1.0 Purpose

This document describes the analytical procedure for the determination of Thymoquinone (TQ) in raw materials and finished products.

2.0 Scope

This procedure applies to the identification and quantification of TQ in raw materials and finished products. This method was validated under Protocol MV-LAB-19-194.

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 **TQ** – Thymoquinone

4.4 **IPA** – Isopropyl Alcohol

4.5 **HPLC** – High Performance Liquid Chromatography

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

5.0 References

5.1 MV-LAB-19-194, Protocol, Thymoquinone Determination Using HPLC with UV/Vis Spectroscopy

5.2 D-793, SOP, Cryogenic Grinding of Chewable Gels

6.0 Supplies

6.1 Chemicals – All reagents are HPLC grade or better

6.1.1 Milli-Q Water

6.1.2 IPA

6.1.3 Methanol

6.1.4 Thymoquinone Reference Standard

6.2 Supplies and Glassware

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

6.2.2 Low actinic volumetric glassware and/or adjustable pipettes and tips

6.2.3 Weigh paper or funnels

6.2.4 10ml Syringes with 0.45µm 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 Wrist Action Shaker

7.0 Procedure

7.1 Mobile Phase & Diluent Preparation

7.1.1 Mobile Phase

7.1.1.1 Combine 500 mL of Water, 450 mL of Methanol and 50mL of IPA. Mix well.

7.1.2 Extraction Solvent and Diluent

7.1.2.1 IPA

7.1.3 Preparations may be scaled as necessary

Note: Use low actinic (red) glassware for all sample and standard preparations

7.2 Standard Prep

7.2.1 Accurately weigh and transfer about 37.5 mg of TQ reference standard into a 100-mL volumetric flask. Add 50 mL of IPA and shake mechanically for 10 min.

7.2.2 Dilute to volume with IPA and mix well – this is the TQ Stock. Dilute the TQ Stock 1:10 with IPA – this is the TQ Intermediate Standard. Dilute the TQ Intermediate Standard 1:10 with IPA – this is the TQ Working Standard.

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 The validated range for the analytical method is 1.9 – 5.7 µg/mL.

7.3.3 The volume chosen must be in the solubility range of TQ (validated at 0.0375 mg/ml). 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 of measurement. Ensure that the stock sample is equilibrated to room temperature prior to performing further dilution.

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 For solid dose finished products: Combine and homogenize no less than 20 dosage units. Based on the label claim and fill weight (for capsules) or tablet weight per dose, accurately weigh and transfer no less than 195 mg of the

homogenized sample into a suitably sized volumetric flask of no less than 25 mL to generate an analyte concentration that is within the validated linearity range. Add Diluent to about 50% of the flask volume, and shake mechanically for 10 min, and then dilute to volume with Diluent.

7.3.6 For liquid dose finished products: Use a TC pipet to transfer no less than 2.0 mL of the product into a suitably sized volumetric flask of no less than 25 mL to generate an analyte concentration that is within the validated linearity range. Wipe the outside of the pipet, and rinse the pipet three times with water collecting the rinses in the volumetric flask. Dilute to volume using Diluent.

7.3.7 For chewable gels (gummies), homogenize at least 10 dosage units according to the procedure outlined in D-793 Cryogenic Grinding of Chewable Gels. Quickly weigh a portion of the homogenized sample into a volumetric flask of no less than 25 mL to generate an analyte concentration that is within the validated linearity range. Add Diluent to about 50% of the flask volume, and shake mechanically for 10 min, and then dilute to volume with Diluent.

7.3.8 The final sample must be filtered or centrifuged before analyzing by HPLC.

7.3.8.1 For filtration: filter a portion through a 0.45µm nylon membrane discarding the first 2-3 mL of filtrate before collecting an aliquot for analysis.

7.3.8.2 For centrifugation: centrifuge for 5 min at 10,000 rpm.

7.4 HPLC Parameters

7.4.1 Column: Agilent InfinityLab Poroshell 120 EC-C18, 4.6 x 100mm, 2.7u

7.4.2 Column Temperature: 40°C

7.4.3 Flow rate: 0.8 mL/min

7.4.4 Wavelength: 258 nm

7.4.5 Injection Volume: 5 µL

7.4.6 Run Time: 10 minutes.

7.4.7 Recommended 3-D Spectral Range (for Identification) - 210nm to 350nm

7.5 Recommended Sequence

7.5.1 Make at least 2 injections of the diluent.

7.5.2 Make five (5) injections of Standard Solution.

7.5.3 Make a single injection of each Sample Preparation.

7.5.4 Make a single injection of the Standard Solution 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 2.0%

7.6.2 The %RSD of all standard injections is NMT 3.0%.

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

$$\%\,\mathrm{TQ} = \frac{\mathrm{R_u}}{\mathrm{R_s}} \times \frac{\mathrm{Wt_{std}} \times \mathrm{P}}{\mathrm{V_{std}}} \times \frac{SS}{SA} \times \frac{\mathrm{V_{spl}}}{\mathrm{LA}} \times 100$$

$\mathrm{R_u}$ Sample peak area

$\mathrm{R_s}$ Mean standard peak area

$\mathrm{Wt_{std}}$ Weight of the 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

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

SS Serving size: Average weight of ten dosage units in mg for tablets, fill weight for capsules, 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 TQ (use 1 for raw materials)

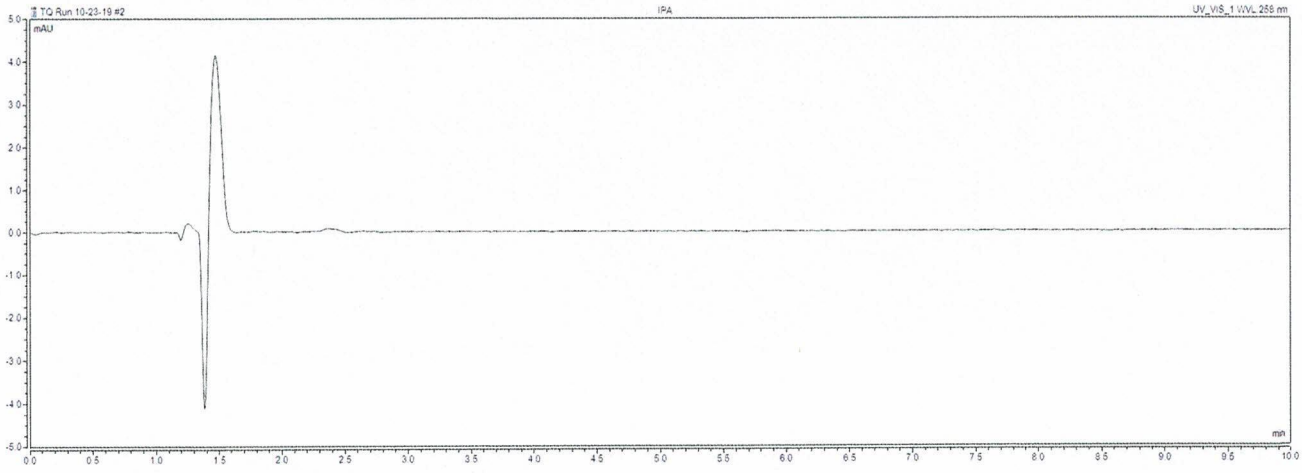
7.8 System Wash, Column Wash and Column Storage

7.8.1 Wash and store the column in 75:25 MeOH / Milli-Q Water.

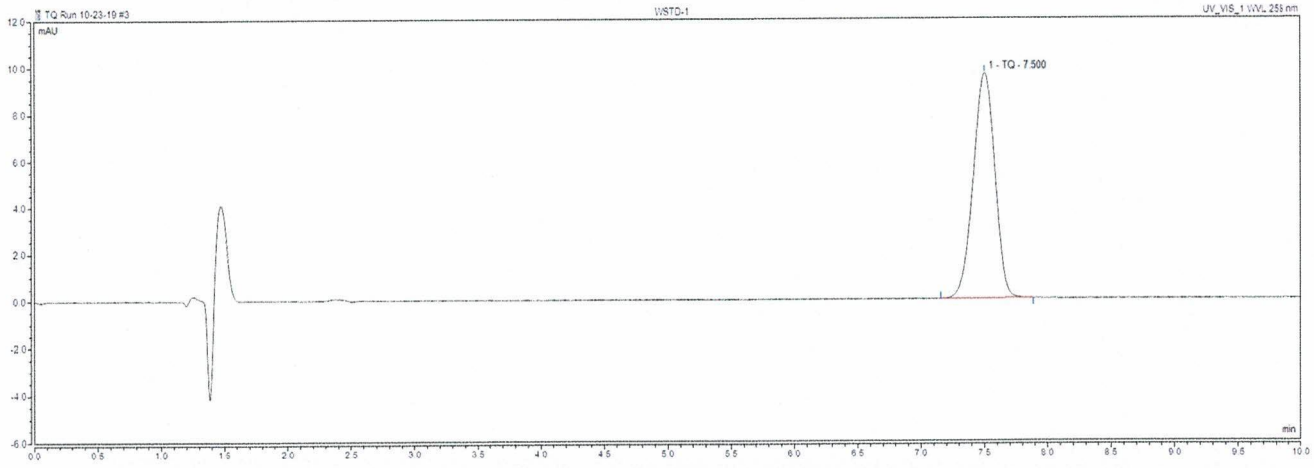
7.8.2 It is recommended to flush the system with IPA, followed by Milli-Q water.

8.0 Chromatograms

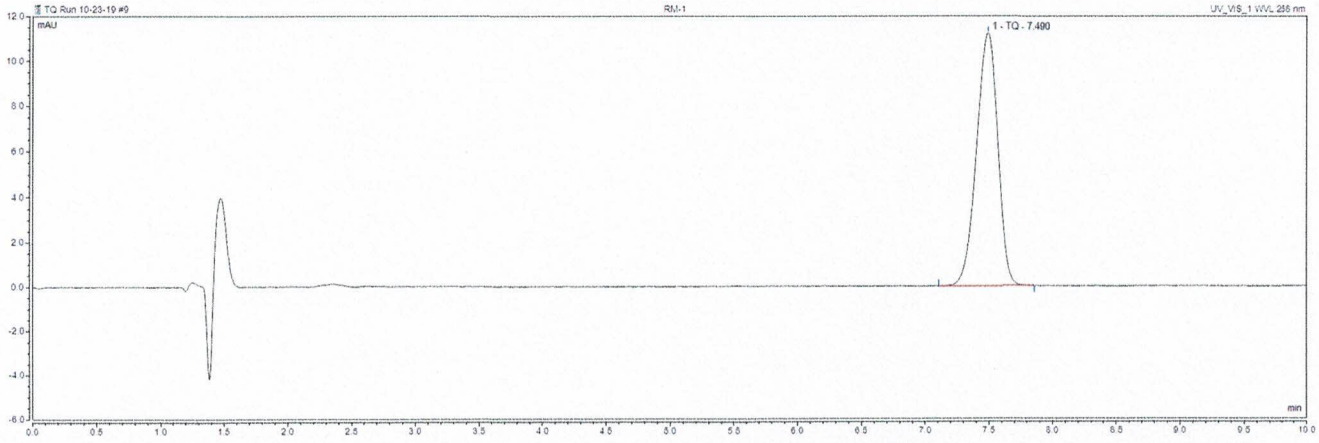
8.1 Typical Diluent Chromatogram



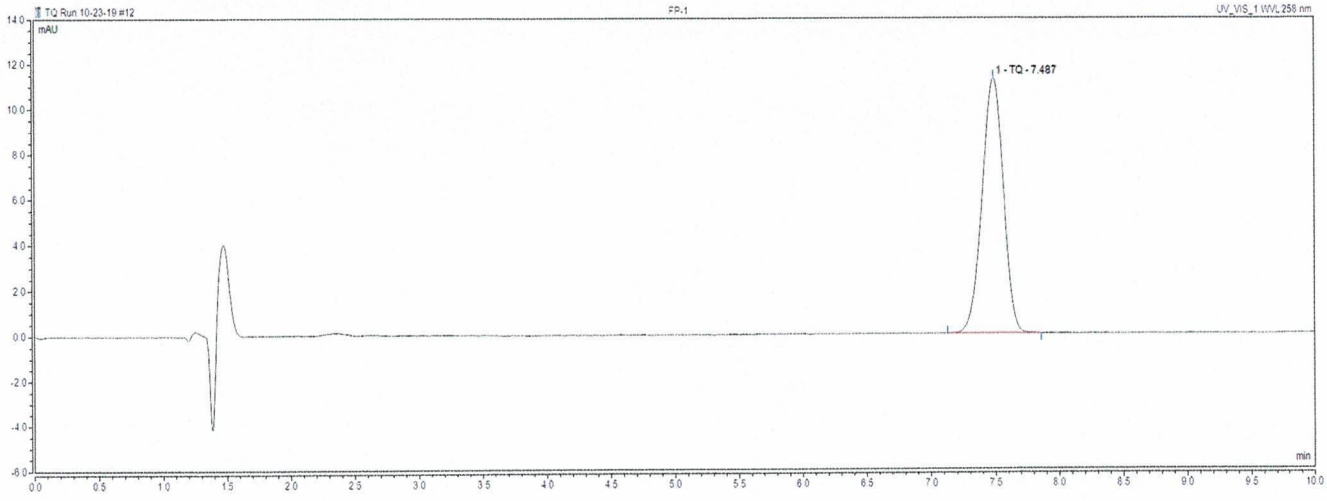
8.2 Typical Working Standard Chromatogram



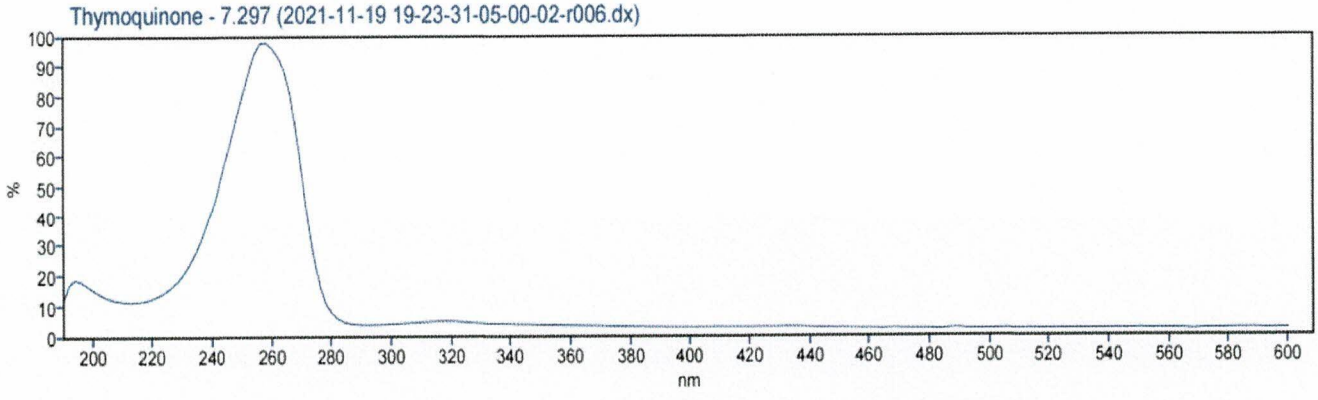
8.4 Typical Raw Material Chromatogram



8.5 Typical Finished Product Chromatogram



8.6 Thymoquinone UV Spectrum



9.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	02/12/20	New	N/A	C. Perry
1	04/04/23	Add instruction to follow test details in the product profile for standard and sample preparation, modify sample preparation section to outline specific instructions for different sample types.	CC-23-0177	S. Sassman