1.0 Purpose

This document describes the analytical procedure for the determination of free Astaxanthin and Fucoxanthin in raw materials.

2.0 Scope

This procedure applies to the identification and quantification of free Astaxanthin and Fucoxanthin in raw materials. Astaxanthin is quantified as the sum of the all-trans, 9-cis and 13-cis geometric isomers of Astaxanthin. This method ***does not*** apply to raw materials containing fatty acid esters of Astaxanthin and/or Fucoxanthin – but only to quantification of the free forms. This method was validated under protocol PRTCL-21-0043.

3.0 Responsibility

3.1 It is the responsibility of QC and Analytical chemists who have verified their ability to execute this procedure to follow this procedure.

3.2 It is the responsibility of QC Laboratory Management to implement this procedure and to ensure that the procedure is being followed.

3.3 It is the responsibility of QC Laboratory Management and/or Analytical Development Personnel to keep this procedure current with the associated monographs and laboratory practices.

4.0 Definitions

4.1 **QC** – Quality Control

4.2 **AXN** – Astaxanthin

4.3 **FXN** – Fucoxanthin

4.4 **MeOH** – Methanol

4.5 **ACS** – American Chemical Society

4.6 **HPLC** – High Performance Liquid Chromatography

4.7 **UV-Vis** – Ultraviolet-Visible (Detection)

5.0 References

5.1 PRTCL-21-0043, Protocol, Validation of an Analytical Method for the Determination of Astaxanthin and Fucoxanthin by HPLC / UV-Vis

5.2 USP Monograph, Astaxanthin Esters

6.0 Supplies

6.1 Chemicals – All reagents are ACS grade or better

6.1.1 Milli-Q Water

6.1.2 MeOH

6.1.3 Acetone

6.1.4 AXN Reference Standard (all-trans, free form)

6.1.5 FXN Reference Standard (all-trans, free form)

6.2 Supplies and Glassware

6.2.1 HPLC vials, 12mm X 32mm with screw cap enclosures w/ septa

6.2.2 Volumetric glassware

6.2.3 Weigh paper

6.2.4 Syringes with 0.45µ glass fiber syringe filters

6.3 Equipment

6.3.1 Suitable gradient HPLC system consisting of a pump, autosampler, column oven and UV-Vis detector with a chromatographic data handling system

6.3.2 Analytical Balance

6.3.3 Analytical Micro Balance

6.3.4 Sonicator Bath

7.0 Procedure

7.1 Mobile Phase & Diluent Preparation

7.1.1 Mobile Phase

7.1.1.1 Mobile Phase A: Water

7.1.1.2 Mobile Phase B: MeOH

7.1.2 Extraction Solvent / Diluent

7.1.2.1 Acetone

Note: Actives spray-dried onto water-soluble carriers require pre-treatment with water, see Sample Preparation below.

7.2 Standard Prep

7.2.1 AXN: Accurately weigh and transfer ~3 mg of reference standard into a 100 mL volumetric flask using ~60 mL of acetone. Swirl to dissolve then QS to volume. Sonicate for 10 minutes then mix well. Sonicate for an additional 10 minutes and mix well.

7.2.2 FXN: Accurately weigh and transfer ~2 mg of reference standard into a 100 mL volumetric flask using ~60 mL of acetone. QS to volume then sonicate for 5 minutes and mix well.

7.3 Sample Preparation

7.3.1 The validated linear ranges for the analytical method are 19.2 – 44.8 µg/mL AXN, 1.37 – 3.13 µg/mL 9-cis-AXN, 4.9 – 10.3 µg/mL 13-cis-AXN and 11.7 – 27.3 µg/mL FXN.

7.3.2 Extract sufficient sample (based on the raw material manufacturer assay value) with Extraction Solvent in order to generate a concentration that is within the validated linear range. Mechanically shake at ~1/2 volume for 15 minutes, then QS to volume and sonicate for 10 minutes. Shake vigorously then filter a 5ml aliquot for analysis, discarding the first 3-4ml of filtrate.

7.3.3 When analyzing samples that are spray-dried onto water-soluble carriers (e.g. maltodextrin), pre-treatment with water is required. Transfer the powder to the bottom of a 100mL volumetric flask and add 5mL of water. Thoroughly wet the material with gentle, intermittent swirling over the course of 5 minutes. QS to volume with Extraction Solvent and sonicate for 10 minutes. Shake vigorously then sonicate for an additional 10 minutes. Shake vigorously then filter a 5ml aliquot for analysis, discarding the first 3-4ml of filtrate.

7.3.4 For materials being analyzed for the first time using this method, an in process validation is required to demonstrate spectral purity and extraction efficiency as a part of system suitability before data can be reported using this method

7.4 HPLC Parameters

7.4.1 Column: HALO C_{30}, 4.6 x 150mm, 160Å, 2.7µm SPP (Or Equivalent)

7.4.2 Column Temperature: 35°C

7.4.3 Flow rate: 0.8 mL/min

7.4.4 Mobile Phase: Gradient

7.4.4.1

Time, min	%B
0.0	90
5.00	90
15.00	100
20.00	100
20.10	90
25.00	90

7.4.5 Wavelength: 477 nm (AXN), 450 nm (FXN)

7.4.6 Injection Volume: 5 µL

7.4.7 Run Time: 25 minutes

7.4.8 3-D Spectral Range (for Identification): 340nm - 650nm

7.5 Recommended Sequence

7.5.1 Make at least 2 injections of the Diluent.

7.5.2 Make at least five (5) injections of Working Standard.

7.5.3 Make a single injection of each Sample Preparation.

7.5.4 Make a single injection of the Working Standard after every ten (10) sample injections or at the end of the run.

7.6 System Suitability Requirements

7.6.1 The %RSD of five (5) consecutive standard injections is NMT 3%.

7.6.2 The %RSD of all standard injections is NMT 5.0%.

7.6.3 If present, any interference in the diluent should be subtracted out of the sample and standard peak areas.

7.7 Example calculations for determining % assay:

7.7.1 $$\% = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times 100$$

R_u Sample peak area

[Note: Add 1.3(13-cis peak area) + 1.1(9-cis peak area) to R_u if present in AXN sample.]

R_s Mean (n=5) standard peak area

Wt_{std} Weight of the reference standard

V_{std} Volume of the standard preparation accounting for dilutions in mL

P Purity of the reference standard in decimal format

SA Sample amount

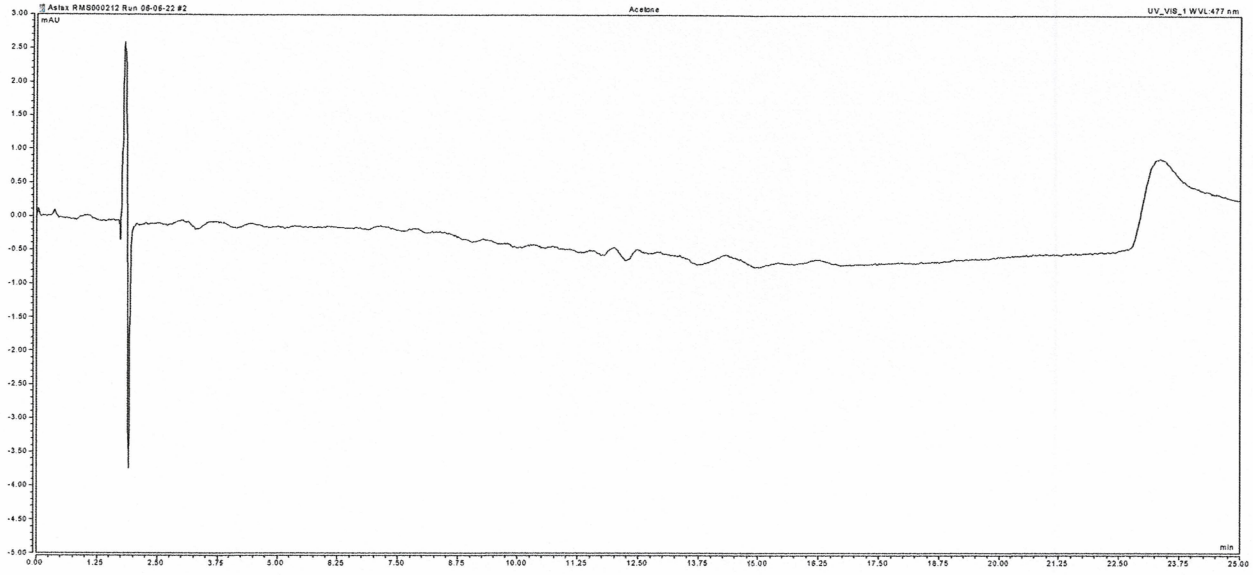
V_{spl} Volume of the sample preparation accounting for dilutions in mL

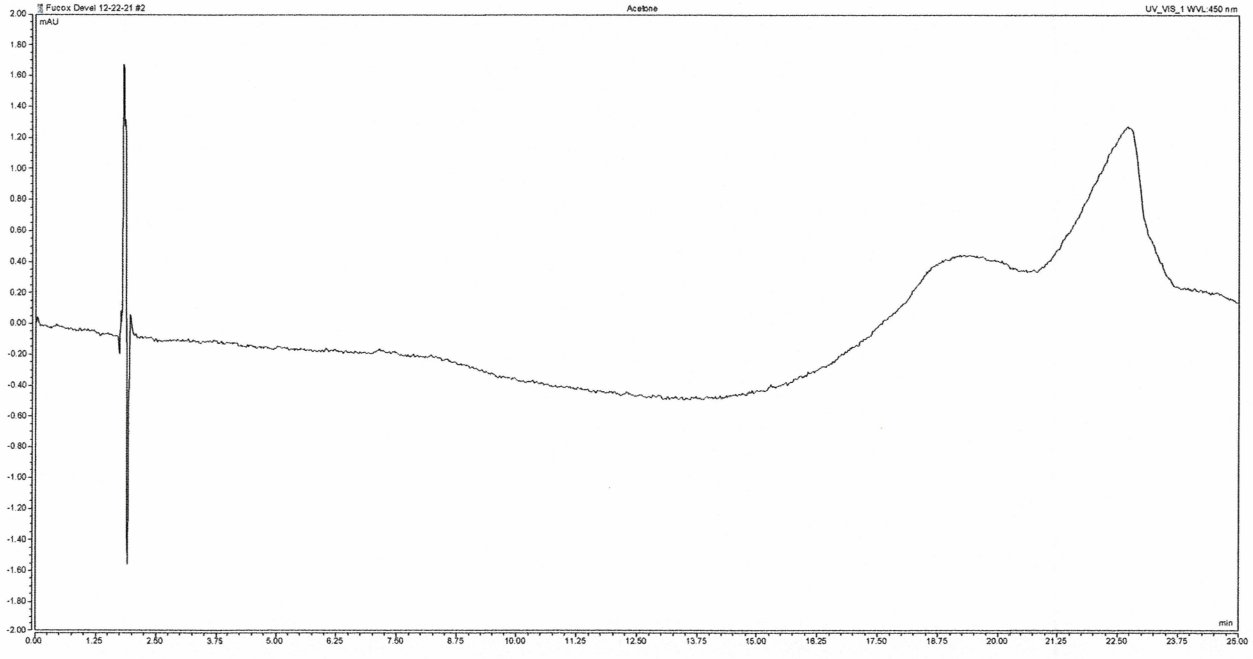
7.8 System Wash, Column Wash and Column Storage

7.8.1 Wash and store the column in 75:25 ACN / Water

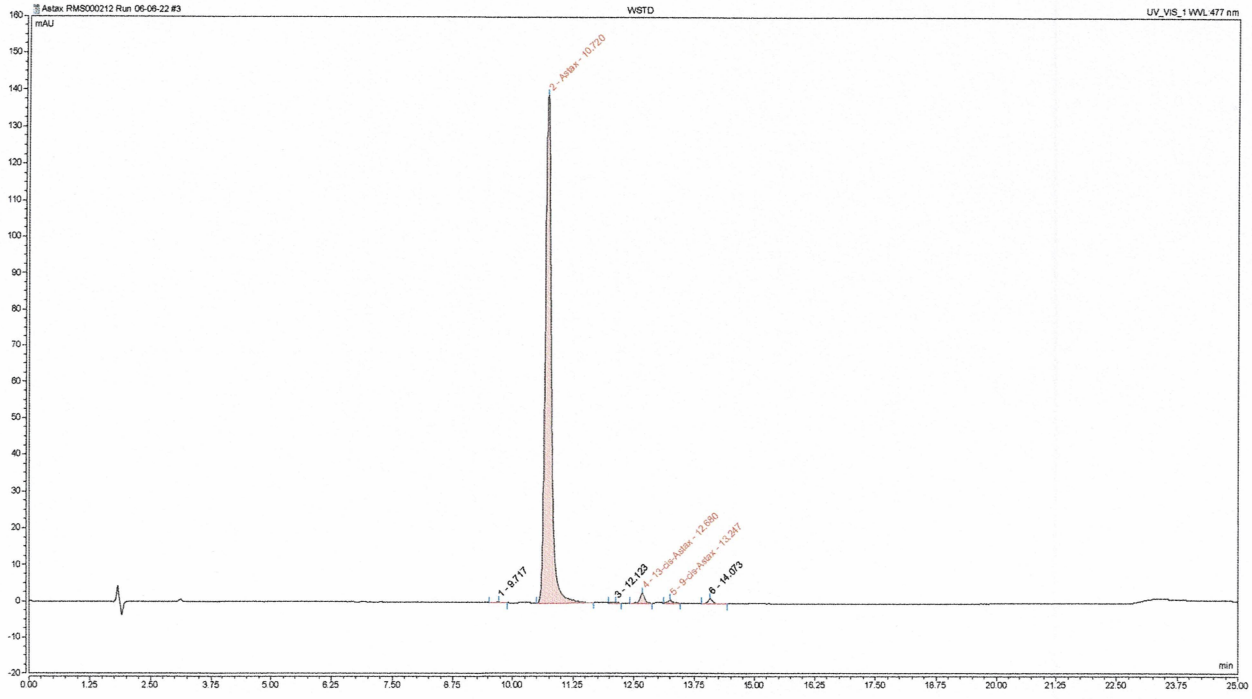
8.0 Chromatograms

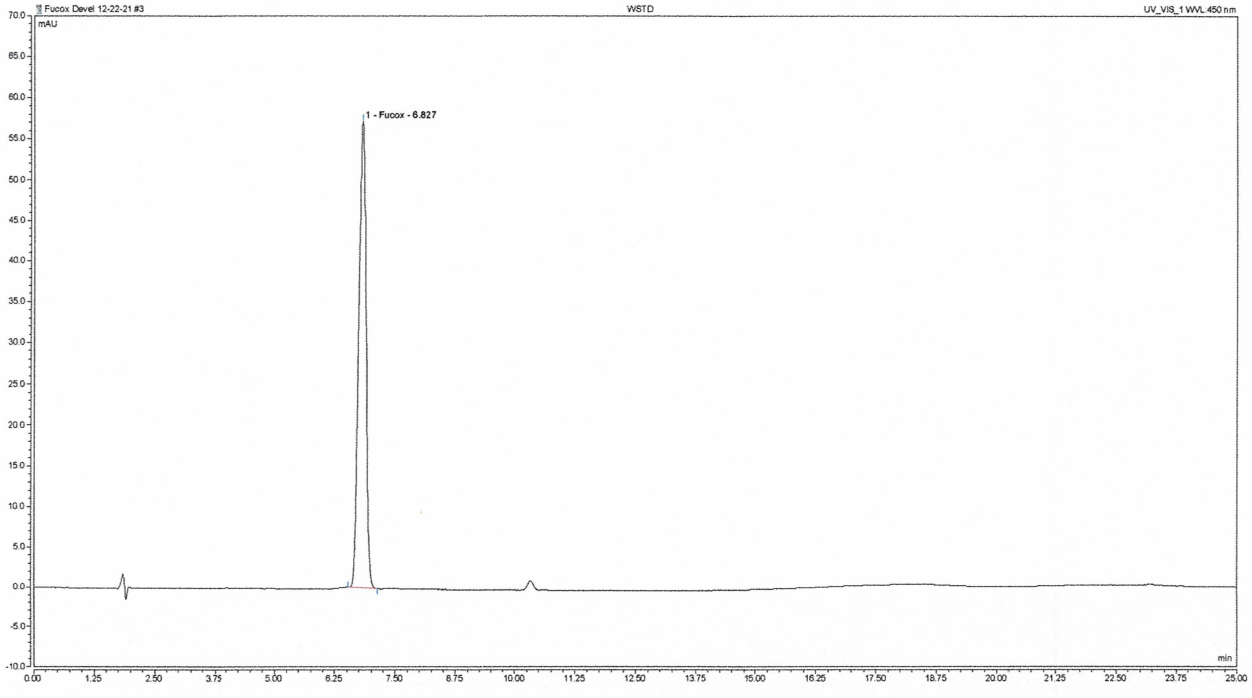
8.1 Typical Diluent Chromatograms



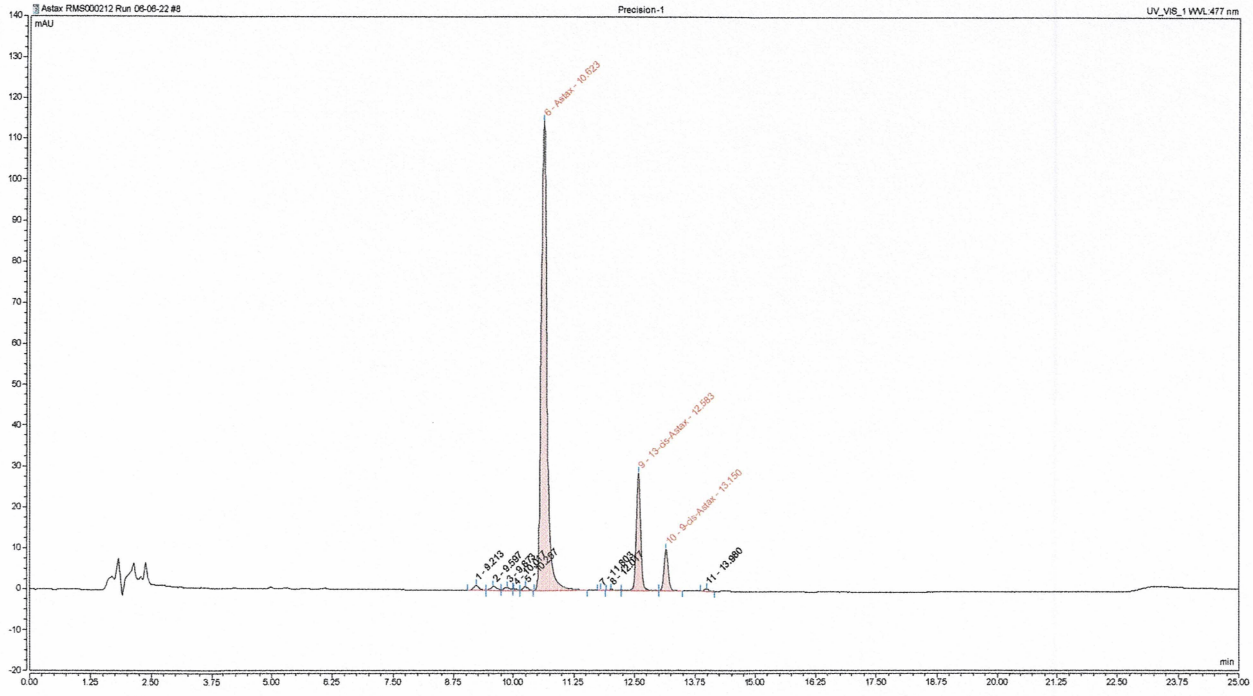


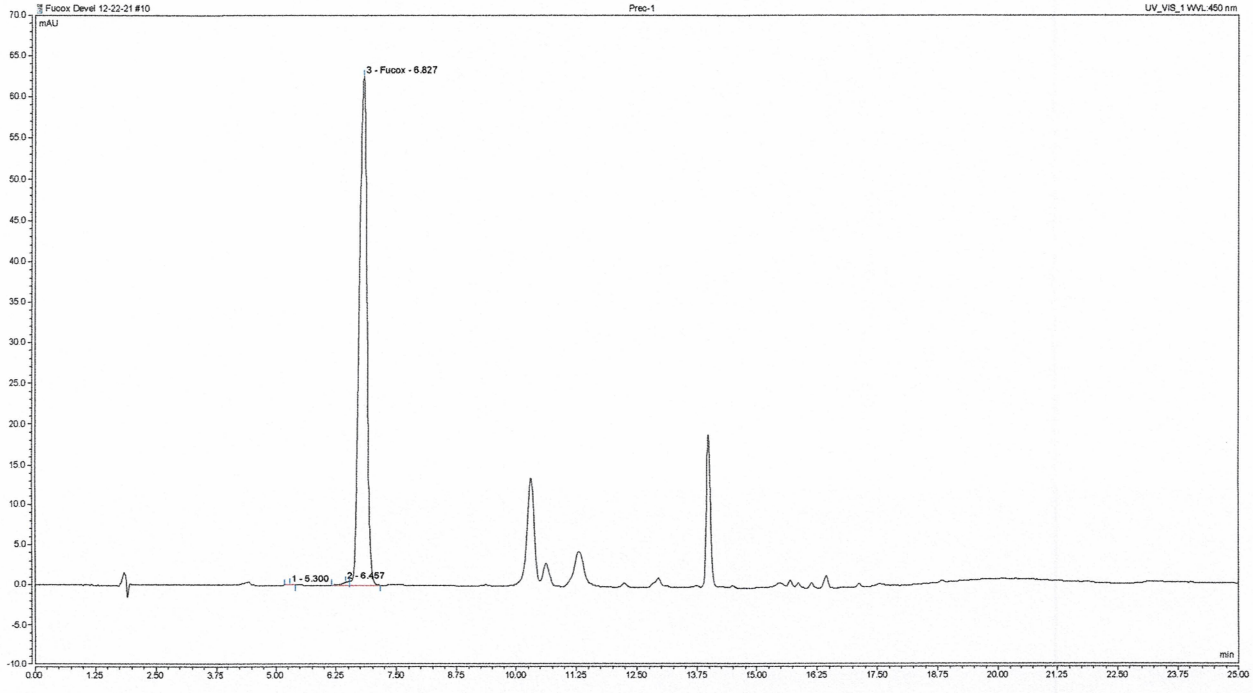
8.2 Typical Standard Chromatograms





8.3 Typical Raw Material Chromatograms





9.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	08/02/22	New procedure.	N/A	C. Perry