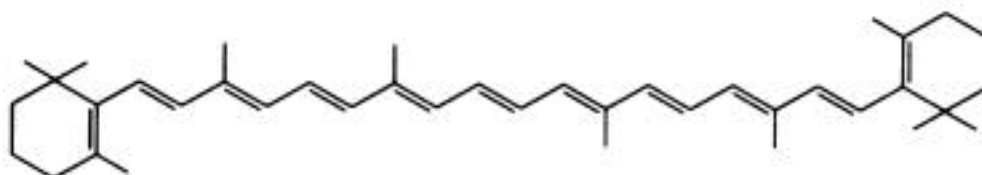
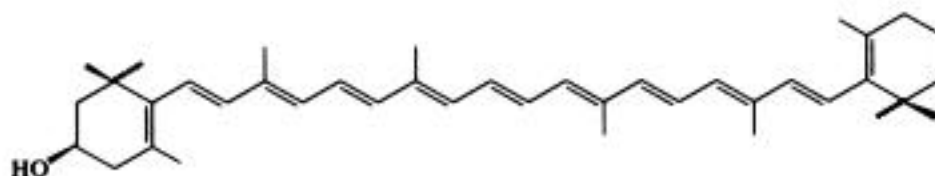


Analysis of Beta-Carotene and Total Carotenoids from Pacific Sea Plasma (Spectrophotometric Method)

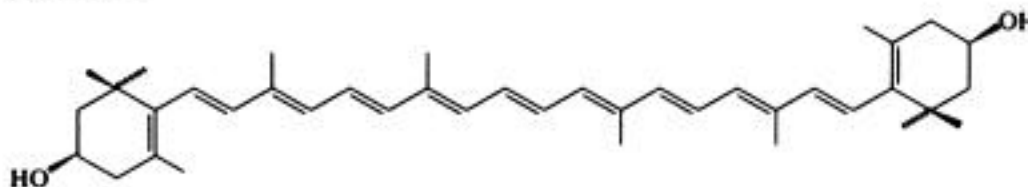
***β*-Carotene**



***β*-Cryptoxanthin**



Zeaxanthin



Background:

Spirulina has several carotenoids, the major components being β -carotene, zeaxanthin, echinenone, myxoxanthophyll and β -cryptoxanthin. Several isomers of beta-carotene also exist. Comparative studies have shown that the A.O.A.C. Official Methods of Analysis (1995); 941.15 does not fully extract or recover beta-carotene and other carotenoids from Spirulina.

This following is an improved spectrophotometer method to quantify beta-carotene and total carotenoids compared to the AOAC method for dried vegetables. It has been validated by HPLC to be accurate within 5%.

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 as low as possible.

Part 1: Extraction in Methanol

Materials

Metric balance
Hach tubes (12 ml) with caps
Glass beads
Water bath (50 degree C.)
Vortexer
Centrifuge
Volumetric flask (25 ml)
DMSO
Methanol (Reagent Grade)
Graduated pipettes
Pasteur pipettes

Procedure

Perform the methanol extraction separately for each sample.

- 1) Weigh approximately 30 mg of Spirulina powder directly into Hach Tubes. Record the weight.
- 2) Add 3 grams of glass beads and 2.5 ml DMSO to each tube.
- 3) Tightly cap the tubes and vortex them briefly for 30 seconds.
- 4) Place tubes into a 50 C degree water bath for 30 minutes. Every 10 minutes remove tubes from the water bath and vortex them for 30 seconds.
- 5) After 30 minutes in the water bath, remove the tubes.
- 6) Add 5 ml of methanol to each tub, cap the tubes and vortex them vigorously for 30 seconds. Centrifuge the tubes at 4200 rpm for 3 minutes.
- 7) With a Pasteur pipette, draw the supernatant from each tube and into volumetric flasks.
- 8) Add 4 ml of methanol, cap the tubes, vortex them for 15-30 seconds and centrifuge them again for three minutes. Collect the supernatant into the volumetric flasks.
- 9) Add just enough methanol to cover the glass beads. Cap the tubes and vortex them vigorously for 30 seconds. Remove the caps and add 4 ml of methanol. Recap the tubes and vortex them for 30 seconds.
- 10) Continue to add methanol, vortex, centrifuge and collect supernatant in the volumetric flask until the methanol is absolutely clear. It is important to note that any color left in the methanol is beta-carotene so it is extremely important to extract all the pigment from the pellet even if it means an extra extraction for verification.

- 11) After all the supernatant is collected in the volumetric flask, bring the volume up to 25 ml with methanol.
- 12) Place the stopper in the volumetric flask and invert gently to mix the contents. If the methanol extract is cloudy it may be necessary to clarify the extract by centrifugation before continuing.

Part 2: Beta-Carotene Analysis

Materials

Methanol extract obtained in Part 1

Heptane

Saturated potassium hydroxide in methanol

DI water

30 ml centrifuge tubes

15 ml conical centrifuge tube (with or without graduations) and caps

8 ml volumetric pipette

Pasteur pipettes

10 ml volumetric flasks

Vortexer

Centrifuge

Scanning spectrophotometer

Dry weight of Spirulina

Procedure

Perform each analysis in duplicate.

- 1) With the 8 ml volumetric pipette remove 8 ml of the methanol extract from the 25 ml volumetric flask and put it into a clean 15 ml centrifuge tube.
- 2) Add 5 ml of heptane and 1.5 ml of saturated KOH in methanol. Cap the tube without mixing.
- 3) Place the tubes in a dark place to saponify. After 15 minutes, lightly vortex the tubes to mix the contents.
- 4) After 30 minutes, vortex the tubes vigorously for 15 seconds and centrifuge them for 3 minutes at 4200 RPM.
- 5) With the Pasteur pipette remove the heptane layer and put it into a 10 ml volumetric flask. Gently add approximately 1 ml of fresh heptane to the tube and wash the interphase. Pipette the heptane into the 10 ml volumetric flask.
- 6) Add 3 ml of fresh heptane to the tube. Cap the tube and invert it 8 times to allow any remaining beta-carotene in the methanol to enter the heptane. Allow the heptane to separate from the methanol for about 2 minutes and then pipette the heptane into the 10 ml volumetric flask.
- 7) Bring the 10 ml volumetric flask up to volume with heptane. Cap the volumetric and invert to mix. (Optional: If an HPLC analysis of beta-carotene is required, remove 3 ml

of heptane extract from the volumetric flask. Evaporate it under nitrogen and re-suspend it in running solvent.)

8) Pour approximately 5 ml of the heptane extract into a clean Hach tube, add an equal amount of DI water and vortex vigorously for 5 seconds. Centrifuge the tubes for 3 minutes at 4200 RPM.

9) On the spectrophotometer, read the Absorbance at 436 nm of the extract against a heptane blank.

Calculation

Beta-Carotene (percent) =

$$\frac{\text{Abs 436}}{196 \times (\text{wt. (mg)} \times \text{dry wt.})} \times 25 \text{ ml} \times 1.25 \times 100 \times 0.84$$

An adjustment factor of 0.84 is used because of the presence of other carotenoids such as echinenone and beta-cryptoxanthin in the heptane extract. This factor has been verified by parallel HPLC analysis of Spirulina and has been found to be consistent for fresh powder samples. This factor should be evaluated periodically with HPLC analysis. Samples of old powders or tablets should have 3 ml aliquots of the heptane extract prepared (as in step 7) be analyzed by HPLC.

Part 3: Total Carotenoids Analysis

Materials

Methanol Extract obtained in Part 1

Methanol (Reagent Grade)

Diethyl ether (Reagent Grade)

Saturated potassium hydroxide in water

DI water

15 ml graduated centrifuge tubes

Vortexer

Centrifuge

Pasteur pipettes

2 ml calibrated pipette

Scanning spectrophotometer

Dry weight of Spirulina

Procedure

Perform each analysis in duplicate.

1) With the 2 ml volumetric pipette remove 2 ml of extract from the 25 ml volumetric flask and put it into a clean 15 ml graduated centrifuge tube.

- 2) Add 4 ml of diethyl ether to the tube.
- 3) Add 0.5 ml saturated KOH in water to the tube.
- 4) Cap the tube and vortex lightly to mix.
- 5) Place tube in a dark place for 30 minutes. Vortex tube lightly every 10 minutes.
- 6) Remove the cap from the tube and add 5 ml of water.
- 7) Cap the tube and vortex briefly to mix.
- 8) Centrifuge the tube at 4200 rpm for 3 minutes. The ether layer should contain all the yellow pigments and the aqueous layer should be a pale blue-green.
- 9) Note the volume of the ether in each tube recording both the graduation mark of the lower and upper most meniscus.
- 10) With the spectrophotometer, read the maximum absorbance of the ether extract at an absorbance of 450-453 nm against an ether blank.

Calculation:

Total Carotenoid (percent)=

$$\frac{\text{Max abs (450-453)}}{259.2 \times (\text{sample wt. (mg)} \times \text{dry wt.})} \times 25 \text{ ml} \times \frac{\text{vol. of ether (ml)}}{2} \times 100$$