1.0 Purpose

This document describes the analytical procedure for the determination of Total Tocotrienols in raw materials and finished products. Tocotrienols occur as four vitamers: D-Alpha, D-Beta, D-Gamma and D-Delta - which differ from one another in the number and position of methyl groups substituted on the chromanol ring. The D-Beta and D-Gamma forms both possess dimethylated benzopyrane ring structures, and are generally unable to be separated by reverse phase HPLC. However, given that both species possess the same chromophore and molecular weight, D-Gamma-Tocotrienol will (somewhat arbitrarily - based on its predominate occurrence in the raw materials utilized) be used to standardize the quantitation of the sum of D-Gamma and D-Beta Tocotrienol. Total Tocotrienols is calculated as the sum of D-Alpha, D-Beta, D-Gamma and D-Delta Tocotrienol.

2.0 Scope

This procedure applies to the identification and quantification of Tocotrienols in raw materials and finished products. The method lacks specificity regarding the identification and quantitation of D-Beta-T3 in the presence of D-Gamma-T3. The method is, however, specific for the identification and quantification D-Alpha-T3 and D-Delta-T3 in the presence of D-Beta-T3 and D-Gamma-T3. The method is suitable for its intended purpose, which is the determination of Total Tocotrienols in raw materials and finished products. This method was validated under protocol PRTCL-20-0123 and reported in RPT-20-0056.

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 **ACN** – Acetonitrile

4.4 **EtOH** – Ethanol

4.5 **DMSO** – Dimethylsulfoxide

4.6 MeOH - Methanol

4.7 **BHT** – Butylated Hydroxy-Toluene (2,6-di-tert-butyl-4-methylphenol)

4.8 **T3** – Tocotrienol

4.9 **ACS** – American Chemical Society

4.10 **HPLC** – High Performance Liquid Chromatography

4.11 **UV** – Ultraviolet Electromagnetic Spectra

5.0 References

5.1 PRTCL-20-0123, Protocol, Validation of an Analytical Method for the Determination of Total Tocotrienols by HPLC-UV

5.2 RPT-20-0056, Report, Validation of an Analytical Method for the Determination of Total Tocotrienols by HPLC-UV

6.0 Supplies

6.1 Chemicals – All reagents are ACS grade or better.

6.1.1 ACN

6.1.2 EtOH

6.1.3 MeOH

6.1.4 BHT

6.1.5 DMSO

6.1.6 D-Alpha-T3 Reference Standard, 50 mg Vial (Preferred Mass)

6.1.7 D-Gamma-T3 Reference Standard, 50 mg Vial (Preferred Mass)

6.1.8 D-Delta-T3 Reference Standard, 50 mg Vial (Preferred Mass)

6.2 Supplies and Glassware

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

6.2.2 Volumetric glassware **(Use Red Glassware!)**

6.2.3 Volumetric pipets and/or adjustable pipettes and tips

6.3 Equipment

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

6.3.2 Analytical Balance

7.0 Procedure

7.1 Mobile Phase & Diluent Preparation

7.1.1 Mobile Phase

7.1.1.1 Mobile Phase A: EtOH

7.1.1.2 Mobile Phase B: ACN

7.1.2 Diluent: 100 mg/L BHT in EtOH

7.1.3 Preparations may be scaled as necessary

7.2 Standard Preparation

7.2.1 **Use red glassware and minimize material exposure to air and light!** Prepare Tocotrienol stock standards at ~1 mg/mL by transferring approximately 50 mg of each into separate 50 mL volumetric flasks. Add ~25 mL of Diluent and swirl thoroughly until completely dissolved. QS to volume with Diluent and mix well.

Note: Tocotrienols are waxy oils that do not lend themselves to simple weigh-offs. As such, quantitatively transfer the contents of scrupulously clean, pre-weighed vials by means of careful DMSO (1X) & Diluent (5X) rinses into the 50 mL volumetric flasks. Carefully rinse the evacuated vials with MeOH (3X) then dry thoroughly prior to obtaining the empty vial masses. Calculate the masses of the standards transferred to the volumetric flasks by difference.

7.2.2 Use the D-Gamma-T3 stock as the D-Gamma-T3 working standard (WS-1).

7.2.3 Combine 7 parts of the D-Alpha-T3 stock standard with 3 parts of the D-Delta-T3 stock standard (WS-2) and mix well.

7.2.4 Alternate standard preparation is acceptable provided that the final working standard concentrations are within the linear range listed below.

7.3 Sample Preparation

7.3.1 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 listed below.

7.3.2 **Use red glassware and minimize material exposure to air and light!** The validated linear ranges for the analytical method are 0.575 – 1.341 mg/mL D-Gamma-T3, 0.426 – 0.994 mg/mL D-Alpha-T3 and 0.166 – 0.386 mg/mL D-Delta-T3.

7.3.3 Extract sufficient sample (based on the raw material manufacturer assay value / finished product label claim) with Diluent in order to generate analyte concentrations within their respective validated ranges.

7.3.4 Samples can be extracted in Diluent at any volume starting from 50mL. The volume chosen must be in the solubility range (validated at ~1 mg/mL D-Gamma-

T3, ~0.3 mg/mL D-Delta-T3 & ~0.7 mg/mL D-Alpha-T3). To manage large volumes, the sample can be initially dissolved in a smaller volume that is within the solubility range and a portion further diluted using Diluent to bring the concentration into the linear range.

7.3.5 Fill the flask to about 50% of the chosen volume with Diluent then swirl thoroughly until completely dissolved. QS to volume with Diluent and mix well.

7.4 HPLC Parameters

7.4.1 Column: Thermo Scientific Acclaim 120 C_{18}, 4.6 x 250mm, 5µm (Or Equiv.)

7.4.2 Column Temperature: 35°C

7.4.3 Flow rate: 2 mL/min

7.4.4 Mobile Phase: Gradient

7.4.4.1 Short Run (Standards)

Time, min	%B (ACN)
0.00	95
2.00	95
8.00	85
8.01	95
12.00	95

7.4.4.2 Long Run (Samples)

Time, min	%B (ACN)
0.00	95
2.00	95
14.00	75
17.00	75
17.10	95
20.00	95

7.4.5 Wavelength: 295 nm

7.4.6 Injection Volume: 5 µL

7.4.7 Run Time: 12 min (Short), 20 min (Long)

7.4.8 Recommended 3-D Spectral Range (for Identification): 210 nm - 550 nm

7.5 Recommended Sequence

7.5.1 Make at least two (2) injections of the Diluent.

7.5.2 Make five (5) injections of the Working Standard 1 (WS-1).

7.5.3 Make five (5) injections of Working Standard 2 (WS-2).

7.5.4 Make a single injection of each Sample Preparation.

7.5.5 Make a single injection of Working Standard 1 (WS-1) after every ten (10) samples and at the end of the run.

7.5.6 Make a single injection of Working Standard 2 (WS-2) after every ten (10) samples and at the end of the run.

7.6 System Suitability Requirements

7.6.1 The %RSD of five (5) consecutive standard injections is NMT 3.0%.

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

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:

7.7.1 $$\%\,D-(Gamma+Beta)-T3 = \frac{R_{D-(Gamma+Beta)-T3}}{R_{D-Gamma-T3}} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{SS}{SA} \times \frac{V_{spl}}{LA} \times 100$$

$R_{D-(Gamma+Beta)-T3}$ Sample D-(Gamma+Beta)-T3 Peak Area

$R_{D-Gamma-T3}$ Average D-Gamma-T3 Peak Area for the Standard Injections

Wt_{std} Weight of the D-Gamma-T3 reference standard

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

P Purity of the reference standard in decimal format

SS Serving Size (Enter "1" for Raw Materials)

SA Sample Amount

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

LA Label Amount (Enter "1" for Raw Materials)

7.7.2 $$\%\,\mathrm{D-Alpha-T3} = \frac{R_{D-Alpha-T3}}{R_{D-Alpha-T3(Std)}} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{SS}{SA} \times \frac{V_{spl}}{LA} \times 100$$

$R_{D-Alpha-T3}$ Sample D-Alpha-T3 Peak Area

$R_{D-Alpha-T3(Std)}$ Average D-Alpha-T3 Peak Area for the Standard Injections

Wt_{std} Weight of the D-Alpha-T3 reference standard

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

P Purity of the reference standard in decimal format

SS Serving Size (Enter "1" for Raw Materials)

SA Sample Amount

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

LA Label Amount (Enter "1" for Raw Materials)

7.7.3 $$\%\,\mathrm{D-Delta-T3} = \frac{R_{D-Delta-T3}}{R_{D-Delta-T3(Std)}} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{SS}{SA} \times \frac{V_{spl}}{LA} \times 100$$

$R_{D-Alpha-T3}$ Sample D-Delta-T3 Peak Area

$R_{D-Alpha-T3(Std)}$ Average D-Delta-T3 Peak Area for the Standard Injections

Wt_{std} Weight of the D-Delta-T3 reference standard

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

P Purity of the reference standard in decimal format

SS Serving Size (Enter "1" for Raw Materials)

SA Sample Amount

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

LA Label Amount (Enter "1" for Raw Materials)

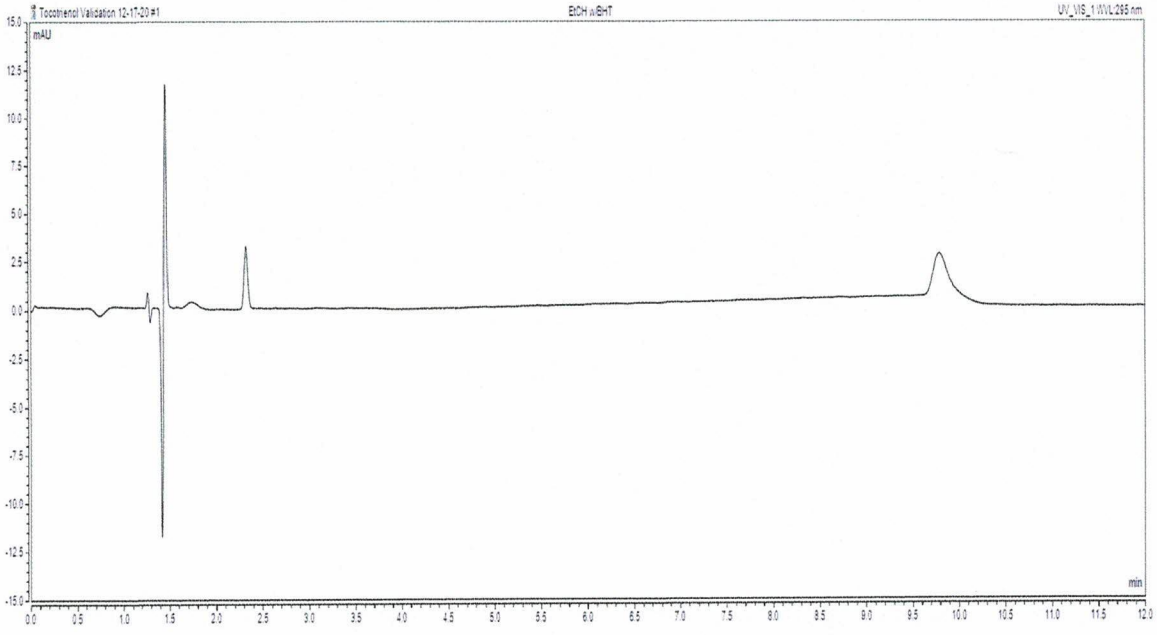
7.7.4 Total Tocotrienols = % D-(Gamma+Beta)-T3 + % D-Alpha-T3 +% D-Delta-T3

7.8 Column Wash and Storage

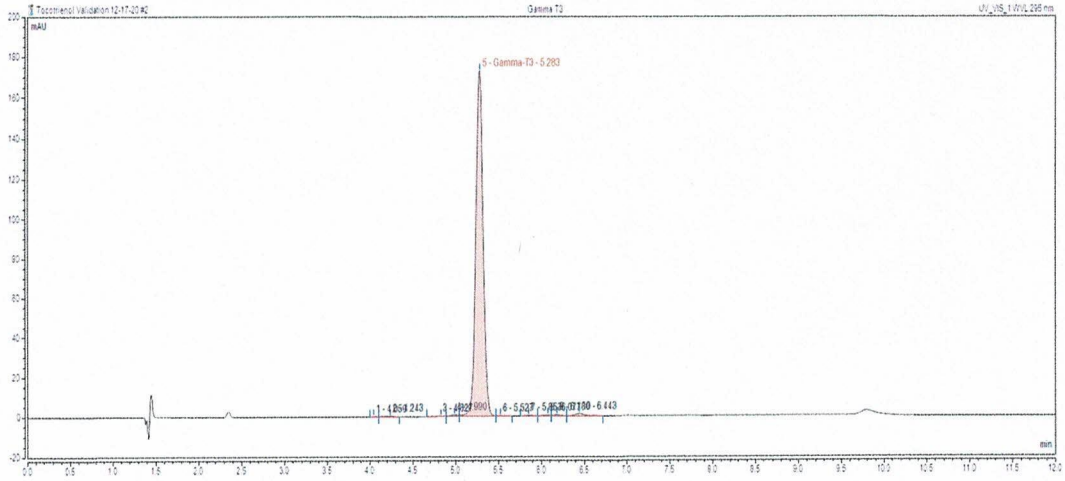
7.8.1 Wash and store the column using 100% ACN.

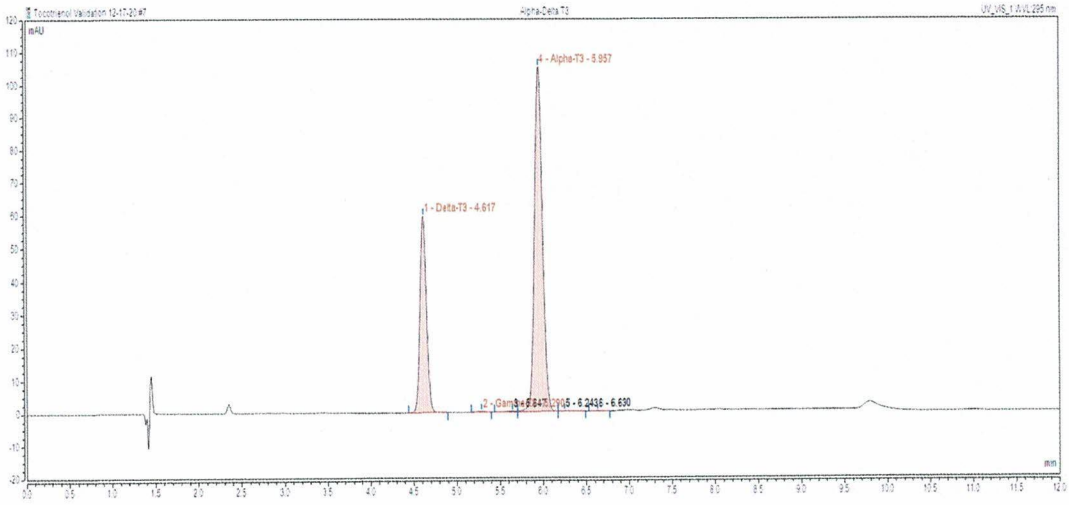
8.0 Chromatograms

8.1 Typical Diluent Chromatogram

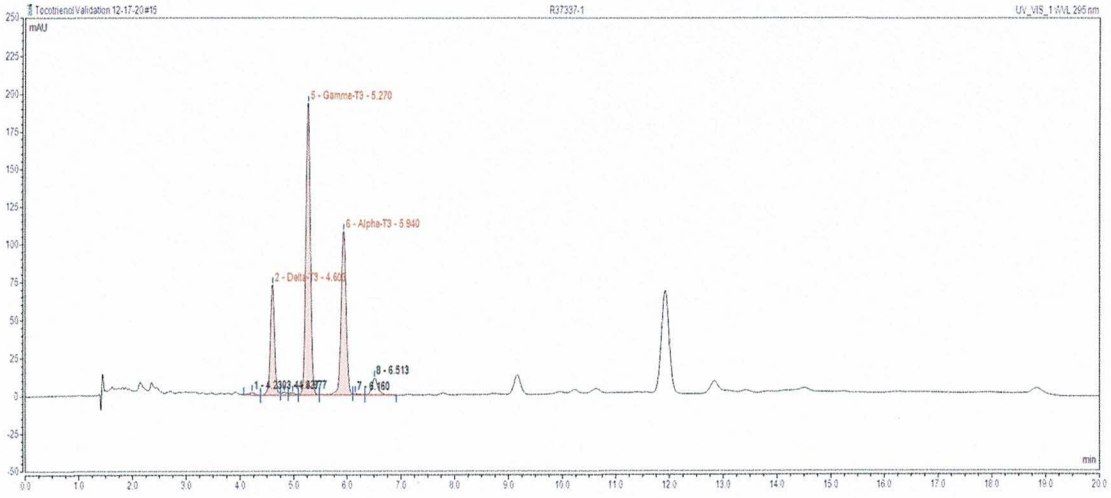


8.2 Typical Working Standard Chromatograms

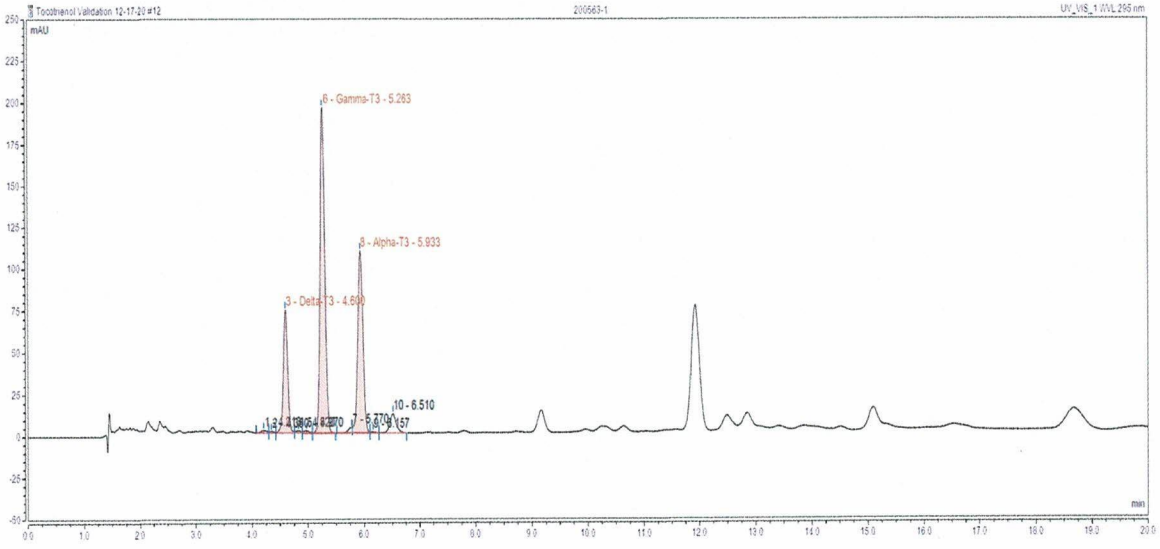




8.3 Typical Raw Material Chromatogram



8.4 Typical Finished Product Chromatogram



9.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	04/14/21	New	N/A	C. Perry
1	05/22/23	Added option for alternate standard preparation. Added instruction to follow product specific test details. Added requirement to run bracketing standard. Updated logo.	CC-23-0253	S. Sassman