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Add the following:

^Haematococcus pluvialis Powder

DEFINITION

Haematococcus pluvialis Powder is cell wall crushed, dried, algal biomass powder from cultures of microalgae *Haematococcus pluvialis* (Fam. Haematococcaceae). It consists mainly of 3S,3'S stereoisomers of astaxanthin in the monoester, diester, and free forms as active compounds. The monoester form is the most abundant, followed by the diester form. The free form is a minor component. It contains NLT 4.5% of total astaxanthin, calculated as free astaxanthin on the anhydrous basis. It may contain added substances as antioxidants.

IDENTIFICATION

• A. [HPTLC FOR ARTICLES OF BOTANICAL ORIGIN \(203\)](#)

Standard solution: 10 mg/mL of [USP Astaxanthin Esters from Haematococcus pluvialis RS](#) in acetone

Sample solution: Transfer 20 mg/mL of *Haematococcus pluvialis* Powder in acetone. Sonicate for 5 min, centrifuge, and use supernatant.

Chromatographic system

(See *Standard Parameters* as defined in [HPTLC for Articles of Botanical Origin \(203\)](#), [Table 1](#).)

Application volume: 2 µL

Developing solvent system: [Cyclohexane](#) and [ethyl acetate](#) (75:25)

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Samples* as bands, and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry the plate at room temperature for 5 min. Examine the plates under white light.

System suitability: The *Standard solution* exhibits an orange band due to astaxanthin diesters in the upper-third section, as well as four broad orange bands due to astaxanthin monoesters in the middle-third section, which form two pairs of increasing intensity with the zone at lowest R_f being the most intense.

Acceptance criteria: The *Sample solution* exhibits five bands corresponding in R_f , color, and intensity to those observed in the *Standard solution*. One or two very faint orange bands due to free astaxanthin may be observed in the lower-third section.

• B. HPLC

Analysis: Proceed as directed in the test for *Content of Total Astaxanthin*.

Acceptance criteria: The *Sample solution* exhibits three major peaks with the retention times corresponding to those of 13-*cis*-astaxanthin, all-*trans*-astaxanthin, and 9-*cis*-astaxanthin peaks in the *Standard solution* as obtained in the test for *Content of Total Astaxanthin*. All-*trans*-astaxanthin is the major constituent followed by 13-*cis*-astaxanthin and 9-*cis*-astaxanthin.

ASSAY

• CONTENT OF TOTAL ASTAXANTHIN

[NOTE—Astaxanthin determined by this method is total astaxanthin, including the free astaxanthin, the monoester, and the diester.]

Buffer solution: Dissolve 6.06 g of [tris\(hydroxymethyl\)aminomethane](#) in 750 mL of [water](#), adjust with [1 N hydrochloric acid](#) to a pH of 7.0, and dilute with [water](#) to 1000 mL.

Cholesterol esterase solution: 4 units/mL of cholesterol esterase¹ in *Buffer solution*. Prepare fresh daily.

Solution A: [Methanol](#)

Solution B: [t-Butylmethylether](#)

Solution C: [Phosphoric acid](#), 1% aqueous

Mobile phase: See [Table 1](#).

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	81	15	4
15	66	30	4
23	16	80	4
27	16	80	4
27.1	81	15	4
35	81	15	4

Internal standard solution: 5 µg/mL of [USP Apocarotenol RS](#) in acetone

Standard stock solution: Transfer 30 mg of [USP Astaxanthin Esters from Haematococcus pluvialis RS](#) to a 100-mL volumetric flask. Dissolve in 30 mL of [acetone](#), shake by mechanical means, and dilute with [acetone](#) to volume. Dilute the resulting solution in acetone to obtain a concentration of total astaxanthin as free astaxanthin at about 5 µg/mL.

Standard solution: Combine 2.0 mL each of the *Standard stock solution* and the *Internal standard solution* in a glass centrifuge tube. Add 4.0 mL of *Cholesterol esterase solution* to the tube, and mix gently by inversion. Place the tube in a block heater set to 37° and allow the reaction to continue for 45 min while gently and slowly inverting the tube every 10 min. After 45 min, add 1 g of [sodium sulfate decahydrate](#) and 2.0 mL of [petroleum ether](#) to the tube. Mix on a vortex mixer for 30 s, and then centrifuge at 3000 rpm for 3 min. Carefully transfer the petroleum ether layer to a 10-mL glass centrifuge tube containing 1 g of [sodium sulfate anhydrous](#). Be careful to avoid pipetting the intermediate emulsive layer. Repeat the extraction with 2.0 mL of petroleum ether and combine in the tube. Evaporate the petroleum ether layer by using a vacuum or a stream of inert gas at room temperature. Add 4.0 mL of [acetone](#), sonicate, and filter the mixture.

Sample stock solution: Transfer 120 mg of *Haematococcus pluvialis* Powder to a glass centrifuge tube. Add 0.5 g of glass beads and 1 mL of water to the tube, and sonicate in an ultrasonic bath at 45° for 5 min. Add 10.0 mL of [acetone](#), mix on a vortex mixer for 10 s, sonicate in an ultrasonic bath at 45° for 5 min, and then centrifuge at 3000 rpm for 3 min. Carefully transfer the supernatant into a 100-mL volumetric flask. Repeat the acetone extraction until the supernatant is colorless, dilute with [acetone](#) to volume, and mix gently by inversion. Dilute the resulting solution in acetone to obtain a concentration of total astaxanthin as free astaxanthin at about 5 µg/mL.

Sample solution: Combine 2.0 mL of the *Sample stock solution* and 2.0 mL of the *Internal standard solution* in a glass centrifuge tube. Add 4.0 mL of *Cholesterol esterase solution* to the tube, and mix gently by inversion. Place the tube in a block heater set to 37° and allow the reaction to continue for 45 min while gently and slowly inverting the tube every 10 min. After 45 min, add 1 g of [sodium sulfate decahydrate](#) and 2.0 mL of [petroleum ether](#) to the tube. Mix on a vortex mixer for 30 s, and then centrifuge at 3000 rpm for 3 min. Carefully transfer the petroleum ether layer to a 10-mL glass centrifuge tube containing 1 g of [sodium sulfate anhydrous](#). Be careful to avoid pipetting the intermediate emulsive layer. Repeat the extraction with 2.0 mL of [petroleum ether](#) and combine in the tube. Evaporate the petroleum ether layer by using a vacuum or a stream of inert gas at room temperature. Add 4.0 mL of [acetone](#), sonicate, and filter the mixture.

Chromatographic system

(See [Chromatography \(621\), System Suitability](#).)

Mode: LC

Detector: UV 474 nm

Column: 4.6-mm × 25-cm, 5-µm packing [L62](#)

Flow rate: 1.0 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

[NOTE—See [Table 2](#) for approximate relative retention times and relative response factors.]

Table 2

Name	Approximate Relative Retention Time	Relative Response Factor
13-cis-Astaxanthin	0.9	1.3

Name	Approximate Relative Retention Time	Relative Response Factor
all- <i>trans</i> -Astaxanthin	1.0	1.0
9- <i>cis</i> -Astaxanthin	1.4	1.1
Apocarotenal (internal standard)	1.7	—

Suitability requirements

Resolution: NLT 2.0 between 13-*cis*-astaxanthin and all-*trans*-astaxanthin

Relative standard deviation: NMT 2.0% for all-*trans*-astaxanthin

Chromatogram similarity: The chromatogram of the *Standard solution* is similar to the reference chromatogram provided with the lot of [USP Astaxanthin Esters from Haematococcus pluvialis RS](#) being used.

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of total astaxanthin as free astaxanthin in the portion of *Haematococcus pluvialis* Powder taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times P$$

R_U = ratio of [(1.3 × peak area of 13-*cis*-astaxanthin) + peak area of all-*trans*-astaxanthin + (1.1 × peak area of 9-*cis*-astaxanthin)] to the internal standard peak area from the *Sample solution*

R_S = ratio of [(1.3 × peak area of 13-*cis*-astaxanthin) + peak area of all-*trans*-astaxanthin + (1.1 × peak area of 9-*cis*-astaxanthin)] to the internal standard peak area from the *Standard solution*

C_S = concentration of [USP Astaxanthin Esters from Haematococcus pluvialis RS](#) in the *Standard solution* (µg/mL)

C_U = concentration of astaxanthin in the *Sample solution* (µg/mL)

P = labeled amount of total astaxanthin as free astaxanthin in [USP Astaxanthin Esters from Haematococcus pluvialis RS](#) (%)

Acceptance criteria: NLT 4.5% on the anhydrous basis

CONTAMINANTS

• LIMIT OF BENZO[a]PYRENE, BENZO[b]FLUORANTHENE, BENZ[a]ANTHRACENE, AND CHRYSENE

Internal standard stock solution: 10 µg/mL each of benzo[a]pyrene (D_{12}), benzo[b]fluoranthene (D_{12}), benz[a]anthracene (D_{12}), and chrysene (D_{12}) in [n-hexane](#)

Internal standard solution:² 0.10 µg/mL each of benzo[a]pyrene (D_{12}), benzo[b]fluoranthene (D_{12}), benz[a]anthracene (D_{12}), and chrysene in [n-hexane](#), by serial diluting the *Internal standard stock solution* with [n-hexane](#)

Standard stock solution:³ 0.10 µg/mL each of benzo[a]pyrene, benzo[b]fluoranthene, benz[a]anthracene, and chrysene in [n-hexane](#)

Standard solution: 0.01 µg/mL each of benzo[a]pyrene, benzo[b]fluoranthene, benz[a]anthracene, and chrysene in [n-hexane](#), by serial diluting the *Standard stock solution* with [n-hexane](#)

Working standard solutions: See [Table 3](#).

Table 3

Working Standard Concentration (ng/mL)	Volume Taken (mL)			Final Volume after Dilution with n-Hexane (mL)
	Stock Standard Solution	Standard Solution	Internal Standard Solution	
10	2.0	—	2.0	20
5.0	1.0	—	2.0	20
2.0	—	4.0	2.0	20

