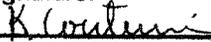
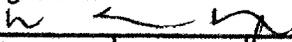
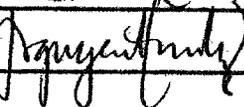
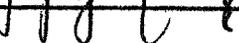


U.S. NUTRA		
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H. PLUVIALIS EXTRACT OFFICIAL ANALYTICAL PROTOCOL		
Document Number: USN-LSOP-10.02.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:		Page: 1

OFFICIAL ANALYTICAL PROTOCOL

H. pluvialis Algae Meal and Carbon Dioxide Extract

USN-LSOP-10.02.0

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Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:		Page: 2

TABLE OF CONTENT

1.	PURPOSE.	3
2.	SCOPE.	3
3.	RESPONSIBILITIES.	3
4.	TRAINING.	3
5.	DEFINITION.	3
6.	CHEMICALS.	3
7.	EQUIPMENT.	4
8.	ASTAXANTHIN ASSAY.	4
	8.1. Spectrophotometric Determination of Astaxanthin.	4
	8.2. Chromatographic Determination of Astaxanthin.	8
9.	OTHER CAROTENOIDS.	14
	9.1. β -Carotene assay.	14
	9.2. Lutein Assay.	17
	9.3. Canthaxanthin Assay.	20
10.	EXTRACTION OF DRIED AND CRACKED <i>H. pluvialis</i> .	23
11.	REFERENCES.	23

U.S. NUTRA		
Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:		Page: 3

1. PURPOSE.

The purpose of this Protocol is to measure the content of astaxanthin, β -carotene, canthaxanthin and lutein using High Performance Liquid Chromatography (HPLC) in the freshwater algae *H. pluvialis* or supercritical CO₂ extracts from the algae.

2. SCOPE.

This Protocol is to be used in the analysis of dried and cracked algae *H. pluvialis*, and carbon dioxide extracts thereof manufactured and/or sold by U.S. Nutra. The methods described below are not intended for other astaxanthin containing products, due to variance in matrices and the related potential interference of another constituents.

3. RESPONSIBILITIES.

It is the responsibility of the analytical manager, or an assigned alternate, to revise and/or update this Protocol when necessary.

4. TRAINING.

4.1. It is the responsibility of the analytical manager, or an assigned alternate, to act as trainer for this Protocol.

4.2. Analyst must read and understand this Protocol; then receive training by the analytical manager, or an assigned alternate.

5. DEFINITION.

Astaxanthin extract (astaxanthin-rich carotenoid oleoresin) is a complex mixture of natural products extracted from algae *H. pluvialis* using supercritical carbon dioxide. Unprocessed, dry algae biomass is extracted with acetone solely for the purpose of analysis as described in Section 10.

6. CHEMICALS.

Name of Chemical	CAS Registry #	Min. Grade and/or Purity
Acetone	67-64-1	HPLC, 99.9%
Ammonium Acetate	631-61-8	HPLC, 98%
Astaxanthin	472-61-7	98%
Butylated Hydroxytoluene	128-37-0	99%
Canthaxanthin	514-78-3	95%
β -Carotene	7235-70-7	99%
Chloroform	67-66-3	ACS, HPLC
Cholesterol Esterase, lyophilized powder	9026-00-0	Activity: >100 unit/mg solid
Hexanes	73513-42-5	HPLC, 99.9%
Lutein	127-40-2	95%
Methyl <i>tert</i> -Butyl Ether (MTBE)	1634-04-4	HPLC, 99%
Nitrogen compressed	7727-37-9	99.99%
Petroleum Ether	68467-50-6	ACS

U.S. NUTRA		
Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:		Page: 4

o-Phosphoric Acid	7664-38-2	HPLC, 85%
Sodium Hydroxide	1310-73-2	ACS, 97%
Name of Chemical	CAS Registry #	Min. Grade and/or Purity
Sodium Sulfate Anhydrous	7757-82-6	ACS, 99.0%
Sodium Sulfate Decahydrate	7727-73-3	USP, 99.0%
Tris-Hydroxymethyl Aminomethane·HCl	1185-53-1	Electrophoresis
Water	7732-18-5	HPLC

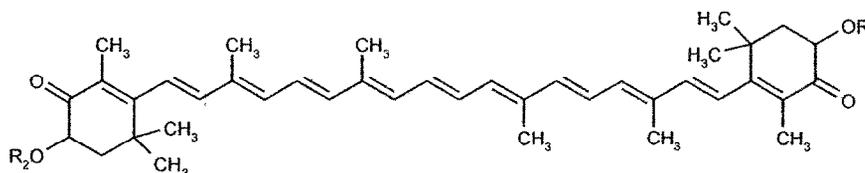
7. EQUIPMENT

Analytical balance with readability not less than 0.1 mg.
Vortex type mixer.
Centrifuge.
50 mL disposable centrifuge tubes.
Vacuum rotary evaporator with temperature controlled water bath.
Water or oil bath.
Spectrophotometer
Quartz cuvettes
Aspirator or vacuum pump.
Assorted volumetric flasks.
Assorted class A volumetric glass pipets.
Transfer pipets.
Test tube rack.
Assorted glassware.
Thermometer (1°C subdivision).
Rubber bulb – pipet filler.
High Performance Liquid Chromatograph (HPLC)
HPLC autosampler vials.
Separation funnel (500-mL).
0.45 µm filters.

8. ASTAXANTHIN ASSAY.

8.1. Spectrophotometric Determination of Astaxanthin.

8.1.1. Analyte.



Where for: Astaxanthin: $R_1=R_2=H$
Astaxanthin monoesters: $R_1=Acyl, R_2=H; R_1=H, R_2=Acyl$
Astaxanthin Diesters: $R_1=R_2=Acyl$

Note 1: Carotenoids are very sensitive to light, oxygen and heat. All analysis must be performed in a darkened

U.S. NUTRA		
Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:		Page: 5

room and using red (amber) glassware, while keeping the temperatures as low as possible.

Note 2: *To reduce degradation of carotenoids 0.2% (w/v) of Butylated Hydroxytoluene (BHT) must be dissolved in absolutely all solvents applied for this protocol.*

8.1.2. Test Definition: The following procedure permits the measurement of the total content of carotenoids, including content of free astaxanthin and its esters in the *H. pluvialis* extract. It is based on the measurement of the light absorption of the chromophore group of carotenoids (polyconjugated double bond sequence).

The method is rather fast and specific enough to run routine analysis of astaxanthin content, especially for the purpose of extraction process control.

8.1.3. Test Preparation.

8.1.3.1. Dissolve ca 20 mg of *H. pluvialis* extract in acetone and to 100 mL. Dilute 6.0 mL of this solution to 25.0 mL with acetone.

8.1.4. Standard Preparations.

8.1.4.1. Basic Standard Solution.

Mix ca 20 mg of astaxanthin with 60 mL of acetone in a 100-mL volumetric flask. Vortex well, then sonicate for 15 minutes keeping the bath temperature below 25°C. Dilute with acetone to volume and mix.

8.1.4.2. Standard Stock Solution.

Transfer 5.0 mL of basic solution into a 50-mL red volumetric flask, dilute to volume with acetone, and mix.

8.1.4.3. Standard Preparation #1.

8.1.4.3.1. Dilute 4.0 mL of standard stock solution to 25.0 mL with acetone.

8.1.4.3.2. Calculate the concentration of astaxanthin C_{S1} in $\mu\text{g/mL}$ for standard preparation #1 by the formula:

$$C_{S1} = \frac{WP}{625}$$

U.S. NUTRA		
Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:		Page: 6

Where: *W* is the weight in mg, of astaxanthin taken for Basic Standard Solution 8.1.4.1.;

P is the astaxanthin assay in % from the manufacturer's Certificate of Analysis.

8.1.4.4. Standard Preparation #2.

8.1.4.4.1. Dilute 6.0 mL of standard stock solution to 25.0 mL with acetone.

8.21.4.2. Calculate the concentration of astaxanthin C_{S2} in $\mu\text{g/mL}$ for standard preparation #2 by the formula:

$$C_{S2} = \frac{6WP}{2500}$$

Where: *W* is the weight in mg, of astaxanthin taken for Basic Standard Solution 8.1.4.1.;

P is the astaxanthin assay in % from the manufacturer's Certificate of Analysis.

8.1.4.5. Standard Preparation #3.

8.1.4.5.1. Dilute 8.0 mL of standard stock solution to 25.0 mL with acetone.

8.1.4.5.2. Calculate the concentration of astaxanthin C_{S3} in $\mu\text{g/mL}$ for standard preparation #3 by the formula:

$$C_{S3} = \frac{2WP}{625}$$

Where: *W* is the weight in mg, of astaxanthin taken for Basic Standard Solution 8.1.4.1.;

P is the astaxanthin assay in % from the manufacturer's Certificate of Analysis.

U.S. NUTRA		
Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:		Page: 7

8.1.4.6. Calibration Curve and System Suitability.

8.1.4.6.1. Turn on the spectrophotometer and setup wavelength to 478.0 nm. Allow to warm up for 1 hour before first measurement.

8.1.4.6.2. Fill up the quartz cuvette with the acetone used for standard preparations, insert in the spectrophotometer and zero the reading.

8.1.4.6.3. Wash and dry the cuvette, fill it with the Standard Preparation #1, insert in the spectrophotometer and record the reading as A_1 .

8.1.4.6.4. Repeat the operation described in **8.1.4.6.3.** for Standard Preparation #2 and record reading as A_2 .

8.1.4.6.5. Repeat the operation described in **8.1.4.6.3.** for Standard Preparation #3 and record reading as A_3 .

8.1.4.6.6. Calculate the slope m for linear regression (absorbance, A versus concentration, C)
 $C=mA+b$, by the formula:

$$m = \frac{3(A_1C_{S1} + A_2C_{S2} + A_3C_{S3}) - (A_1 + A_2 + A_3)(C_{S1} + C_{S2} + C_{S3})}{3(A_1^2 + A_2^2 + A_3^2) - (A_1 + A_2 + A_3)^2}$$

8.1.4.6.7. Calculate intercept b for linear regression (absorbance, A versus concentration, C)
 $C=mA+b$, by the formula:

$$b = \frac{(C_{S1} + C_{S2} + C_{S3}) - m(A_1 + A_2 + A_3)}{3}$$

8.1.4.6.8. Calculate the Coefficient of Correlation r by the formula:

$$r = \frac{3(A_1C_{S1} + A_2C_{S2} + A_3C_{S3}) - (A_1 + A_2 + A_3)(C_{S1} + C_{S2} + C_{S3})}{\sqrt{3[(A_1 + A_2 + A_3)^2 - (A_1^2 + A_2^2 + A_3^2)][3(C_{S1} + C_{S2} + C_{S3})^2 - (C_{S1}^2 + C_{S2}^2 + C_{S3}^2)]}}$$

8.1.4.6.9. In the suitable system r is not less than 0.998.

U.S. NUTRA		
Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:		Page: 8

8.1.4.7. Test Measurement.

8.1.4.7.1. Fill up the quartz cuvette with the acetone used for Test Preparation, insert in the spectrophotometer and zero the reading.

8.1.4.7.2. Wash and dry the cuvette, fill it with the Test Preparation, insert in the spectrophotometer and record the reading as A_T .

8.1.4.7.3. Calculate concentration C_T in $\mu\text{g/mL}$ of astaxanthin in the Test Preparation by the formula:

$$C_T = mA_T + b$$

Where: m is the slope from the calibration curve equation (8.1.4.6.6.);

b is the intercept from the calibration curve equation (8.1.4.6.7.).

8.1.4.7.4. Calculate astaxanthin **Assay** in % by the formula:

$$\% \text{Assay} = \frac{C_T \times 100\text{mL} \times 25\text{mL}}{W \times 6\text{mL}} \times 100\%$$

Where: W is the weight in μg of extract taken for Test Preparation.

8.2. Chromatographic Determination of Astaxanthin.

8.2.1. **Test Definition:** The following procedure permits the measurement of total content of *trans*-astaxanthin, as well as 9-*cis*- and 13-*cis*-astaxanthin in *H. pluvialis* extract or algae biomass. It is based on the enzymatic hydrolysis of astaxanthin esters followed by HPLC separation on normal phase silica column.

8.2.2. Mobile Phase.

Mix 140 mL of acetone and 860 mL of hexanes, and filter through 0.45 μm filter.

U.S. NUTRA		
Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:		Page: 9

8.2.3. Column Parameters.

Length: 150 mm.
Internal diameter: 4.6 mm.
Packing: Silica .
Particle size: 3 µm.
Packing closest USP equivalent: L3.

8.2.4. Column Conditioning.

- 8.2.4.1. Mix 10 mL of o-phosphoric acid with water, dilute to 1000 mL and filter through 0.45 µm filter.
- 8.2.4.2. Pump the solution (8.2.4.1.) through the column (8.2.3.) at 0.5 mL/min for 2 hours.
- 8.2.4.3. Run mobile phase (8.2.2.) at 0.5-1 mL/min for 6-8 hours.

Note: *Column needs to be conditioned only once prior to the first astaxanthin analysis.*

8.2.5. Typical Instrument setup.

Injection Volume: 10 µL.
Mobile phase flow: 1.0 mL/min (isocratic).
Runtime: 25 min.
Wavelength: 477 nm.
Temperature: ambient.

8.2.6. Test Preparation.

8.2.6.1. 0.5 N Sodium hydroxide solution.

- 8.2.6.1.1. Dissolve 2 g of sodium hydroxide in 50 mL of water, cool to room temperature and dilute with water to 100 mL.
- 8.2.6.1.2. Store at room temperature in a plastic container for one year.

8.2.6.2. 0.05 M Tris-HCl pH 7.0 buffer solution.

- 8.2.6.2.1. Dissolve 7.9 g of tris-hydroxymethyl aminomethane-HCl in 950 mL of water, then adding 0.5 N sodium hydroxide solution dropwise adjust to pH 7.0. Dilute with water to 1000 mL.
- 8.2.6.2.2. Store at room temperature for one year.

U.S. NUTRA		
Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:		Page: 10

8.2.6.3. Cholesterol esterase stock solution.

- 8.2.6.3.1.** Dissolve the content of vial of cholesterol esterase in 1mL of 0.05 M tris-HCl pH 7.0 buffer solution. Accurately transfer this solution using Pasteur pipet into a bigger container, washing the initial vial with 1 mL portions of same buffer 5 times. Perform necessary calculations and dissolve solution to have ca 8-10 unit/mL.
- 8.2.6.3.2.** Distribute prepared solution into the 1.5-2.0 mL vials, and store below -10°C for 6 months.

8.2.6.4. Hydrolysis and isolation.

- 8.2.6.4.1.** Accurately weigh ca 40 mg of extract, dissolve in acetone and dilute with acetone to 100.0 mL.
- 8.2.6.4.2.** Dilute 5.0 mL of this solution from **8.2.6.4.1.** with acetone to 50.0 mL.
- 8.2.6.4.3.** In a screw capped 20-mL tube mix 3.0 mL of this solution from **8.2.6.4.2.** with 2.0 mL of 0.05 M tris-HCl pH 7.0 buffer solution and the content of 1 vial of cholesterol esterase stock solution (**8.2.6.3.2.**).
- 8.2.6.4.4.** Cap the tube and heat for 90 minutes in the water bath at 37°C, vortexing the mixture every 15 minutes.
- 8.2.6.4.5.** Add to the tube 1 g of sodium sulfate decahydrate and 2 mL of petroleum ether. Shake and allow layers to separate, then transfer the upper (organic) layer into the other tube containing 0.5 g of anhydrous sodium sulfate. Repeat this operation until the organic layer is colorless.
- 8.2.6.4.6.** Vortex the second tube, allow to settle, then transfer the clear supernatant into the third tube. Add another 2 mL of petroleum ether in the second tube, vortex, allow to settle and transfer the solvent into the third tube. Repeat this operation until solvent is colorless. Evaporate petroleum ether with the steam of nitrogen and dissolve residue in 2.0 mL of mobile phase.

U.S. NUTRA		
Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:		Page: 11

8.2.7. Standard Preparations.

8.2.7.1. Weigh ca 20 mg of astaxanthin standard and transfer it to an amber 100-mL volumetric flask. Add 60 mL of acetone, mix well and sonicate at least 15 minutes at room temperature, then bring to volume with acetone.

8.2.7.2. Dilute 5.0 mL of basic standard solution (8.2.7.1.) to 50.0 mL with acetone.

8.2.7.3. Standard Preparation #1.

8.2.7.3.1. Dilute 1.0 mL of stock solution from 8.2.7.2. to 10.0 mL with acetone.

8.2.7.3.2. Calculate the concentration of astaxanthin C_{S1} in $\mu\text{g/mL}$ for standard preparation #1 by the formula:

$$C_{S1} = \frac{WP}{1000}$$

Where: *W* is the weight in mg, of astaxanthin taken in 8.2.7.1.;

P is the astaxanthin assay in % from the manufacturer's Certificate of Analysis.

8.2.7.4. Standard Preparation #2.

8.2.7.4.1. Dilute 2.0 mL of solution from 8.2.7.2. to 10.0 mL with acetone.

8.2.7.4.2. Calculate the concentration of astaxanthin C_{S2} in $\mu\text{g/mL}$ for standard preparation #2 by the formula:

$$C_{S2} = \frac{WP}{500}$$

Where: *W* is the weight in mg, of astaxanthin taken in 8.2.7.1.;

P is the astaxanthin assay in % from the manufacturer's Certificate of Analysis.

U.S. NUTRA		
Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:		Page: 12

8.2.7.5. Standard Preparation #3.

8.2.7.5.1. Dilute 3.0 mL of solution from 8.2.7.2. to 10.0 mL with acetone.

8.2.7.5.2. Calculate the concentration of astaxanthin C_{S3} in $\mu\text{g/mL}$ for standard preparation #3 by the formula:

$$C_{S3} = \frac{3WP}{1000}$$

Where: *W* is the weight in mg, of astaxanthin taken in 8.2.7.1.;

P is the astaxanthin assay in % from the manufacturer's Certificate of Analysis.

8.2.8. Test.

8.2.8.1. Inject each standard preparation three times, then integrate astaxanthin peak.

8.2.8.2. Calculate an average peak area \bar{A}_n for each standard preparation by the formula:

$$\bar{A}_n = \frac{A_1 + A_2 + A_3}{3}$$

Where: A_1 , A_2 and A_3 are the astaxanthin peak areas for separate injections of standard preparation #n,

8.2.8.3. Calculate the standard deviation S_n by the formula:

$$S_n = \sqrt{\frac{(A_1 - \bar{A}_n)^2 + (A_2 - \bar{A}_n)^2 + (A_3 - \bar{A}_n)^2}{2}}$$

8.2.8.4. Calculate relative standard deviation RSD_n in percents by the formula:

$$RSD_n = \frac{S_n}{A_n} \times 100\%$$

8.2.8.5. **RSD** for each standard preparation must be not more than 5.0%. Otherwise the system is considered not suitable.

U.S. NUTRA		
Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0	Document Level: 3	
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:	Page: 13	

- 8.2.8.6. Calculate the slope **m** for linear regression (peak area versus concentration) **C=ma+b** by the formula:

$$m = \frac{3(\bar{A}_1 C_{S1} + \bar{A}_2 C_{S2} + \bar{A}_3 C_{S3}) - (\bar{A}_1 + \bar{A}_2 + \bar{A}_3)(C_{S1} + C_{S2} + C_{S3})}{3(\bar{A}_1^2 + \bar{A}_2^2 + \bar{A}_3^2) - (\bar{A}_1 + \bar{A}_2 + \bar{A}_3)^2}$$

- 8.2.8.7. Calculate intercept **b** by the formula:

$$b = \frac{(C_{S1} + C_{S2} + C_{S3}) - m(\bar{A}_1 + \bar{A}_2 + \bar{A}_3)}{3}$$

- 8.2.8.8. Inject test preparation, identify and integrate peaks.

- 8.2.8.9. The major peak observed in the chromatogram corresponds to trans-astaxanthin.

- 8.2.8.10. Other peaks usually present in the chromatogram are:

Peak of	Approximate Relative Retention Time
Astacene	0.65
Di-trans-Astaxanthin	0.96
trans-Astaxanthin (E-astaxanthin)	1
9-cis-Astaxanthin (9Z-astaxanthin)	1.15
13-cis-Astaxanthin (13Z-astaxanthin)	1.25

- 8.2.8.11. Calculate the total area of astaxanthins **A_{TA}** by the formula:

$$A_{TA} = A_E + 1.3 \times A_{9Z} + 1.6 \times A_{13Z}$$

Where: *A_E* is the peak area of trans-astaxanthin;

A_{9Z} is the peak area of 9-cis-astaxanthin;

A_{13Z} is the peak area of 13-cis-astaxanthin;

1.3 and 1.6 are response coefficients for corresponding cis-isomers of astaxanthin.

- 8.2.8.12. Apply **A_{TA}** in the linear regression as **a** and calculate the total concentration in µg/mL of astaxanthins **C_{TA}**.

$$C_{TA} = mA_{TA} + b$$

Where: *m* is slope found in 8.2.8.6.,

U.S. NUTRA		
Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0	Document Level: 3	
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:	Page: 14	

b is intercept found in 8.2.8.7.

8.2.8.13. Calculate the assay in % for total astaxanthins in the sample taken for test preparation by the formula:

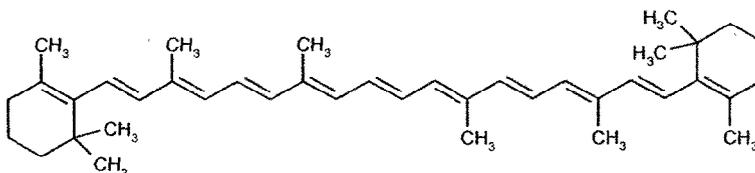
$$\% \text{Assay} = \frac{200C_{TA}}{3W}$$

Where: *W* is the weight in mg, of sample taken for analysis.

9. OTHER CAROTENOIDS.

9.1. β -Carotene assay.

9.1.1. Analyte.



9.1.2. Test Definition: The following procedure permits the measurement of content of β -carotene in *H. pluvialis* biomass or its CO₂ extract. It is based on HPLC separation on C30 reverse phase column.

9.1.3. Chromatographic Conditions.

9.1.3.1. Mobile Phase.

9.1.3.1.1. Dissolve 77.1 g of ammonium acetate in 500 mL of water and dilute with water to 1 L.

9.1.3.1.2. Mix 40 mL of the solution from 9.1.3.1.1. with 460 mL of methyl alcohol, add 500 mL of MTBE, then filter through 0.45 μ m filter.

9.1.3.1.3. Column Parameters.

Length: 250 mm.
Internal diameter: 4.6 mm.
Packing: C30/silica.
Particle size: 5 μ m.

U.S. NUTRA		
Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:		Page: 15

9.1.3.1.4. Typical Instrument Setup.

Injection Volume: 20 μ L.
 Mobile phase flow: 1.5 mL/min (isocratic).
 Runtime: 15 min.
 Wavelength: 455 nm.
 Temperature: ambient.

9.1.4. Test Preparation.

9.1.4.1. If testing β -carotene in *H. pluvialis* biomass, extract ca 250 mg of cracked *H. pluvialis* according to procedure, described in 10.

9.1.4.1.1. Evaporate the solvent using vacuum rotary evaporator to ca 30 mL, the dilute to 50.0 mL with acetone.

9.1.4.2. If testing β -carotene in *H. pluvialis* extract dissolve ca 100 mg of *H. pluvialis* extract in 30 mL of acetone and dilute to 50 mL with same solvent.

9.1.5. Standard Preparations.

9.1.5.1. Weigh ca 20 mg of β -carotene standard and transfer it to an amber 50-mL volumetric flask. Add 30 mL of chloroform mix well and sonicate at least 15 minutes at room temperature, then bring to volume with chloroform.

9.1.5.2. Dilute 5.0 mL of basic standard solution (9.1.5.1.) to 50.0 mL with acetone.

9.1.5.3. Standard Preparation #1.

9.1.5.3.1. Dilute 1.0 mL of stock solution from 9.1.5.2. to 10.0 mL with acetone.

9.1.5.3.2. Calculate the concentration of β -carotene C_{S1} in μ g/mL for standard preparation #1 by the formula:

$$C_{S1} = \frac{WP}{1000}$$

Where: *W* is the weight in mg, of β -carotene taken in 9.1.5.1.;

P is the β -carotene assay in % from the manufacturer's Certificate of Analysis.

U.S. NUTRA	
Laboratory Standard Operational Procedure	
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol	
Document Number: USN-LSOP-10.02.0	Document Level: 3
Revision No.: 0	Revision Date:
Effective Date:	Total pages: 24
	Page: 16

9.1.5.4. Standard Preparation #2.

9.1.5.4.1. Dilute 2.0 mL of solution from **9.1.5.2.** to 10.0 mL with acetone.

9.1.5.4.2. Calculate the concentration of β -carotene C_{S2} in $\mu\text{g/mL}$ for standard preparation #2 by the formula:

$$C_{S2} = \frac{WP}{500}$$

Where: *W* is the weight in mg, of β -carotene taken in **9.1.5.1.**;

P is the β -carotene assay in % from the manufacturer's Certificate of Analysis.

9.1.5.5. Standard Preparation #3.

9.1.5.5.1. Dilute 3.0 mL of solution from **9.1.5.2.** to 10.0 mL with acetone.

9.1.5.5.2. Calculate the concentration of β -carotene C_{S3} in $\mu\text{g/mL}$ for standard preparation #3 by the formula:

$$C_{S3} = \frac{3WP}{1000}$$

Where: *W* is the weight in mg, of β -carotene taken in **9.1.5.1.**;

P is the β -carotene assay in % from the manufacturer's Certificate of Analysis.

9.1.6. Test.

9.1.6.1. Inject each standard preparation three times, then integrate β -carotene peak.

9.1.6.2. Calculate an average peak area \bar{A}_n for each standard preparation by the formula:

$$\bar{A}_n = \frac{A_1 + A_2 + A_3}{3}$$

U.S. NUTRA		
Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:		Page: 17

Where: A_1 , A_2 and A_3 are the β -carotene peak areas for separate injections of standard preparation #n;

9.1.6.3. Calculate the standard deviation S_n by the formula:

$$S_n = \sqrt{\frac{(A_1 - \bar{A}_n)^2 + (A_2 - \bar{A}_n)^2 + (A_3 - \bar{A}_n)^2}{2}}$$

9.1.6.4. Calculate relative standard deviation RSD_n in percents by the formula:

$$RSD_n = \frac{S_n}{A_n} \times 100\%$$

9.1.6.5. RSD for each standard preparation must be not more than 5.0%. Otherwise the system is considered not suitable.

9.1.6.6. Calculate the slope m for linear regression (peak area versus concentration) $C = ma + b$ by the formula:

$$m = \frac{3(\bar{A}_1 C_{S1} + \bar{A}_2 C_{S2} + \bar{A}_3 C_{S3}) - (\bar{A}_1 + \bar{A}_2 + \bar{A}_3)(C_{S1} + C_{S2} + C_{S3})}{3(\bar{A}_1^2 + \bar{A}_2^2 + \bar{A}_3^2) - (\bar{A}_1 + \bar{A}_2 + \bar{A}_3)^2}$$

9.1.6.7. Calculate intercept b by the formula:

$$b = \frac{(C_{S1} + C_{S2} + C_{S3}) - m(\bar{A}_1 + \bar{A}_2 + \bar{A}_3)}{3}$$

9.1.6.8. Inject test preparation identify and integrate β -carotene peak, and express its area as A_U .

9.1.6.9. Apply A_U in the linear regression as a and calculate the total concentration in $\mu\text{g/mL}$ of β -carotene C_U .

$$C_U = mA_U + b$$

Where: m is slope found in 9.1.6.6.;

b is intercept found in 9.1.6.7.

9.1.6.10. Calculate the assay in % for β -carotene in the sample taken for test preparation by the formula:

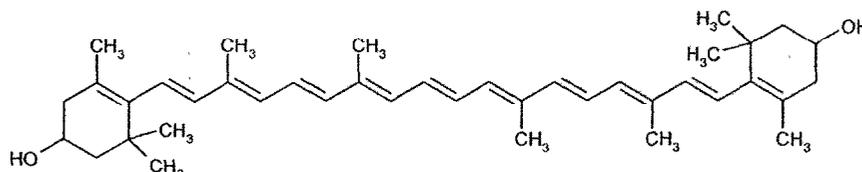
$$\% \text{ Assay} = \frac{5C_U}{W}$$

U.S. NUTRA	
Laboratory Standard Operational Procedure	
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol	
Document Number: USN-LSOP-10.02.0	Document Level: 3
Revision No.: 0	Revision Date:
Effective Date:	Total pages: 24
	Page: 18

Where: *W* is the weight in mg, of sample taken for analysis.

9.2. Lutein Assay.

9.2.1. Analyte.



9.2.2. Test Definition:

The following procedure permits the measurement of the content of lutein in *H. pluvialis* or its CO₂ extract. It is based on HPLC separation on C30 reverse phase column.

9.2.3. Chromatographic Conditions.

9.2.3.1. Mobile Phase.

9.2.3.1.1. Dissolve 77.1 g of ammonium acetate in 500 mL of water and dilute with water to 1 L.

9.2.3.1.2. Mobile Phase A (MP_A).

Mix 80 mL of the solution from 9.2.3.1.1. with 1800 mL of methyl alcohol, add 120 mL of MTBE, then filter through 0.45 μm filter.

9.2.3.1.3. Mobile Phase B (MP_B).

Mix 40 mL of the solution from 9.2.3.1.1. with 60 mL of methyl alcohol, add 900 mL of MTBE, then filter through 0.45 μm filter and transfer into the separation funnel. If water separates remove it carefully, then use the organic (upper) layer.

9.2.3.1.4. Column Parameters.

Length: 250 mm.
Internal diameter: 4.6 mm.
Packing: C30/silica.
Particle size: 5 μm.

U.S. NUTRA		
Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:		Page: 19

9.2.3.1.5. Typical Instrument Setup.

Gradient program:

1. 1.0 min.: MPhA – 100%.
2. 73.0 min.: MPhA – from 100% to 0%,
MPhB – from 0% to 100%.
with degree of curvature 3,
3. 10.0 min.: MPhB – 100%.

Mobile phase flow: 1.0 mL/min.

Runtime: 84 min.

Injection Volume: 20 µL.

Wavelength: 445 nm.

Temperature: ambient.

9.2.4. Test Preparation.

9.2.4.1. If testing lutein in *H. pluvialis*, extract ca 180 mg of cracked *H. pluvialis* according to procedure 10.

9.2.4.1.1. Evaporate the solvent using vacuum rotary evaporator to ca 30 mL, the dilute to 50.0 mL with acetone.

9.2.4.2. If testing lutein in *H. pluvialis* extract dissolve ca 70 mg of *H. pluvialis* extract in 30 mL of acetone and dilute to 50 mL with same solvent

9.2.5. Standard Preparation.

Use commercially available solution of lutein in ethyl alcohol with concentration ca 1.5 µg/mL (Example: ChromaDex, Product # 12450-A).

9.2.6. Test.

9.2.6.1. Consecutively inject each of standard preparation and test preparation.

9.2.6.2. Identify and integrate lutein peak (retention time is about 22 min.) in both chromatograms.

9.2.6.3. If necessary, make a new test preparation with the amount adjusted to have lutein peak area within ±10% margin of that in the standard preparation.

9.2.6.4. Perform 6 consecutive injections of standard preparation and integrate lutein peak.

9.2.6.5. Calculate an average lutein peak area \bar{A}_S in standard preparation by the formula:

$$\bar{A}_S = \frac{A_1 + A_2 + A_3 + A_4 + A_5 + A_6}{6}$$

U.S. NUTRA	
Laboratory Standard Operational Procedure	
H. <i>pluvialis</i> Algae Meal and Extract Official Analytical Protocol	
Document Number: USN-LSOP-10.02.0	Document Level: 3
Revision No.: 0	Revision Date:
Effective Date:	Total pages: 24
	Page: 20

Where: A_1, A_2, A_3, A_4, A_5 and A_6 are the peak areas of lutein for separate injections of standard preparation.

9.2.6.6. Calculate the standard deviation **S** by the formula:

$$S = \sqrt{\frac{(A_1 - \bar{A}_S)^2 + (A_2 - \bar{A}_S)^2 + (A_3 - \bar{A}_S)^2 + (A_4 - \bar{A}_S)^2 + (A_5 - \bar{A}_S)^2 + (A_6 - \bar{A}_S)^2}{5}}$$

9.2.6.7. Calculate relative standard deviation **RSD** in percents by the formula:

$$RSD = \frac{S}{A_S} \times 100\%$$

9.2.6.8. **RSD** for each standard preparation must be not more than 5.0%. Otherwise the system is considered as not suitable.

9.2.6.9. Inject test preparation, integrate lutein peak, and express its area as A_U .

9.2.6.10. Calculate the assay in % for lutein in the sample taken for test preparation by the formula:

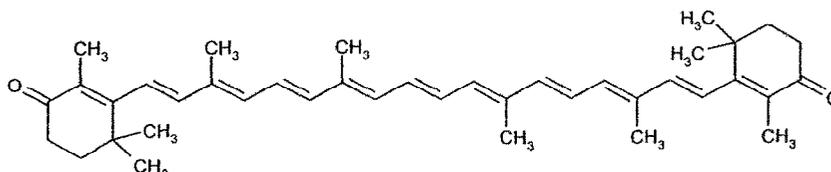
$$\% \text{ Assay} = \frac{5A_U C_S}{A_S W}$$

Where: **W** is the weight in mg, of sample taken for analysis;

C_S is the concentration in $\mu\text{g/mL}$ of lutein in the standard preparation from the manufacturer's Certificate of Analysis.

9.3. Canthaxanthin Assay.

9.3.1. Analyte.



9.3.2. **Test Definition:** The following procedure permits the measurement of the content of

U.S. NUTRA			
Laboratory Standard Operational Procedure			
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol			
Document Number: USN-LSOP-10.02.0		Document Level: 3	
Revision No.: 0	Revision Date:	Total pages: 24	
Effective Date:		Page: 21	

canthaxanthin in *H. pluvialis* and/or its extract. It is based on HPLC separation on C30 reverse phase column.

9.3.3. Chromatographic Conditions.

9.3.3.1. Mobile Phase.

9.3.3.1.1. Dissolve 77.1 g of ammonium acetate in 500 mL of water and dilute with water to 1 L.

9.3.3.1.2. Mobile Phase A (MPhA).

Mix 80 mL of the solution from 9.3.3.1.1. with 1800 mL of methyl alcohol, add 120 mL of MTBE, then filter through 0.45 µm filter.

9.3.3.1.3. Mobile Phase B (MPhB).

Mix 40 mL of the solution from 9.2.3.1.1. with 60 mL of methyl alcohol, add 900 mL of MTBE, then filter through 0.45 µm filter and transfer into the separation funnel. If water separates remove it carefully, then use an organic (upper) layer.

9.3.3.1.4. Column Parameters.

Length: 250 mm.
Internal diameter: 4.6 mm.
Packing: C30/silica.
Particle size: 5 µm.

9.3.3.1.5. Typical Instrument Setup.

Gradient program:

1. 1.0 min.: MPhA – 100%.
2. 73.0 min.: MPhA – from 100% to 0%,
MPhB – from 0% to 100%.
with degree of curvature 3,
3. 10.0 min.: MPhB – 100%.

Mobile phase flow: 1.0 mL/min.

Runtime: 84 min.

Injection Volume: 20 µL.

Wavelength: 445 nm.

Temperature: ambient.

9.3.4. Test Preparation.

9.3.4.1. If testing lutein in *H. pluvialis*, extract ca 180 mg of cracked *H. pluvialis* according to procedure 10.

U.S. NUTRA	
Laboratory Standard Operational Procedure	
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol	
Document Number: USN-LSOP-10.02.0	Document Level: 3
Revision No.: 0	Revision Date:
Effective Date:	Total pages: 24
	Page: 22

9.3.4.1.1. Evaporate the solvent using vacuum rotary evaporator to ca 30 mL, the dilute to 50.0 mL with acetone.

9.3.4.2. If testing lutein in *H. pluvialis* extract dissolve ca 70 mg of *H. pluvialis* extract in 30 mL of acetone and dilute to 50 mL with same solvent.

9.3.5. Standard Preparation.

Use commercially available solution of canthaxanthin in ethyl alcohol with concentration ca 1.5 µg/mL (Example: ChromaDex, Product # 03116-A).

9.3.6. Test.

9.3.6.1. Consecutively inject each of standard preparation and test preparation.

9.3.6.2. Identify and integrate canthaxanthin peak (retention time is about 36 min.) in both chromatograms.

9.3.6.3. If necessary, make a new test preparation with the amount adjusted to have canthaxanthin peak area within ±10% margin of that in the standard preparation.

9.3.6.4. Perform 6 consecutive injections of standard preparation and integrate canthaxanthin peak.

9.3.6.5. Calculate an average canthaxanthin peak area \bar{A}_S in standard preparation by the formula:

$$\bar{A}_S = \frac{A_1 + A_2 + A_3 + A_4 + A_5 + A_6}{6}$$

Where: A_1, A_2, A_3, A_4, A_5 and A_6 are the peak areas of canthaxanthin for separate injections of standard preparation.

9.3.6.6. Calculate the standard deviation **S** by the formula:

$$S = \sqrt{\frac{(A_1 - \bar{A}_S)^2 + (A_2 - \bar{A}_S)^2 + (A_3 - \bar{A}_S)^2 + (A_4 - \bar{A}_S)^2 + (A_5 - \bar{A}_S)^2 + (A_6 - \bar{A}_S)^2}{5}}$$

9.3.6.7. Calculate relative standard deviation **RSD** in percents by the formula:

$$RSD = \frac{S}{\bar{A}_S} \times 100\%$$

U.S. NUTRA			
Laboratory Standard Operational Procedure			
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol			
Document Number: USN-LSOP-10.02.0		Document Level: 3	
Revision No.: 0	Revision Date:	Total pages: 24	
Effective Date:		Page: 23	

- 9.3.6.8.** RSD for each standard preparation must be not more than 5.0%. Otherwise the system is considered as not suitable.
- 9.3.6.9.** Inject test preparation, integrate canthaxanthin peak, and express its area as A_U .
- 9.3.6.10.** Calculate the assay in % for canthaxanthin in the sample taken for test preparation by the formula:

$$\% \text{Assay} = \frac{5A_U C_S}{A_S W}$$

Where: **W** is the weight in mg, of sample taken for analysis;

C_S is the concentration in $\mu\text{g/mL}$ of canthaxanthin in the standard preparation from the manufacturer's Certificate of Analysis.

10. EXTRACTION OF DRIED AND CRACKED *H. pluvialis*.

- 10.1.** Place ca 100 mg of *H. pluvialis* in a 50 mL-centrifuge tube and add ca 40 mL of Acetone.
- 10.2.** Cap the tube firmly and vortex for 1 min.
- 10.3.** Centrifuge for 10 min at 10,000 rpm.
- 10.4.** Decant supernatant into the round bottom flask.
- 10.5.** Add 20 mL of acetone into the same centrifuge tube and repeat steps **10.2** – **10.4**
- 10.6.** If necessary repeat step **10.5** until supernatant becomes almost colorless.

Note: ***If more than 100 mg of *H. pluvialis* is necessary to extract, use several centrifuge tubes and do not change the algae/acetone ratio mentioned in 10.1.***

U.S. NUTRA		
Laboratory Standard Operational Procedure		
<i>H. pluvialis</i> Algae Meal and Extract Official Analytical Protocol		
Document Number: USN-LSOP-10.02.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 24
Effective Date:		Page: 24

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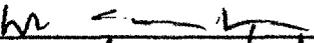
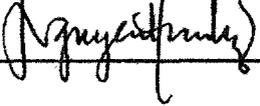
U.S. NUTRA		
Laboratory Standard Operational Procedure		
FATTY ACID PROFILE		
Document Number: USN-LSOP-10.03.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 5
Effective Date:		Page: 1



FATTY ACID PROFILE

USN-LSOP-10.03.0

u.s. nutra

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U.S. NUTRA		
Laboratory Standard Operational Procedure		
FATTY ACID PROFILE		
Document Number: USN-LSOP-10.03.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 5
Effective Date:		Page: 2

TABLE OF CONTENTS

1.	PURPOSE	3
2.	RESPONSIBILITIES	3
3.	TRAINING	3
4.	DEFINITION	3
5.	EQUIPMENT	3
6.	CHEMICALS	4
7.	FATTY ACID PROFILE	4
8.	REFERENCES	4

U.S. NUTRA		
Laboratory Standard Operational Procedure		
FATTY ACID PROFILE		
Document Number: USN-LSOP-10.03.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 5
Effective Date:		Page: 3

1. PURPOSE.

This Protocol defines and describes the analytical procedure for fatty acid profile determination in the various products manufactured by U.S. Nutra.

2. RESPONSIBILITIES.

It is the responsibility of the analytical manager, or an assigned alternate, to revise and/or update this Protocol when necessary.

3. TRAINING.

5.1. It is the responsibility of the analytical manager, or an assigned alternate, to act as trainer for this Protocol.

5.2. Analyst must read and understand this Protocol; then receive training by the analytical manager, or an assigned alternate.

4. DEFINITION.

Fatty Acid Profile is the content of individual fatty acids in the sample expressed as a percentage of total amount of identified fatty acids. The following method is based on acid hydrolysis of the fats in the sample, followed by esterification of the free fatty acids and GC analysis of the final mixture of methyl esters of fatty acids.

5. EQUIPMENT.

Analytical balance with readability not less than 0.1 mg.
Hot plate or heating mantle.
Vortex type mixer.
Water or oil bath.
Extrelut QE20 cartridge, EM Science, Cat. # 901020-1.
Gas chromatograph equipped with flame-ionization detector (FID).
20 ml pressure proof glass tubes with screw caps.
Assorted class A volumetric glass pipets.
Test tube rack.
Assorted glassware.
Water bath with temperature controller.
Thermometer (1°C subdivision).
Transfer pipettes.
Rubber bulb – pipet filler.
Capillary column
GC autosampler vials.
Luer type needles.
Centrifuge

U.S. NUTRA Laboratory Standard Operational Procedure FATTY ACID PROFILE		
Document Number: USN-LSOP-10.03.0		Document Level: 3
Revision No.: 0	Revision Date:	Total pages: 5
Effective Date:		Page: 4

6. CHEMICALS.

Name of Chemical	CAS Registry #	Min. Grade and/or Purity
Air compressed	N/A	Ultra Zero
Helium compressed	7440-59-7	99.99%
Hexanes	73513-42-5	ACS, HPLC
Hydrogen compressed	1333-74-0	99.99%
Methyl Alcohol	67-56-1	HPLC
Nitrogen compressed	7727-37-9	99.99%
Sodium Chloride	7647-14-5	ACS
Sulfuric Acid	7664-93-9	ACS
Water	7732-18-5	HPLC

7. FATTY ACID PROFILE.

7.1. Typical gas chromatograph setup.

- 7.1.1. Column: Capillary column made from fused silica. Length: 30 m; OD: 32 mm, film thickness: 0.25 µm; liquid phase: G16 by USP nomenclature.
- 7.1.2. Carrier gas: helium.
- 7.1.3. Carrier gas flow rate: 1.0 mL/min.
- 7.1.4. Split ratio: 70:1.
- 7.1.5. Injection volume: 2.0 µL.
- 7.1.6. Injector port temperature: 300°C.
- 7.1.7. Flame Ionization Detector port temperature: 300°C.
- 7.1.8. Column preconditioning: 1 hour at 240°C with 2 ml/min of carrier gas.
- 7.1.9. Oven temperature program:
 - Temperature 1: 60°C.
 - Hold time 1: 3.00 min.
 - Rate: 1.5°C/min.
 - Temperature 2: 240°C.
 - Total run time: 123.00 min.
 - Equilibration time: 1.00 min.

7.2. Standard Preparation.

- 7.2.1. Use commercially available standard mixture of Fatty Acid Methyl Esters (FAME). Example of standard mixture: Supelco 37 Component FAME Mix, Cat. # 47885-U.

U.S. NUTRA	
Laboratory Standard Operational Procedure	
FATTY ACID PROFILE	
Document Number: USN-LSOP-10.03.0	Document Level: 3
Revision No.: 0	Revision Date:
Effective Date:	Total pages: 5
	Page: 5

7.3. Test Preparation.

- 7.3.1. Measure 5.0 mL of sulfuric acid and transfer into a 100.0-mL volumetric flask. Add 90 mL of methanol, mix, cool to room temperature, bring to volume with methanol and mix again.
- 7.3.2. Accurately weigh 100-500 mg of sample (test amount depends on actual fatty acid content) in a pressure-proof, screw-capped vial.
- 7.3.3. Add 3.0 mL of sulfuric acid-methanol solution and tighten cap firmly.
- 7.3.4. Heat the vial in a water or oil bath for two hours at ca. 100°C shaking or vortexing the vial every 20-30 minutes during heating
- 7.3.5. Cool to room temperature, carefully unscrew the cap and quickly add 10 mL of water, 1 g of sodium chloride and 5.0 mL of hexanes.
- 7.3.6. Tighten cap firmly and shake the vial for 2 minutes.
- 7.3.7. Unscrew cap and allow layers to separate completely.
- 7.3.8. Transfer ca 1.5 mL of upper (hexanes) layer into the autosampler vial and close it.

7.4. Test Procedure.

- 7.4.1. Inject 2.0 mL of standard preparation and identify FAME peaks according to manufacturer Cert. of Analysis and supplied sample chromatogram.
- 7.4.2. The system is considered suitable if the number of FAME peaks in the sample chromatogram is equal to those in the actual chromatogram obtained (7.4.1.).
- 7.4.3. Inject 2.0 µL of test preparation, identify peaks and integrate

7.5. Calculations.

- 7.5.1. Calculate total area A_T of all identified FAME by adding areas A_i of all individual FAME peaks.
- 7.5.2. Calculate percentage of each individual FAME by the formula:

$$\%FAME = \frac{A_i}{A_T} \times 100\%$$

The profile of FAME corresponds to the fatty acid profile of the sample.

8. REFERENCES.

- 1. *United States Pharmacopoeia*, 2003, Saw Palmetto. USP-26, NF-21.

END OF DOCUMENT