1.0 Purpose

This document describes the analytical procedure for the determination of Lutein & Zeaxanthin in raw materials and finished products.

2.0 Scope

This procedure applies to the identification and quantification of Lutein & Zeaxanthin in raw materials and finished products. This method was validated under protocol PRTCL-23-0009.

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 **ACN** – Acetonitrile

4.3 **THF** – Tetrahydrofuran

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-23-0009, Protocol, Validation of an Analytical Method for the Determination of Lutein & Zeaxanthin by HPLC-UV

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 ACN

6.1.4 THF

6.1.5 Lutein Reference Standard – **USE SIGMA-ALDRICH P/N PHR-1699**

6.2 Supplies and Glassware (Note: **USE RED GLASSWARE**)

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

6.2.2 Volumetric glassware (red low actinic stained glass)

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 WITH NEEDLE WASH, COLUMN COMPARTMENT CAPABLE OF MAINTAINING 10°C,** and UV-Vis detector with a chromatographic data handling system

6.3.2 Analytical Balance

6.3.3 Wrist Action Shaker

7.0 Procedure

7.1 Mobile Phase & Diluent Preparation

7.1.1 Mobile Phase

7.1.1.1 Combine 990mL MeOH + 10mL water and mix well.

7.1.2 Extraction Solvent

7.1.2.1 Use THF.

7.1.3 Diluent

7.1.3.1 Use MeOH.

7.2 Standard Prep (Prepare in red glassware.)

7.2.1 Prepare Lutein standard stock at ~0.5 mg/mL in THF. Shake stock for 15 minutes at ~ ½ volume then QS to volume. Dilute stock 2:50 with Diluent using volumetric glassware. Shake vigorously then filter a 5mL aliquot for analysis, discarding the first 3-4mL of filtrate.

7.2.2 A Zeaxanthin standard is not prepared. Zeaxanthin is quantified against Lutein at 446 nm, taking advantage of a coincident detector response at this wavelength.

7.2.3 Be sure to use Sigma-Aldrich P/N PHR-1699. This material contains Zeaxanthin impurity in such quantity as to facilitate its identification in raw materials and finished products.

7.3 Sample Preparation (Prepare in red glassware.)

7.3.1 Specific sample testing details are provided in each product profile. If a specific testing details section is not available, follow preparation procedure as described below, maintaining concentration within the linear range of this method.

7.3.2 The validated linear ranges for the analytical method are 13.7 – 36.5 µg/mL Lutein and 2.8 – 7.4 µg/mL Zeaxanthin.

7.3.3 Pool at least 10 dosage units and homogenize as appropriate (e.g. grind tablets / capsule fill / powders / stick pack contents by mortar and pestle, cryogenically powder and dissolve gummies, etc.) Extract sufficient sample (based on the raw material manufacturer assay value / product profile) with Extraction Solvent in order to generate a sample stock that is ~0.2 mg/mL Lutein. Shake for 15 minutes at ~ ½ volume then QS to volume. Dilute stock 1:10 with Diluent using volumetric glassware. Shake vigorously then filter a 5mL aliquot for analysis, discarding the first 3-4mL of filtrate.

7.4 HPLC Parameters

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

7.4.2 Column Temperature: 10°C

7.4.3 Flow rate: 0.8 mL/min

7.4.4 Mobile Phase: Isocratic

7.4.5 Wavelength: 446 nm

7.4.6 Injection Volume: 5 µL (Use 1500µl ACN Needle Wash)

7.4.7 Run Time: 15 minutes

7.4.8 3-D Spectral Range (for Identification): 350nm - 550nm

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 2%.

7.6.2 The %RSD of all standard injections is NMT 3%.

7.7 Example calculations for determining % Assay / LC:

7.7.1 $$\% = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times 100 \text{ x SS/LA}$$

R_u Sample peak area

R_s Mean (All) standard peak area (Lutein)

Wt_{std} Weight of the reference standard, mg

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

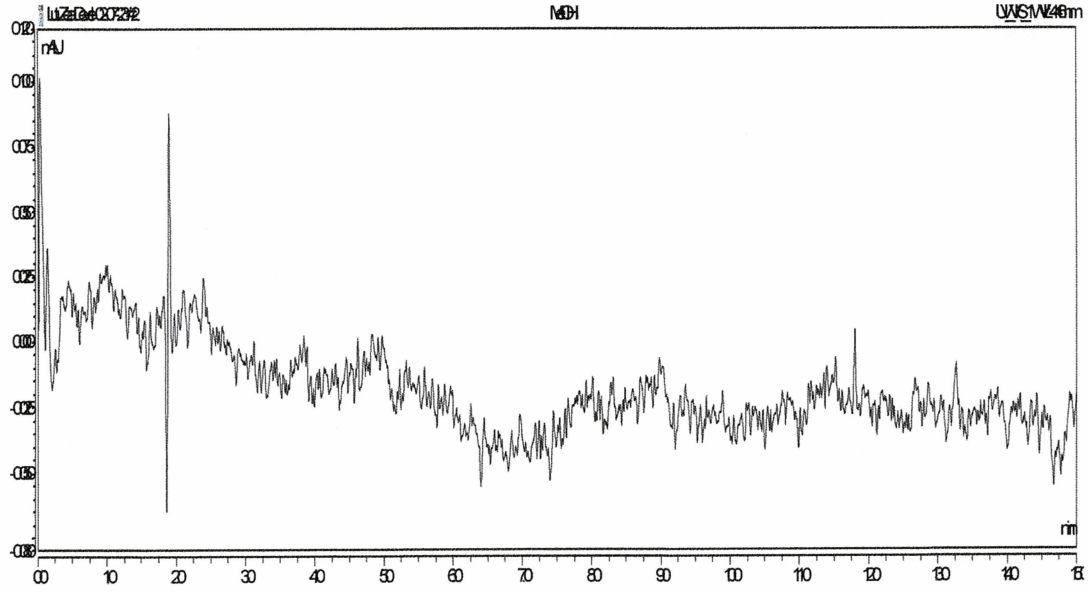
P	Purity of the reference standard in decimal format
SA	Sample amount, g
V_{spl}	Volume of the sample preparation accounting for dilutions, mL
SS	Serving Size from Product Profile, mg (Use "1g" for Raw Materials.)
LA	Label Amount, mg (Use "1" for Raw Materials.)

7.8 System Wash, Column Wash and Column Storage

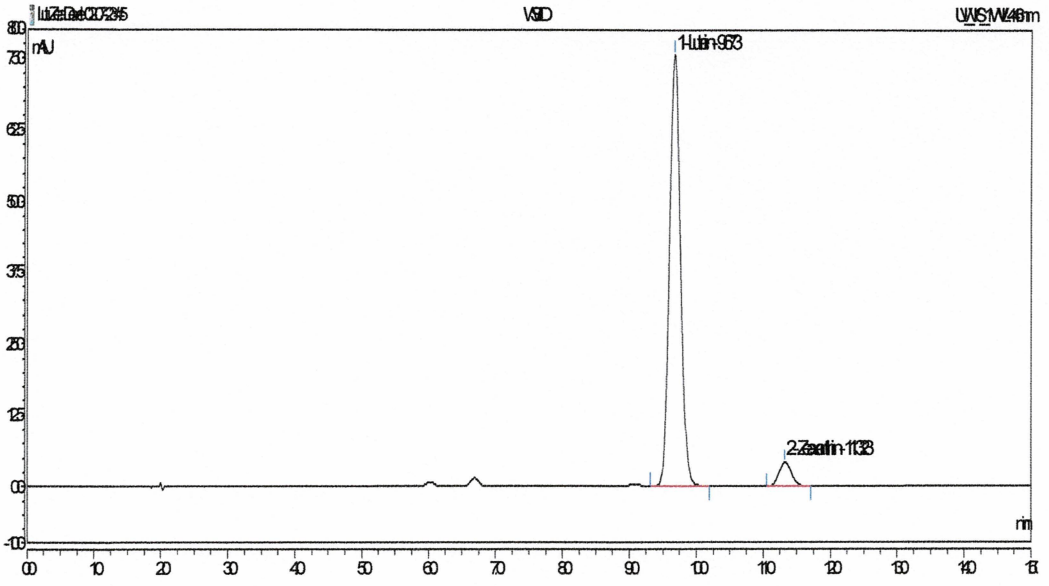
7.8.1 Wash and store the column in Mobile Phase

8.0 Chromatograms

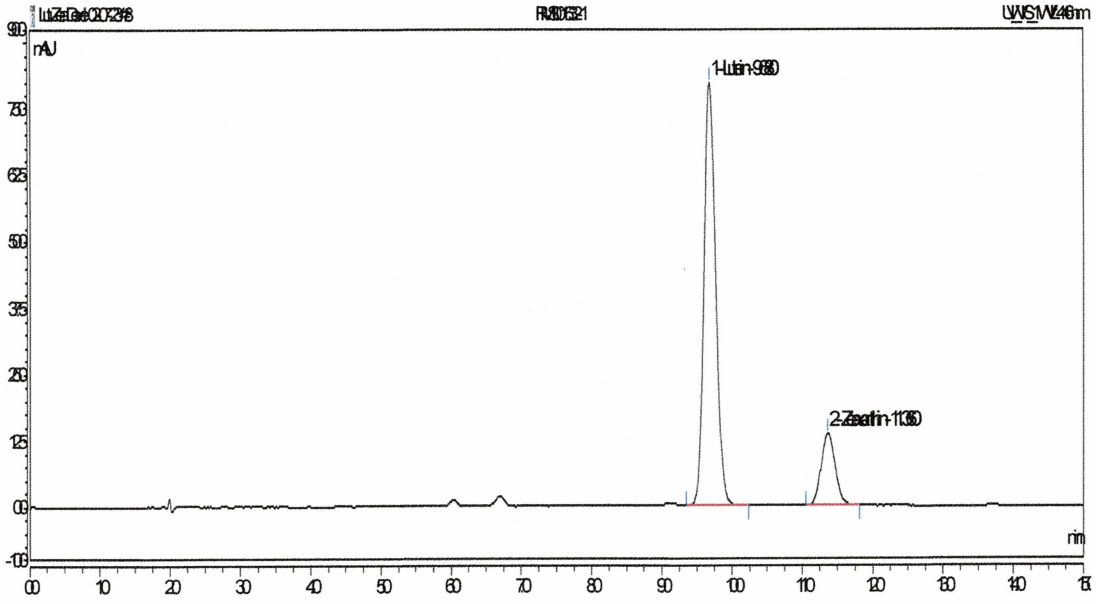
8.1 Typical Diluent Chromatogram



8.2 Typical Standard Chromatogram



8.3 Typical Raw Material Chromatogram



9.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	03/09/23	New procedure.	N/A	C. Perry