1.0 Purpose

The purpose of this procedure is to define the method for the quantitation and/or identification of Beta Carotene in raw materials and finished products using HPLC and UV/VIS spectrophotometry.

2.0 Scope

This procedure applies to the quantification and identification of Beta Carotene in raw materials and finished products. Beta Carotene is an excellent chromophore and was measured at 454nm. Other wavelengths can be used with justification if interferences are present.

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 **AD** – Analytical Development

4.3 **UV/VIS** – Ultraviolet and Visible Electromagnetic Spectrums

4.4 **CofA** – Certificate of Analysis

5.0 References

5.1 MV-LAB-19-121, Protocol, Beta Carotene Determination by HPLC using UV/Vis Spectroscopy.

6.0 Supplies

6.1 Chemicals: All reagents are HPLC grade or better.

6.1.1 Ethanol (denatured with 5% isopropanol and 5% methanol)

6.1.2 Acetonitrile

6.1.3 Methanol

6.1.4 Chloroform

6.1.5 Beta-Carotene reference standard

6.2 Glassware

6.2.1 HPLC vials, 12mm x 32mm with screw cap enclosures with septa

6.2.2 Scintillation Vials

6.2.3 1L Mobile Phase Container

6.2.4 50mL Volumetric Flask

6.2.5 100mL Volumetric Flask

6.3 Disposables

6.3.1 10mL Pipette Tips

6.3.2 1mL Pipette Tips

6.3.3 200µL Pipette Tips

6.3.4 1.5mL microfuge tubes

6.3.5 16mL Test Tubes

6.3.6 Disposable Plastic Luer Lock Syringe – 3mL, 6mL, or 10mL

6.3.7 Nylon Syringe Filters, 0.45µm

6.3.8 Weigh paper

6.4 Equipment

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

6.4.2 Analytical Balance

6.4.3 Ultrasonic bath

6.4.4 Vortex

6.4.5 Stir Plate

6.4.6 Eppendorf Centrifuge

6.4.7 10mL Pipette

6.4.8 1mL Pipette

6.4.9 200µL Pipette

7.0 Preparation of Mobile Phase, Dissolution Buffer, Samples, and Standards

7.1 Mobile Phase (Acetonitrile:Methanol:Chloroform 60:25:15)

7.1.1 Transfer 600 mL acetonitrile to a 1000-mL mobile phase bottle.

7.1.2 Add 250 mL methanol.

7.1.3 Add 150 mL chloroform, and mix well.

7.2 Diluent (Ethanol:Acetonitrile:Methanol:Chloroform 70:18:7.5:4.5)

7.2.1 Transfer 700 mL ethanol to a 1000-mL mobile phase bottle.

7.2.2 Add 180 mL acetonitrile.

7.2.3 Add 75 mL methanol.

7.2.4 Add 45 mL chloroform, and mix well.

7.3 Standard Preparation

7.3.1 **Beta-carotene is light sensitive. Prepare solutions in low-actinic glassware.**

7.3.2 The linear range of the method is 0.2 – 20 mcg/mL. All standard and sample preparations must be within the linear range.

7.3.3 Use the actual purity from the CofA or the standard certification for Beta Carotene reference material for calculations. The stock standard preparation reflects 100% content for the analyte assayed.

7.3.4 The standard is prepared by weighing no less than the minimum weight of the analytical balance, then bringing up to the final volume using Diluent in an appropriately sized volumetric flask, then sonicating for 10 minutes.

7.3.5 Dilutions are prepared using Diluent. Dilutions can be made using volumetric flasks or using 10mL, 1mL, and 200µL variable pipettes. Specific standard concentrations will approximate the concentration expected to be found in the product being tested based on the sample dilution and calculated from the label claim or raw material potency. Dilutions can be prepared in HPLC vials.

7.3.6 Alternative standard preparations are acceptable as long as the preparations are within the linear range of this method, 0.2 – 20 mcg/mL.

7.4 Sample Preparation

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

7.4.2 For finished products, at least 10 dosage units are pooled and ground by mortar and pestle as necessary. **Work quickly to avoid exposure to light.**

7.4.3 Samples can be dissolved in Diluent at any volume starting from 50 mL and any weight greater than the minimum weight of the analytical balance..

7.4.4 Based on the label claim and fill or tablet weight for finished products or expected potency for raw materials, accurately weigh and transfer an amount of sample into an appropriately sized low-actinic volumetric flask to generate a concentration that is within the linear range of the method.

7.4.5 Add H_2O to 10% of the flask volume, and swirl or vortex to ensure that the entire sample is wetted.

7.4.6 Dilute to the final volume using Diluent, and sonicate for 10 minutes.

7.4.7 Before injection, insoluble matter should be removed via filtration using a nylon syringe filter. Discard at least 0.5mL of the sample before collecting filtrate. Dilute filtrate as needed then add 1mL of the final dilution to an HPLC vial for analysis.

7.4.7.1 Alternatively, samples and standards can also be centrifuged at 6000 RPM for 5 minutes in an Eppendorf centrifuge to pellet insoluble matter.

8.0 Test Conditions

8.1 Isocratic

Time	%A	%B	Gradient Type
0.00	100	0.0	
20.00	100	0.0	Isocratic

8.1.1 Column – Kinetex XB-C18, 5µm, 100Å, LC column, 250mm x 4.6mm, or equivalent

8.1.2 Flow Rate – 1.0mL/min

8.1.3 UV Detection – 454nm

8.1.4 Injection Volume - 10µL

8.1.5 Column Temperature – 25°C

8.1.6 Recommended 3-D Spectral Range – 300 to 600 nm

8.2 Recommended Sequence

8.2.1 Make at least 2 injections of a Blank (Diluent).

8.2.2 Make five injections of the Working Standard.

8.2.3 Make a single injection of each Sample Preparation.

8.2.4 Make a single injection of the Working Standard after every six samples and at the end of the run.

8.3 System Suitability

8.3.1 The %RSD of five consecutive injections of Working Standard is NMT 5.0%.

8.3.2 The %RSD of all Working Standard injections is NMT 5%.

8.4 Column Wash and Storage

8.4.1 Column wash is not required.

8.4.2 Store the column with mobile phase.

9.0 Example Calculations

9.1 $$\% \text{ assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times \frac{SS}{LA} \times CF \times 100$$

R_u Sample peak area

R_s Mean standard peak area

Wt_{std} Weight of reference standard in mg

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

P Purity of the reference standard in decimal format

SA Sample amount in mg (solids) or mL (liquids)

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

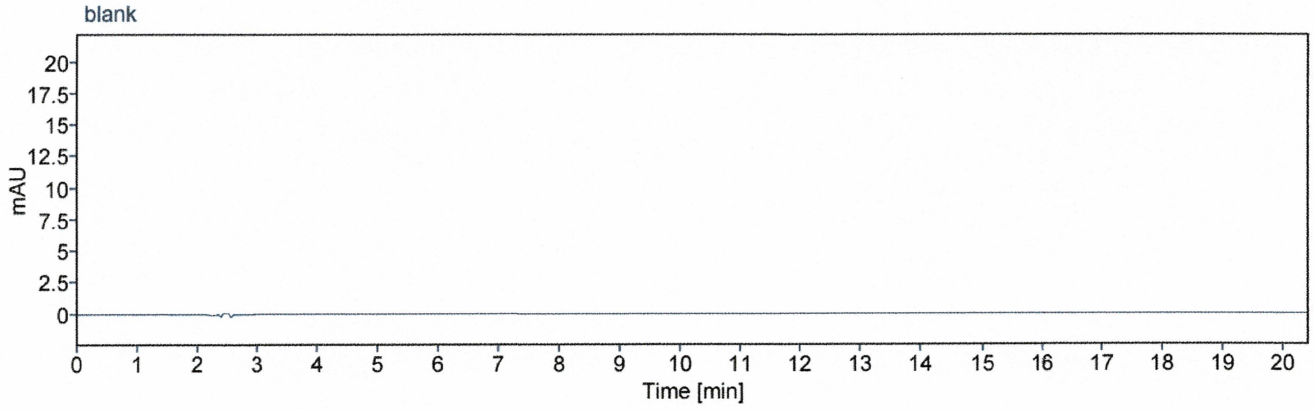
SS Serving size: Average Weight of a single dosage unit in mg for tablets, capsules, and gummies. Volume of a single serving from the theoretical formula in mL for liquids or mg for powders, or 1 for raw materials.

LA Label amount in mg per dose or 1 for raw materials

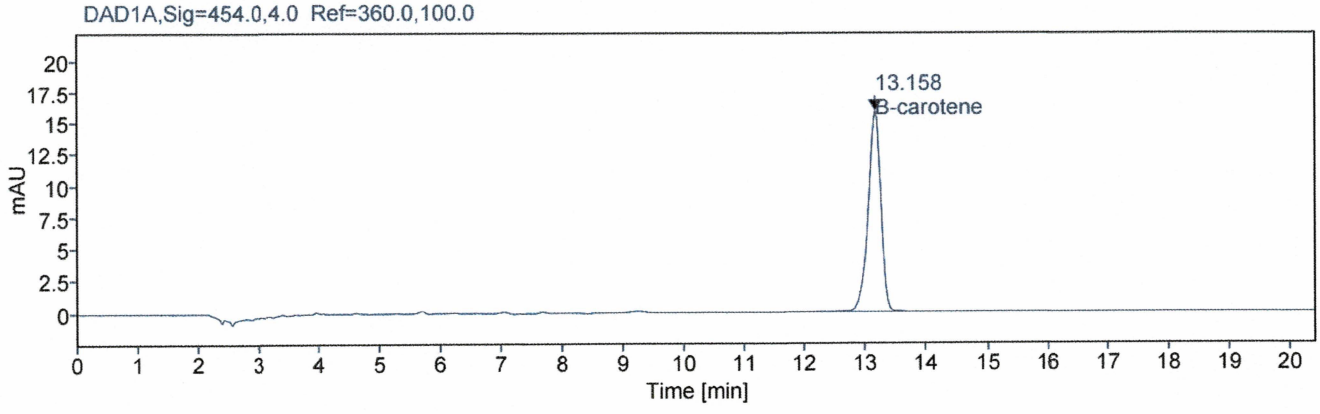
CF Conversion factor (1 if label claim is for Beta Carotene, 0.5 is label claim is for Retinol A Equivalents or Vitamin A)

10.0 Example Chromatography

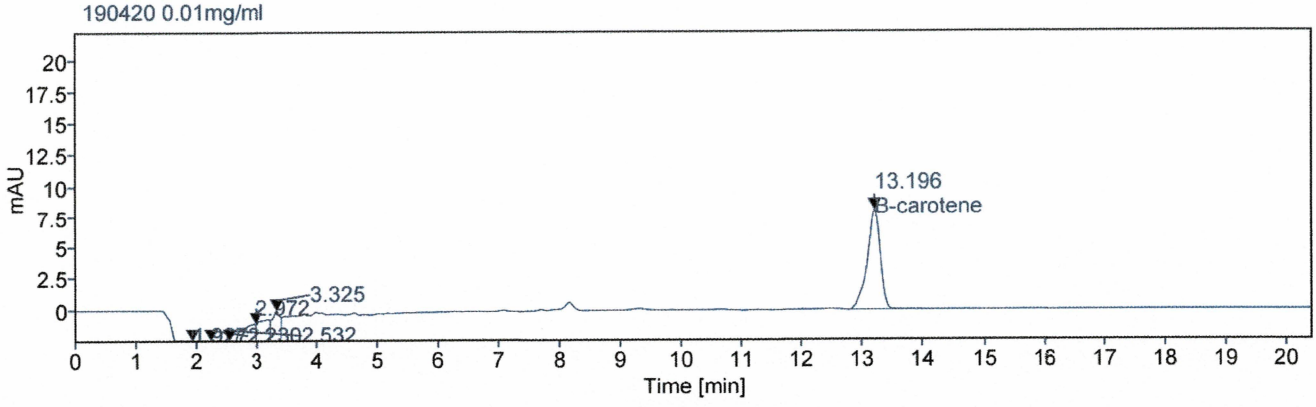
10.1 Blank



10.2 Standard



10.3 Sample



11.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	02/20/20	New	N/A	J. Maignan
1	06/20/22	Update for consistency with current methods, add linear range, add note about light sensitivity, make treatment of sample with water mandatory, add recommended sequence, replace requirements with system suitability, add conversion from beta-carotene to retinol equivalents, add example chromatography.	CC-22-0281	S. Sassman
2	12/20/22	Added test details. Minor edits.	CC-22-0475	J. Sassman