1.0 Purpose

This document describes the analytical procedure for the determination of Ascorbic Acid (AA) in raw materials and finished products.

2.0 Scope

This procedure applies to the identification and quantification of AA in raw materials and finished products. This method was validated under Protocol PRTCL-20-0002.

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 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 **AA** – Ascorbic Acid

4.4 **ACN** – Acetonitrile

4.5 **TFA** – Trifluoroacetic Acid

4.6 **EDTA** – Ethylenediaminetetraacetic Acid

4.7 **ACS** – American Chemical Society

4.8 **HPLC** – High Performance Liquid Chromatography

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

5.0 References

5.1 PRTCL-20-0002, Protocol, Ascorbic Acid Determination by HPLC using UV/Vis Spectroscopy

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

6.0 Supplies

6.1 Chemicals – All reagents are ACS grade or better.

6.1.1 Milli-Q Water

6.1.2 ACN

6.1.3 TFA

6.1.4 Ascorbic Acid Reference Standard

6.1.5 EDTA Disodium Dihydrate

6.1.6 Sodium Phosphate Monobasic Dihydrate

6.1.7 Phosphoric Acid

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 or Glass Fiber Syringe Filters (Note: It is recommended that chewable gel samples be filtered using glass fiber 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 Mobile Phase A: Add 200 µL of TFA to 1000 mL of water and mix well.

7.1.1.2 Mobile Phase B: ACN

7.1.2 Extraction Solvent / Diluent

7.1.2.1 Dissolve 2.34g Sodium Phosphate Monobasic Dihydrate and 0.56g ETDA Disodium Dihydrate in 1000ml water then pH to 3.0 with Phosphoric Acid.

7.1.3 Preparations may be scaled as necessary

7.2 Standard Prep

7.2.1 Accurately weigh and transfer about 45 mg of AA reference standard into a 100-mL volumetric flask. Add 50mL of Diluent and briefly sonicate until all AA is dissolved. (**Caution:** Perform this step quickly as AA is air sensitive.)

7.2.2 Dilute to volume with Diluent and mix well – this is the AA Stock. Dilute the AA Stock 1:10 with Diluent – this is the AA Working Standard.

7.2.3 Alternative standard preparations are acceptable as long as the preparations are within the linear range of this method, 0.01856 – 0.09280 mg/mL

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 for the analytical method is 0.01856 – 0.09280 mg/mL.

7.3.3 For finished products, extract sufficient sample with Diluent in order to generate an AA concentration that is within the validated linearity range. (**Caution:** Some finished products contain alkaline excipients in quantities capable of overwhelming the buffer capacity of the Diluent. Make sure that extraction sample solutions never exceed a pH of 3.0.) When analyzing gummies, powder and dissolve dosage forms as detailed in D-793 Cryogenic Grinding of Chewable Gels.

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 Samples can be dissolved in Diluent at any volume starting from 100mL. The volume chosen must be in the solubility range of AA (validated at approximately 0.4 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 AA concentration into the linear range.

7.3.6 Fill the flask to about 50% of the calculated volume with Diluent and shake mechanically for 10 minutes. (Note: finished products that are liquids, along with chewable gel samples that have been previously powdered and dissolved in Diluent as per D-793 Cryogenic Grinding of Chewable Gels do not require mechanical shaking.) QS to volume with Diluent.

7.3.7 Perform further dilutions as required using Diluent. Filter a 5ml aliquot for analysis, discarding the first 3-4ml of filtrate.

7.4 HPLC Parameters

7.4.1 Column: Phenomenex Kinetex XB-C18, 4.6 x 150mm, 2.6µm (Or Equivalent)

7.4.2 Column Temperature: 30°C

7.4.3 Flow rate: 0.7 mL/min

7.4.4 Mobile Phase Gradient:

Time, min	% A	% B
0.0	100	0
1.0	100	0
6.0	95	5
6.1	100	0
12.0	100	0

7.4.5 Wavelength: 254 nm

7.4.6 Injection Volume: 20 µL

7.4.7 Run Time: 12 minutes (Note: Some finished products may require intermittent blank or flush injections in order to remove more highly retentive formulation constituents that could interfere with subsequent injections.)

7.4.8 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 at least five (5) injections of AA Working Standard.

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 and/or at the end of a run.

7.6 System Suitability Requirements

7.6.1 The %RSD of five (5) consecutive 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 claim or raw material % assay:

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

R_u Sample peak area

R_s Mean (n=5) standard peak area

Wt_{std} Weight of the reference standard (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 (mg)

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

SS Serving size: Average weight of ten dosage units for tablets and chewable gels, fill weight for capsules, mass of a single serving for powders, volume of a single serving from the theoretical formula in mL for liquids, or 1 for raw materials.

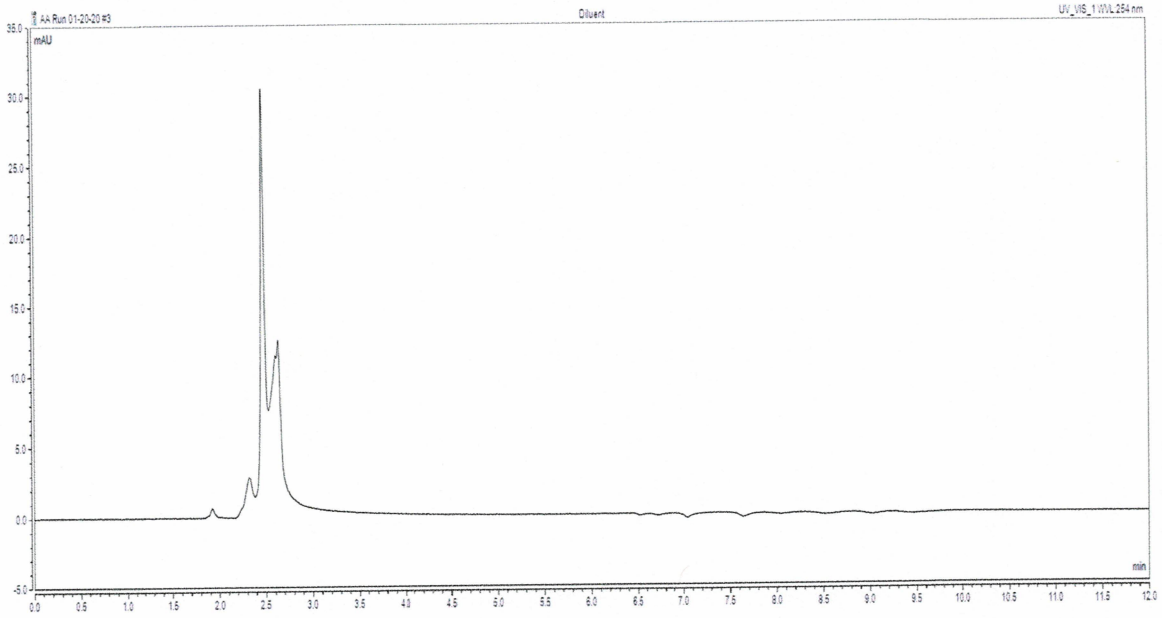
LA Label amount of AA (use 1 for raw materials)

7.8 System Wash, Column Wash and Column Storage

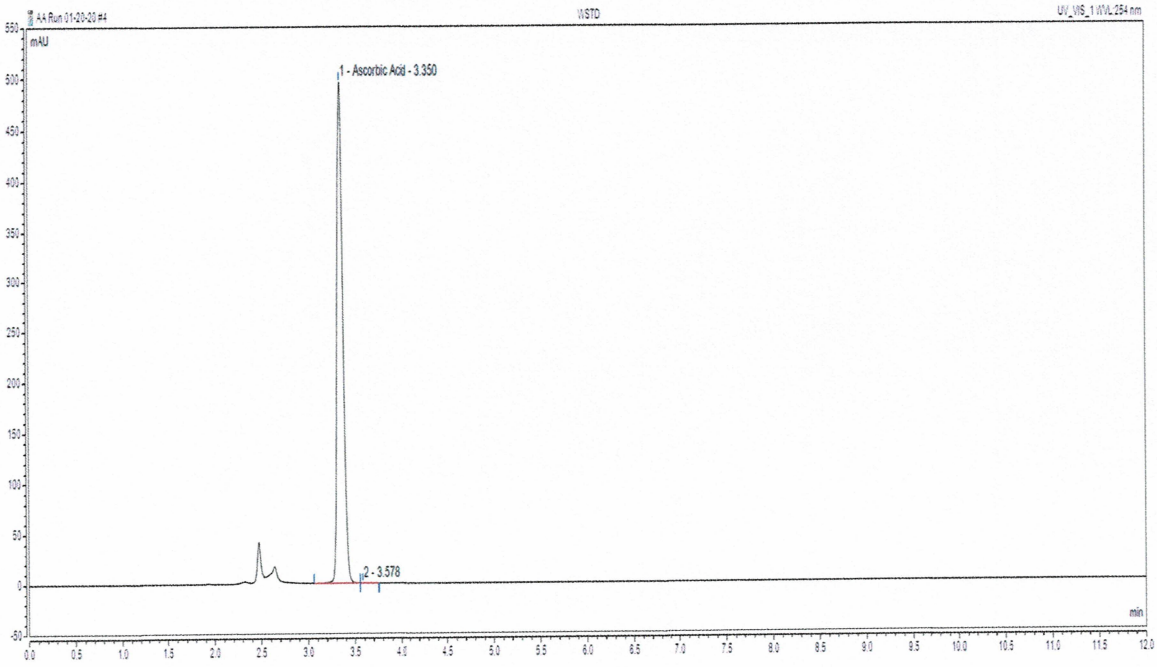
7.8.1 Wash and store the column in 50:50 ACN / Water.

8.0 Chromatograms

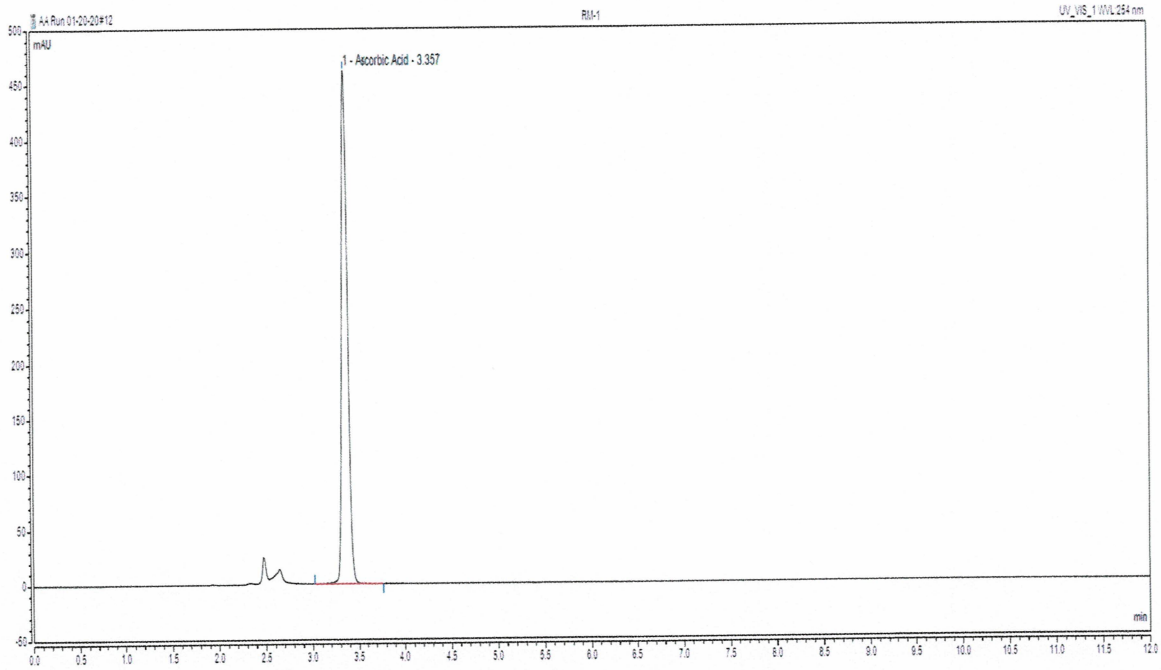
8.1 Typical Diluent Chromatogram



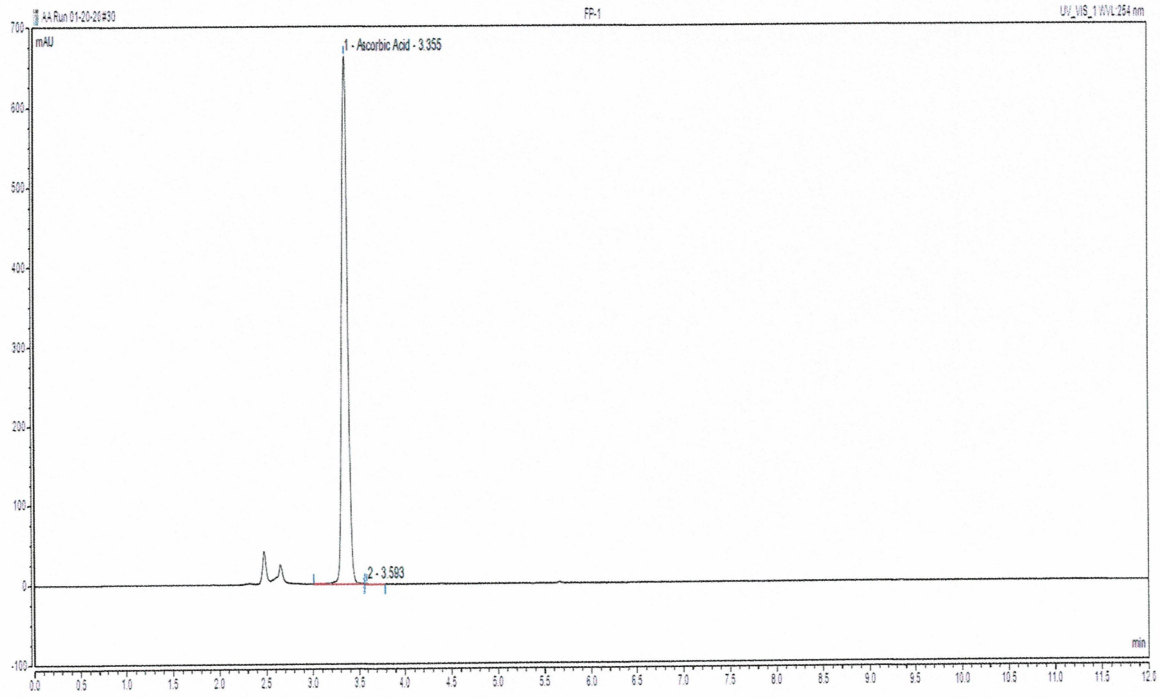
8.2 Typical Working Standard Chromatogram



8.3 Typical Raw Material Chromatogram



8.4 Typical Finished Product Chromatogram



9.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	02/12/20	New	N/A	C. Perry
1	12/20/22	Add sample test details information. Minor edits.	CC-22-0475	J. Sassman