STANDARD OPERATING PROCEDURE	**Analytical Test Method**
"Determination of Fucoxanthin in fucopure (Wakame Extract)"	

SOP No.	REVISION No.	EFECTIVE DATE	PAGE No.
AHPL/QC/MOA/UP01	03	**1/04/2011**	**01 OF 06**
REVIEW PERIOD	PREPARED BY	CHECKED BY	APPROVED BY
Every 2 yrs			

1. OBJECTIVE

To determine the levels of Fucoxanthin and its isomers , carotenoids in fucopure (Wakame extract) standardized extracts by HPLC.

2. SCOPE

This method describes a broad scale HPLC-UV procedure suitable for the analysis and quantifications of 13-cis-Fucoxanthin, all-trans Fucoxanthin, 9-cis-Fucoxanthin, Fucoxanthinol and other carotenoids in Undaria Pinnatifida Extract.

3. STANDARDS

1. Fucoxanthin ASB0000 6292 By Chromadex.
2. Undaria carotenoids :
(Mixture of 9-cis-Fucoxanthin, all-trans-Fucoxanthin, 3-cis- Fucoxanthin & Fucoxanthinol).

4. REAGENTS

4.1 Milli-Q Water
4.2 Acetonitrile-HPLC Grade
4.3 Ethanol-HPLC Grade
4.4 Chloroform-HPLC Grade
4.5 Aceton HPLC Grade

5. EQUIPMENT

5.1 HPLC equipped with UV-Vis detector
5.2 Intersil ODS 2, C18, 5µ, 250mmX 4.6 mm ID(Make- GL Science)
5.3 1 L Mobile phase containers
5.4 Analytical Balance
5.5 1000-, 500- and 250- mL graduated cylinders
5.6 10- and 50- mL low-actinic glass (LAG) volumetric flasks
5.7 100- mL volumetric flasks
5.8 2- and 5- mL volumetric pipets
5.9 Ultrasonication Bath
5.10 0.45 µm PTFE syringe filter

6. PREPARATION OF SOLUTIONS

6.1 Mobile Phase

6.1.1 Mobile Phase (1*)

To 400 mL of acetonitrile, add 80 mL methanol and 20 mL chloroform. Mix thoroughly and degas. Allow to equilibrate to room temperature.

6.2 Diluents

Ethanol (HPLC)/ Methanol (HPLC)

6.3 Stock Standard Solutions

(1)Dissolve Undaria carotenoids in such a way that the final concentration will be 0.1 mg/ml. Protect this solution from direct light.
(2) Adjust Fucoxanthin (solution By Chromadex) in such a way that final concentration will be 1mg/Lt.using diluant.
Mix 99 ml (1) and 1ml(2) to make stock standard solution.

6.4 Sample Solution

Accurately weigh about 50 mg of sample extract and transfer in to 25ml volumetric flask. Add about 20 ml of diluents and sonicate for approximately 5 minutes to disappear green color of sample also take precaution that temperature of solution will not rise more than room temprature. Dilute to 25ml by diluents. Protect whole procedure from light. Filter an aliquot through 0.45 micron membrane filter. Dilute by further diluents so that final concentration is 1.0 mg/ml. Protect all procedure from light.

7. CHROMATOGRAPHY

7.1 Instrument

A suitable HPLC system equipped with at least:

1) a UV-Vis detector capable of monitoring at 405 nm
2) a sample injection system
3) a pump capable of delivering constant flow up to 4000 psi, and
4) a computing data processor.

7.2 Column

Intersil ODS2, C18, 4.6 x 250 mm. 5 µm particle size, operated at ambient temperature.

7.3 Mobile Phase

ACN 100 + MeOH 20 : 5ml $CHCl_3$

7.4 Flow Rate

1.2mL/minute

7.5 Injection Volume

20 µl

7.6 Detection

405 nm

7.7 Injection Needle Wash Solvent

Diluent

7.8 Run Time

30-40 minutes

8. PROCEDURE

After equilibrating the HPLC system with the starting mobile phase conditions for at least 10 minutes, make duplicate injections of each of the Standard Solutions (stock standard solution and 2 linearity standard solutions). Determine the peak areas and retention times for each fucoxanthins (ie. Fucoxanthin, 9-Cis Fucoxanthin, all-trans Fucoxanthin ,Fucoxanthinol etc.) in chromatogram. Verify the system meets the suitability requirements in Section 9.

Inject, in duplicate, each Sample Solution and determine the peak area and retention time of all fucoxanthins in each resultant chromatogram.

9. System Suitability Requirement :

After every 20th sample injection, and at the end of each sequence, make duplicate injections of each of the Standard Solutions.

10. Calculation :

01. Indivisual Fucoxanthin isomers :

Compare Peak area of standard and Sample. Calculate % of fucoxanthin isomers Accordingly

02. Total Fucoxanthins content:

As Undaria pinnatifida contains natural forms of fucoxanthin and its isomers . All have various UV max (2*). Estimation of Total isomers calculated at 405nm in terms of fucoxanthin by below procedure.

" Weight accurately 50 mg FUCOPURE and sonicate in 50 ml Ethanol for 15 min. Filter and dilute 100 ml Dilute sample solution at 0.5mg/ml, Measure the absorbance of the solutions at 405 nm using Ethanol as blank with 1cm pathlength quartz cuvette "

$$\% \text{ Total Fucoxanthin} = \frac{A \times VXN \times 100}{4.01 \times M}$$

Where,
A= Absorbance
V= Volume
N = Dilution
M= Sample quantity in mg
4.01 = Average coefficient digest at 405nm

11. Reference:

1*

Thesis: MARINE HARPACTICOID COPEPOD CULTURE FOR THE PRODUCTION OF LONG CHAIN HIGHLY UNSATURATED FATTY ACIDS AND CAROTENOID PIGMENTS by ADELAIDE CUTTER EVEREST RHODES PAGE No 111

2*

Comparative evaluation of growth inhibitory effect of stereoisomers of fucoxanthin in human cancer cell lines **Y. Nakazawa[a], T. Sashima[b], M. Hosokawa[a] and K. Miyashita[a] , J of Functional Food Jan 2009, Vol 1.**