text{dry, empty gelcap weight} = \text{total content of gelcap (mg)}$$

**Gelcaps only: Mg Astaxanthin per 1g oil**

$$\text{Mg Astaxanthin per 1g oil} = \frac{\text{average astaxanthin concentration per gelcap (HPLC result)}}{\text{average weight of gelcap content (mg)}} \times 1000$$

**7.2 Hydrolysis of carotenoids from *Haematococcus* algae products**

1.1 **Scanning spectrophotometer:** Acetone extract of *Haematococcus* algae product—  
Part 1

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**Materials:**

Cholesterol esterase from *Pseudomonas fluorescens* (Sigma C-9281, 10,000 units per gram, or equivalent).  
 Petroleum ether  
 0.05 M Tris-HCl buffer pH 7.0 (store refrigerated)  
 Sodium sulfate decahydrate (Na<sub>2</sub>SO<sub>4</sub>-10H<sub>2</sub>O)  
 37 degree Celsius water bath  
 Pipettes  
 4 glass test tubes with caps  
 Nitrogen manifold  
 Fume hood  
 Vortex

**Cholesterol esterase preparation**

**2 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 Tris buffer. 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**

- 1) Transfer 3 ml of the extract to a test tube and add 2 ml of 0.05 M Tris HCl buffer.
- 2) Equilibrate the tube in 37 C degree water bath for 2 minutes.
- 3) Add 600 ul of enzyme stock solution to the test tube and cap. React in 37 C degree water bath for 45 minutes with gentle mixing (Note: 600ul of enzyme stock solution contains two units of enzyme)
- 4) Add 1.0 gram of sodium sulfate decahydrate and 4 ml of petroleum ether to tube and vortex for 30 seconds.
- 5) 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.
- 6) Remove the petroleum ether layer containing the carotenoid mixture with a pipette to a clean tube.
- 7) Add 3-4 ml of petroleum ether to tube. Vortex than centrifuge the tube.
- 8) Pipette the petroleum ether layer to the tube containing the carotenoid mixture.
- 9) Dry down under nitrogen gas.
- 10) Re-dissolve into 3 ml of mobile phase for HPLC analysis (82/18 hexane:acetone).
- 11) Relative percent difference of HPLC titer for duplicates should be within 5% to assure quality of test results.

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**Calculations:**

**Relative percent difference (RPD) of duplicates**

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

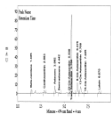
|R1 - R2| = absolute difference between the duplicate HPLC titers  
R = arithmetic mean of the two values

**7.3 HPLC analysis of carotenoids from *Haematococcus* algae meal.**

**Materials:**

Haematococcus algal product acetone extract prepared according Part 2.  
HPLC instrument capable of integration and detection at 470-476 and peripherals  
HPLC grade running solvent : 82/18 hexane: acetone, v/v (not containing BHT)  
Spectrophotometer

**Typical chromatogram of carotenoids from NatuRose or BioAstin powder**



2.0 ATTACHMENT(S)  
None

**3.0 SAFETY**

Proper safety precautions should be followed. Protective gear should include safety glasses and latex gloves. Extractions should be performed in a ventilation hood. In addition, 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.

Approved by \_\_\_\_\_<SIGNED>\_\_\_\_\_ Date\_\_4/4/08\_\_\_\_\_  
Quality Control/Assurance Department