1.0 Purpose

This document describes the analytical procedure for the determination of 5-Hydroxytryptophan, Tryptophan and Melatonin in raw materials and finished products.

2.0 Scope

This procedure applies to the identification and quantification of 5-Hydroxytryptophan, Tryptophan and Melatonin in raw materials and finished products. This method was validated under protocol PRTCL-23-0013.

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 **ACN** – Acetonitrile

4.3 **H_2O** – Milli-Q Water

4.4 **H_3PO_4** – Phosphoric Acid

4.5 **ACS** – American Chemical Society

4.6 **HPLC** – High Performance Liquid Chromatography

4.7 **UV-Vis** – Ultraviolet-Visible (Detection)

5.0 References

5.1 PRTCL-23-0013, Validation of an Analytical Method for the Determination of 5-Hydroxytryptophan, Tryptophan and Melatonin by HPLC-UV

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 H_3PO_4

6.1.4 5-Hydroxytryptophan Reference Standard

6.1.5 Tryptophan Reference Standard

6.1.6 Melatonin 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 (Adjustable pipets may also be used.)

6.2.3 Weigh paper

6.2.4 Syringes with 0.45µ nylon syringe filters

6.3 Equipment

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

6.3.2 Analytical Micro Balance

6.3.3 Analytical Balance

6.3.4 Wrist Action Shaker

7.0 Procedure

7.1 Mobile Phase, Extraction Solvent & Diluent Preparation

7.1.1 Mobile Phase

7.1.1.1 Mobile Phase A: Combine 1000mL H_2O + 1000µL H_3PO_4 and mix well.

7.1.1.2 Mobile Phase B: Combine 1000mL ACN + 1000µL H_3PO_4 and mix well.

7.1.2 Extraction Solvent

7.1.2.1 Combine 600mL ACN + 400mL H_2O and mix well. Allow solution to warm to ambient temperature prior to use.

7.1.3 Diluent

7.1.3.1 Use H_2O.

7.2 Standard Prep

7.2.1 Prepare standard stock at ~0.1 mg/mL analyte in Extraction Solvent. Shake stock for 15 minutes at ~ ½ volume then QS to volume. Shake vigorously then dilute stock 1:10 with Diluent. Shake vigorously then filter a 5mL aliquot for analysis, discarding the first 3-4mL of filtrate.

7.3 Sample Preparation

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

7.3.2 The validated linear ranges for the analytical method are 0.00614 – 0.0143 mg/mL 5-Hydroxytryptophan, 0.00622 – 0.0145 mg/mL Tryptophan and 0.00614 – 0.0143 mg/mL Melatonin.

7.3.3 Pool at least 10 dosage units and homogenize as appropriate (e.g. grind tablets / capsule fill / powders / stick pack contents by mortar and pestle, cryogenically powder and dissolve gummies, etc.) Extract sufficient sample (based on the raw material manufacturer assay value / product profile) with Extraction Solvent in order to generate a sample stock that is ~0.1 mg/mL analyte. Shake for 15 minutes at ~ ½ volume then QS to volume. Dilute stock 1:10 with Diluent. Shake vigorously then filter a 5mL aliquot for analysis, discarding the first 3-4mL of filtrate.

7.4 HPLC Parameters

7.4.1 Column: Supelco Ascentis Express C_8, 2.7µm (SPP), 90Å, 4.6 x 100mm

7.4.2 Column Temperature: 30°C

7.4.3 Flow rate: 0.5 mL/min

7.4.4 Run Time: 20 minutes

7.4.5 Mobile Phase: Gradient

Time, min	%A	%B
0	95	5
5	95	5
15	50	50
15.1	95	5
20	95	5

7.4.6 Wavelength: 220 nm

7.4.7 Injection Volume: 5 µL

7.4.8 Suggested 3-D Spectral Range (for Identification): 210nm - 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 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 the run.

7.6 System Suitability Requirements

7.6.1 The %RSD of five (5) consecutive standard injections is NMT 2%.

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

7.7 Example calculations for determining % Assay / LC:

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

R_u Sample peak area

R_s Mean (All) standard peak area

Wt_{std} Weight of the reference standard, mg

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

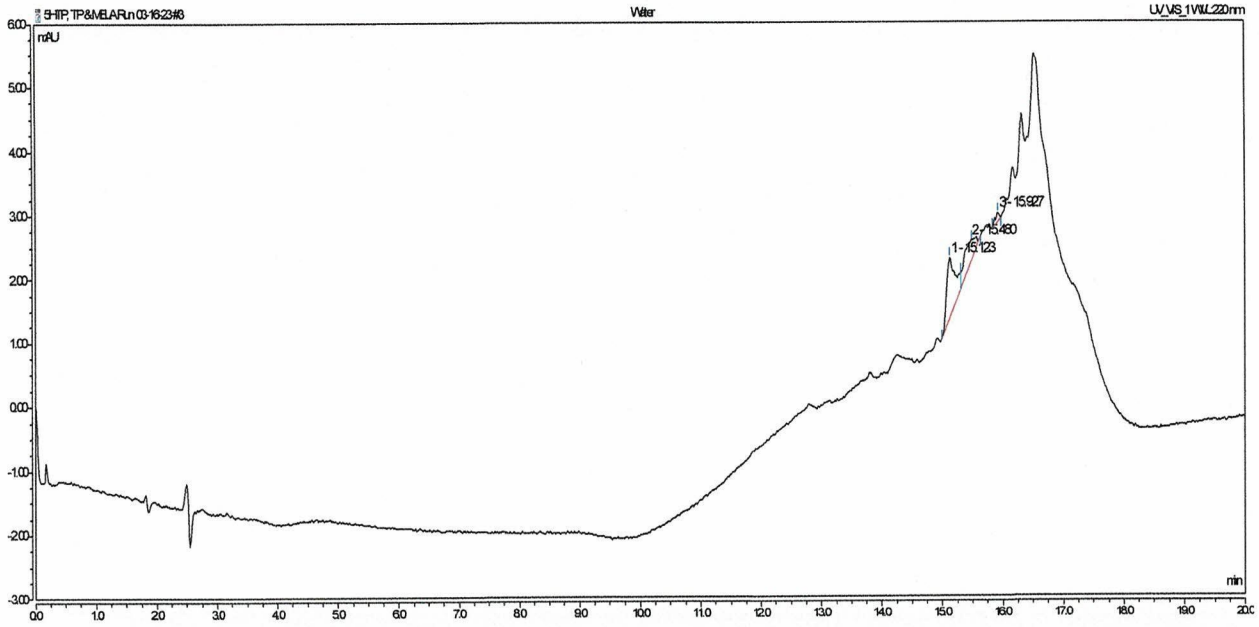
P	Purity of the reference standard in decimal format
SA	Sample amount, g
V_{spl}	Volume of the sample preparation accounting for dilutions, mL
SS	Serving Size, g (Use "1g" for Raw Materials.)
LA	Label Amount, mg (Use "1mg" for Raw Materials.)

7.8 System Wash, Column Wash and Column Storage

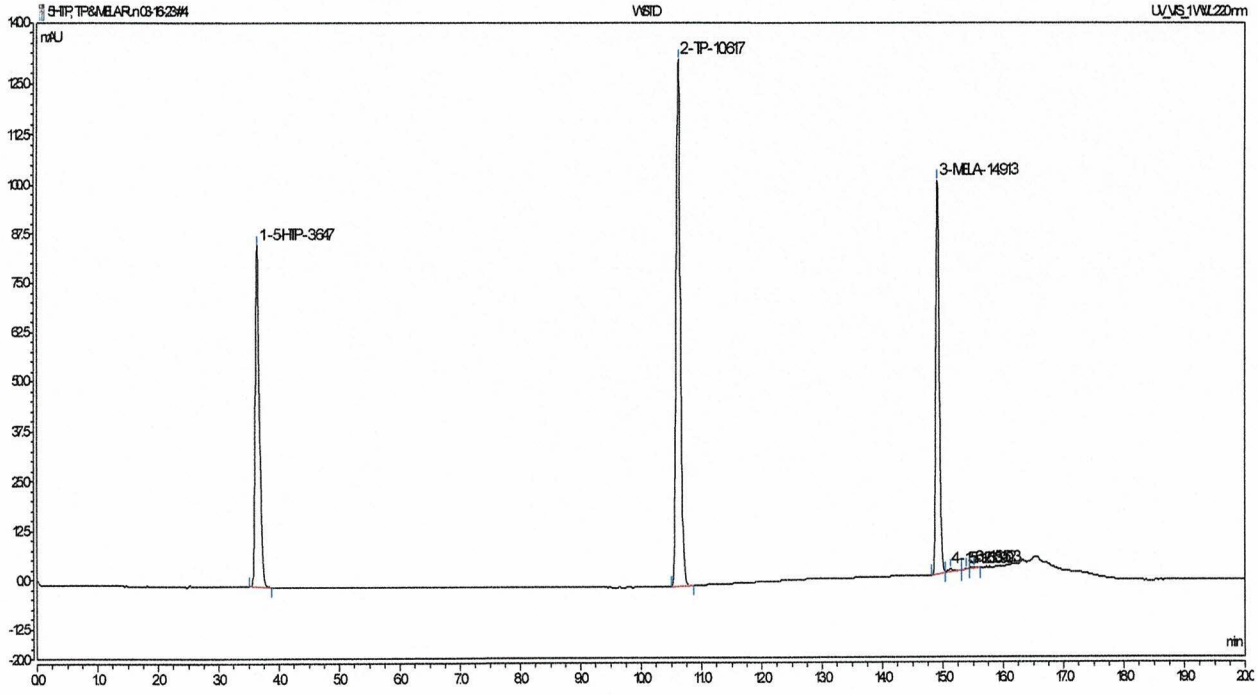
7.8.1 Wash and store the column in 60% ACN.

8.0 Chromatograms

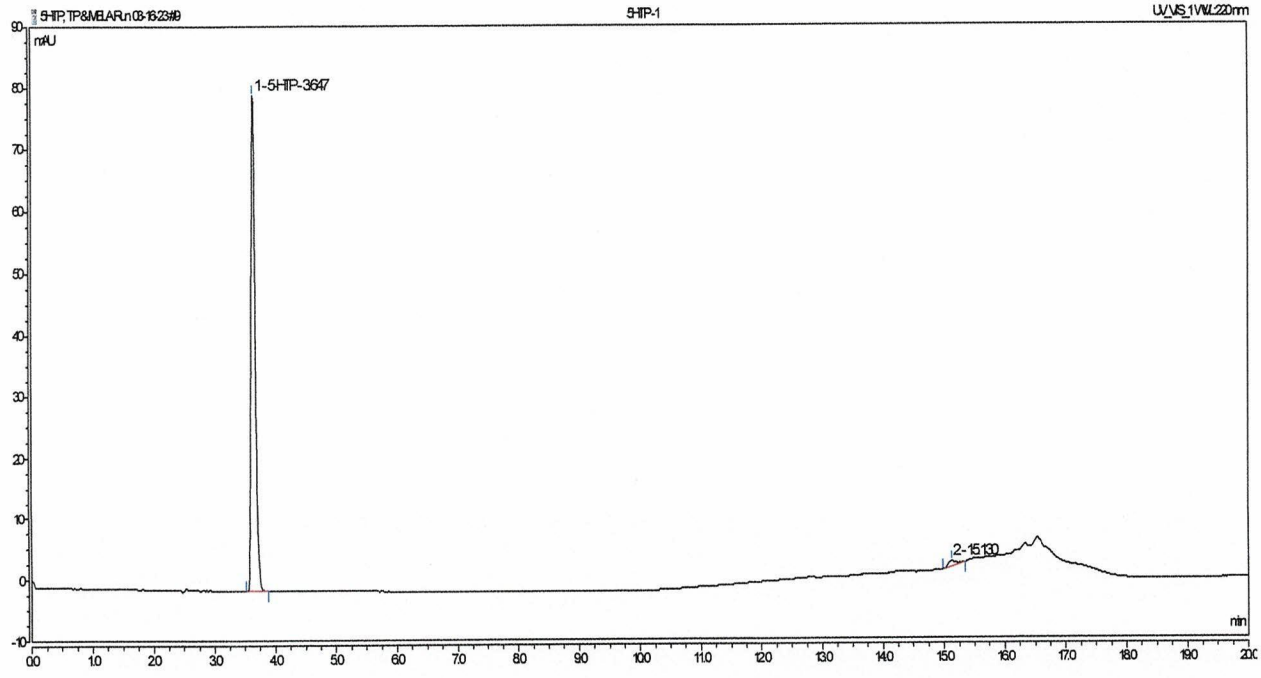
8.1 Typical Diluent Chromatogram

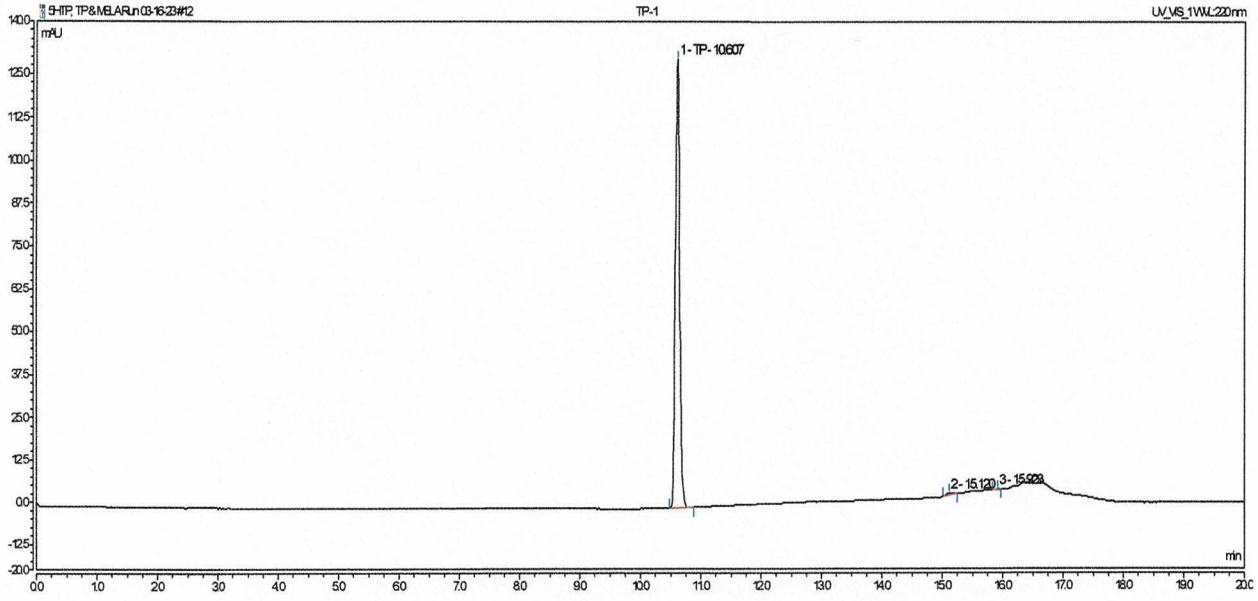


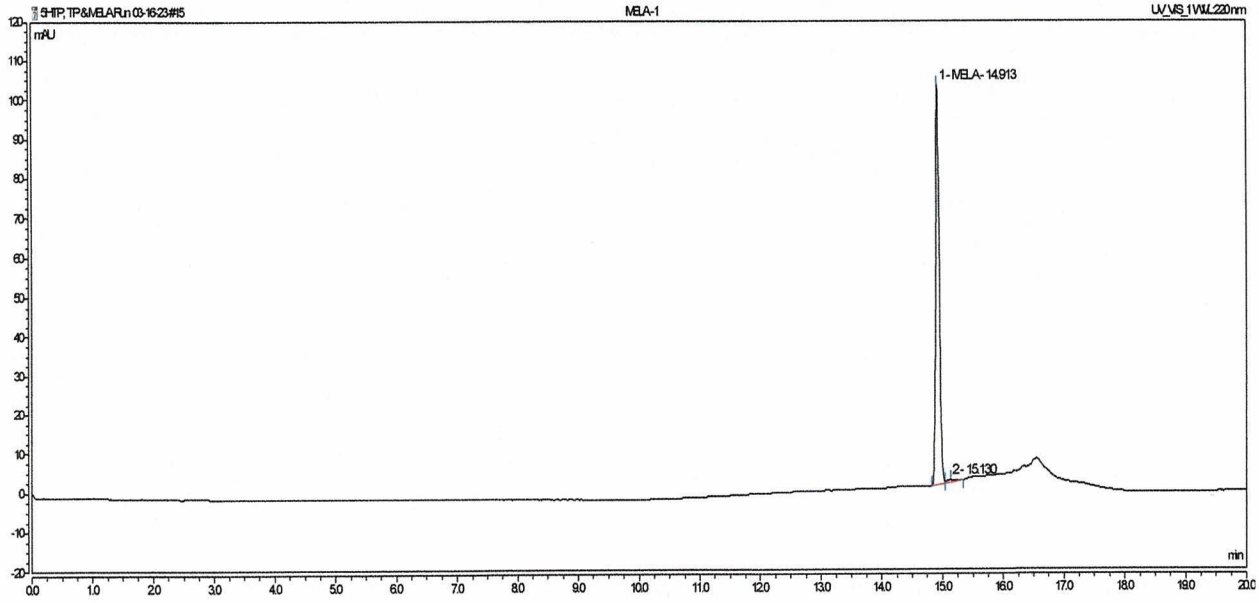
8.2 Typical Standard Chromatogram



8.3 Typical Raw Material Chromatograms







9.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	03/28/23	New procedure.	N/A	C. Perry