1.0 Purpose

This document describes the analytical procedure for the determination of the lipid soluble vitamins: Tocopheryl Acetate, Tocopheryl Succinate, Retinyl Acetate, and Menoquinone 7 in raw materials and finished products.

2.0 Scope

This procedure applies to the identification and quantification of the lipid soluble vitamins, listed above, in raw materials and finished products. This method was validated under Protocol MV-LAB-19-122.

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 the QC Laboratory Management to implement this procedure and to ensure that the procedure is being followed.

3.3 It is the responsibility of the QC Laboratory Management and AD 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 **Vit ARA** – Retinyl Acetate; Vitamin A Acetate

4.4 **Vit ETA** – Tocopheryl Acetate; Vitamin E Acetate

4.5 **Vit ETS** – Tocopherol Succinate; Vitamin E Succinate

4.6 **Vit K2$_{MK7}$** – Menoquinone 7; MQ7; MK7

4.7 **ACN** – Acetonitrile

4.8 **Ethanol** – Ethanol

4.9 **HPLC** – High Performance Liquid Chromatography

4.10 **UV/Vis** – Ultraviolet & Visible Electromagnetic Spectra

5.0 References

5.1 MV-LAB-19-122, Protocol, Lipid Soluble Vitamin Determination Using HPLC with UV/Vis Spectroscopy

6.0 Reagents, Supplies, Glassware, and Equipment

6.1 Chemicals – All reagents are HPLC grade or better

6.1.1 Milli-Q Water

6.1.2 ACN

6.1.3 EtOH

6.1.4 Appropriate Reference Standard

6.2 Supplies and Glassware

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

6.2.2 Volumetric glassware and/or adjustable pipettes and tips

6.2.3 Weigh paper or funnels

6.2.4 10ml Syringes with 17mm x 0.45u Nylon Syringe Filters

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

6.3.3 Sonicator bath

6.3.4 Wrist Action Shaker

7.0 Procedure

7.1 Mobile Phase, Extraction Solvent & Diluent Preparation

7.1.1 Mobile Phase A – 100% ACN

7.1.2 Mobile Phase B – EtOH

7.1.3 Diluent – EtOH

7.2 Standard Prep

7.2.1 Accurately weigh and transfer reference standard into a 50-mL volumetric flask. Add 5mL of DI H_2O and vortex ensuring all solid has come into contact with the water. Sonicate for 2min, cool, then add about 15 mL of EtOH and put on wrist action shaker for 10min. Add about 15 more mL of EtOH and shake for another 10min. QS with EtOH.

7.2.2 Dilute reference standard into linear range for the analyte being run to obtain "Working Standard" used for the assay.

7.2.3 If more than one analyte is being run, standards can be mixed and run simultaneously (be sure to ensure all standards are within the linear range for that analyte).

7.2.4 Other flask sizes may be used however ratio of water to EtOH should remain constant.

7.2.5 Alternative standard preparations are acceptable as long as the preparations are within the linear range of this method.

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 of this method.

7.3.2 The validated range is as follows for each analyte:

7.3.2.1 Vit A_{RA} is 2.64ng – 264 ng (0.000528 – 0.0528mg/mL for 5µL injections) on column.

7.3.2.2 Vit E_{TA} is 22ng – 220ng (0.0044- 0.044mg/mL for 5µL injections) on column

7.3.2.3 Vit E_{TS} is 32.6ng – 326ng (0.00652 – 0.0652mg/mL for 5µL injections) on column

7.3.2.4 MK7 is 23.6ng – 236ng (0.00472 – 0.0472mg/mL for 5uL injections) on column

7.3.3 For finished products, at least 10 dosage units are pooled and/or ground by mortar and pestle. Based on the fill weight (for capsules) or tablet weight per dose and the label claim, weigh the pooled dosages into a suitably sized volumetric flask to generate an analyte concentration that is within the validated linearity range for the analyte being tested.

7.3.4 Prepare raw materials like standards. (However, be sure to consult the specification for expected potency, as raw material samples may not be 100%.)

7.3.5 To the flask, containing the finished product or raw material, add DI H_2O equal to 10% of the volume of the flask and vortex to ensure all powder has come into contact with the water. Sonicate for 2min, cool, then add about 15 mL of EtOH and put on wrist action shaker for 10min. Add about 15 more mL of EtOH and shake for another 10min. QS with EtOH.

7.3.6 Filter a portion for use in subsequent dilutions.

7.3.7 Perform further dilutions as required using Diluent.

7.3.8 Samples can be extracted in diluent at any volume starting from 25mL. 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 to bring the analyte concentration into the linear range.

7.4 HPLC Parameters

7.4.1 Column: Thermo Scientific Acclaim™ 120 C18, 4.6 x 250mm, 5µm

7.4.2 Column Temperature: 35°C

7.4.3 Flow rate: 2 mL/min

7.4.4 Wavelength: 270 nm

7.4.5 Injection Volume: 5 µL

7.4.6 Run Time: 12 minutes.

7.4.7 Recommended 3-D Spectral Range (for Identification) - 210nm to 550nm

7.4.8 Mobile Phase: Isocratic 50:50 ACN:EtOH

7.5 Recommended Sequence

7.5.1 Make an injection of the Diluent.

7.5.2 Make 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 a run.

7.6 System Suitability Requirements

7.6.1 The %RSD of the first five (5) standard injections is NMT 5.0%

7.6.2 The %RSD of all standard injections is NMT 6.0%.

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 finished product % label or raw material % purity

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

R_u Sample peak area

R_s Mean (n=5) standard peak area

Wt_{std} Weight of the reference standard in mg (corr. for water if applicable)

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 in mg for tablets, capsules, and gummies. Mass of a single serving in mg for powders, volume of a single serving from the theoretical formula in mL for liquids, or 1 for raw materials.

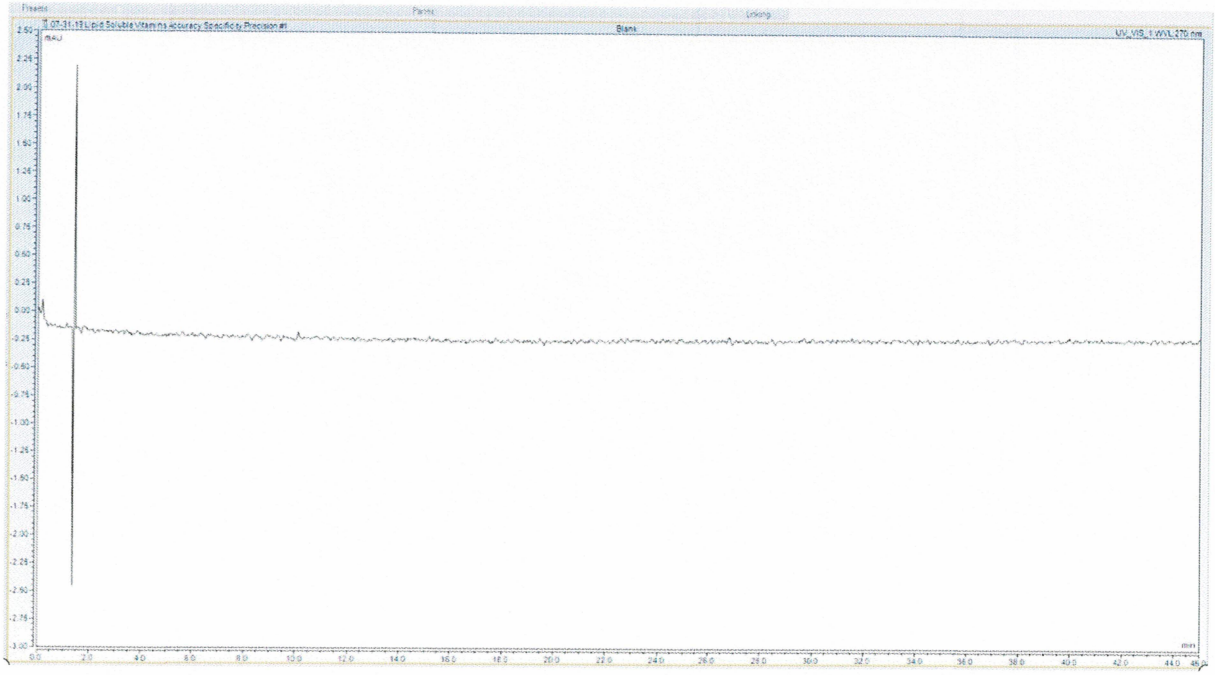
LA Label amount in mg of analyte (use 1 for raw materials)

7.8 Column Wash and Storage

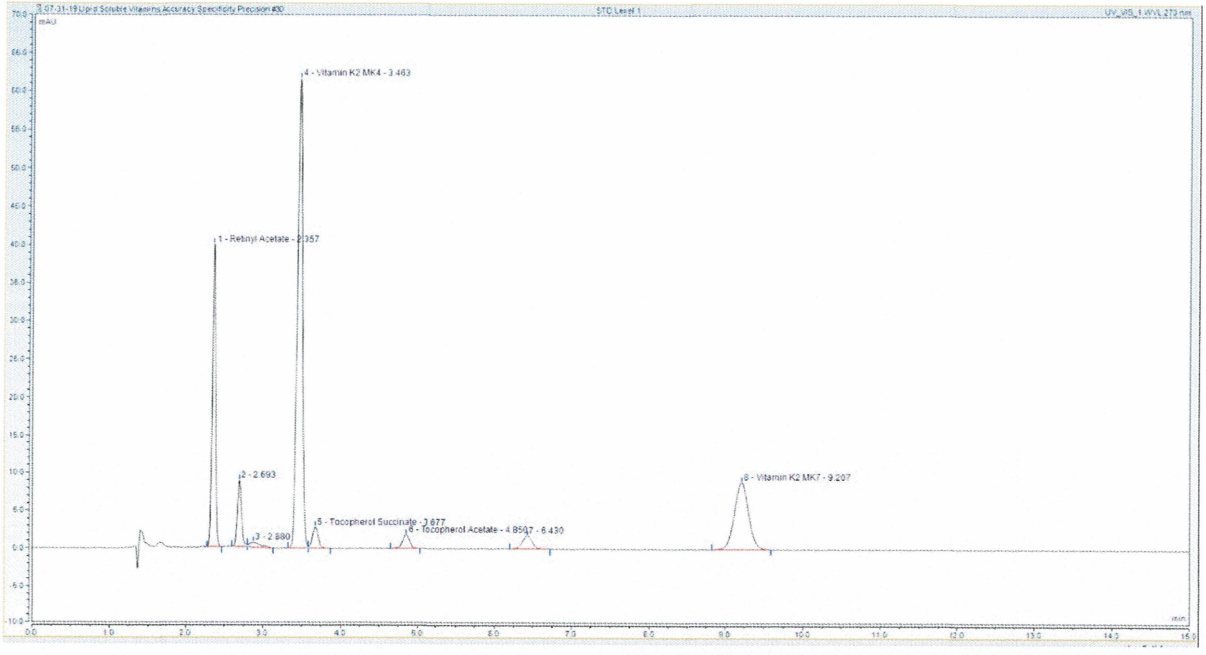
7.8.1 Wash and store the column in 50:50 ACN / Milli-Q Water.

8.0 Chromatograms

8.1 Typical Diluent Chromatogram



8.2 Typical Working Standard Chromatogram



9.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	09/11/19	New	N/A	J. Maignan
1	12/20/22	Added Test Details. Minor edits.	CC-22-0475	J. Sassman