1.0 Purpose

The purpose of this procedure is to define the method for the quantitation and/or identification of melatonin in raw materials and finished product dietary supplements using HPLC and UV/VIS spectrophotometry.

2.0 Scope

This procedure applies to the quantification and identification of melatonin in raw materials and finished products. Melatonin is a good chromophore and was measured at 222 nm. Other wavelengths should not be used without justification.

3.0 Responsibility

3.1 It is the responsibility of QC and Analytical Chemists to follow this procedure.

3.2 It is the responsibility of QC Laboratory Management to ensure that this procedure is being followed.

3.3 It is the responsibility of QC Laboratory Management and/or Analytical Development to keep this procedure aligned with current practices.

4.0 Definitions

4.1 **HPLC** – High Performance Liquid Chromatography

4.2 **UV/VIS** – Ultraviolet and Visible Electromagnetic Spectrums

4.3 **KH_2PO_4** – Monobasic Potassium Phosphate

4.4 **H_3PO_4** – Phosphoric Acid

4.5 **ACN**– Acetonitrile

4.6 **H_2O** – Water (≥18.2 MΩ·cm)

4.7 **Melatonin** – N-acetyl-5-methoxy tryptamine

5.0 References

5.1 USP41-NF36

6.0 Supplies

6.1 Chemicals: All reagents are HPLC grade or better.

6.1.1 H_2O

6.1.2 ACN

6.1.3 H_3PO_4

6.1.4 KH_2PO_4

6.1.5 Melatonin reference standard

6.2 Glassware

6.2.1 Volumetric glassware as required for standard and sample preparations

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 Centrifuge

6.3.4 Adjustable Pipette

6.4 Disposables (as required for standard and sample preparations)

6.4.1 10 mL, 1 mL, and 200 µL Pipette Tips

6.4.2 Microcentrifuge tubes

6.4.3 Disposable Plastic Luer Lock Syringe – 3mL, 6mL, or 10mL

6.4.4 Nylon Syringe Filters, 0.45 µm

6.4.5 Weigh paper or Weigh boat

7.0 Preparation of Mobile Phase, Dissolution Buffer, Samples, and Standards

7.1 Mobile Phase A – Acetonitrile

7.2 Mobile Phase B – 0.5g/L of KH_2PO_4 (aq) adjusted to pH 3.5 with H_3PO_4

7.2.1 Transfer 500 mg of KH_2PO_4 to a 1L volumetric flask.

7.2.2 Add about 950 mL H_2O, and adjust the pH to 3.5 with H_3PO_4.

7.2.3 Dilute to final volume with water.

7.3 Diluent– Mobile Phase A and Mobile Phase B (25:75)

7.4 Standard Preparation

7.4.1 Accurately weigh and transfer about 25 mg of reference standard into a 50-mL volumetric flask, dilute to volume using Diluent, and sonicate for 10 min. **Allow to equilibrate to room temperature before performing further dilutions.** This is the Stock Standard.

7.4.2 Transfer 5.0 mL Stock Standard into a 25-mL volumetric flask, and dilute to volume using Diluent. This is the Working Standard.

7.5 Sample Preparation

7.5.1 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.

7.5.2 Immediate Release Melatonin

7.5.2.1 Raw materials can be dissolved in Diluent at any volume starting from 50 mL and any weight greater than the minimum weight of the analytical balance.

7.5.2.2 The sample is suspended in the final volume and put in the sonicator bath for 10 minutes.

7.5.2.3 Before injection, insoluble matter should be removed via filtration using a nylon syringe filter. Discard at least 0.5 mL of the filtered

sample before collecting filtrate. Dilute filtrate as needed then add 1 mL of the final dilution to an HPLC vial for analysis.

7.5.2.3.1 Alternatively, samples and standards can also be centrifuged at 10,000 RPM for 5 minutes to pellet insoluble matter.

7.5.3 Sustained Release Melatonin (Microactive Melatonin)

7.5.3.1 Raw materials and finished products can be dissolved in 100% ACN at any volume starting from 50 mL and any weight greater than the minimum weight of the analytical balance. Before further dilution, filter using a nylon syringe filter. Discard at least 0.5 mL of the filtered sample before collecting filtrate. This is the stock sample preparation.

7.5.3.2 The filtered stock sample preparation is suspended in the final volume at a 1:3 ratio of ACN: Mobile Phase B. **The working sample preparation MUST be prepared in a diluent matrix of 25:75 ACN: Phosphate Buffer.** For example, 25mL of Stock sample preparation (in 100% ACN) is transferred to a 100-mL volumetric flask, 65 mL of Mobile Phase B added, the solution equilibrated to room temperature, and the solution is diluted to the final volume with Mobile Phase B.

8.0 Test Conditions

8.1 Gradient

Time	%A	%B
0.00	25	75
9.00	25	75

8.2 Column – Phenomenex Luna, C18 (2), 5um, 100Å, LC column, 150mm x 4.6mm, or equivalent.

8.3 Flow Rate – 1.0 mL/min

8.4 UV Detection – 222 nm

8.5 Injection Volume – 10 µL

8.6 Column Temperature – 25 °C

8.7 Suggested Sequence

8.7.1 Perform at least two injections of a Blank (Diluent)

8.7.2 Perform five injections of the Working Standard.

8.7.3 Perform a single injection of each Sample Preparation

8.7.4 After every six samples and at the end of the run, perform a single injection of the Working Standard.

8.8 Column Wash and Storage

8.8.1 Rinse the column with at least 15 mL of H_2O / ACN (75/25).

8.8.2 Rinse the column with at least 10 mL of H_2O / ACN (50/50).

8.8.3 Store the column with H_2O / ACN (50/50).

9.0 System Suitability

9.1 The %RSD of the first five (5) standard injections is NMT 2.0%

9.2 The %RSD of all Working Std A injections is NMT 2%.

10.0 Example Calculations

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

R_u Sample peak area

R_s Mean standard peak area

Wt_{std} Weight of 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 (solids) or mL (liquids)

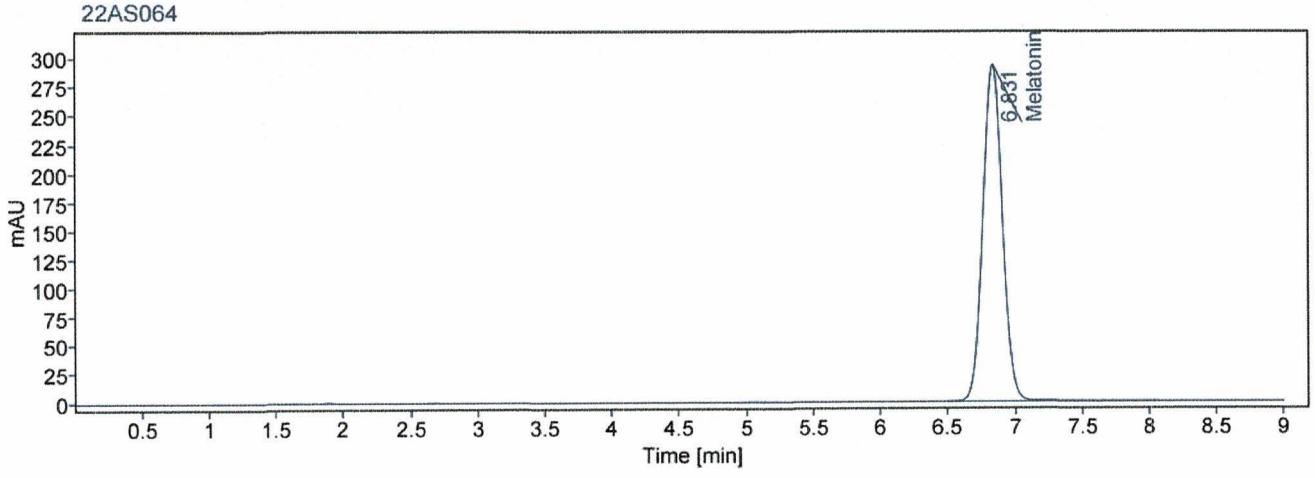
V_{spl} Volume of the sample preparation accounting for dilutions in mL

SS Serving size: Weight of a single dosage unit in mg for tablets and capsules, volume of a single serving from the theoretical formula in mL for liquids, or 1 for raw materials.

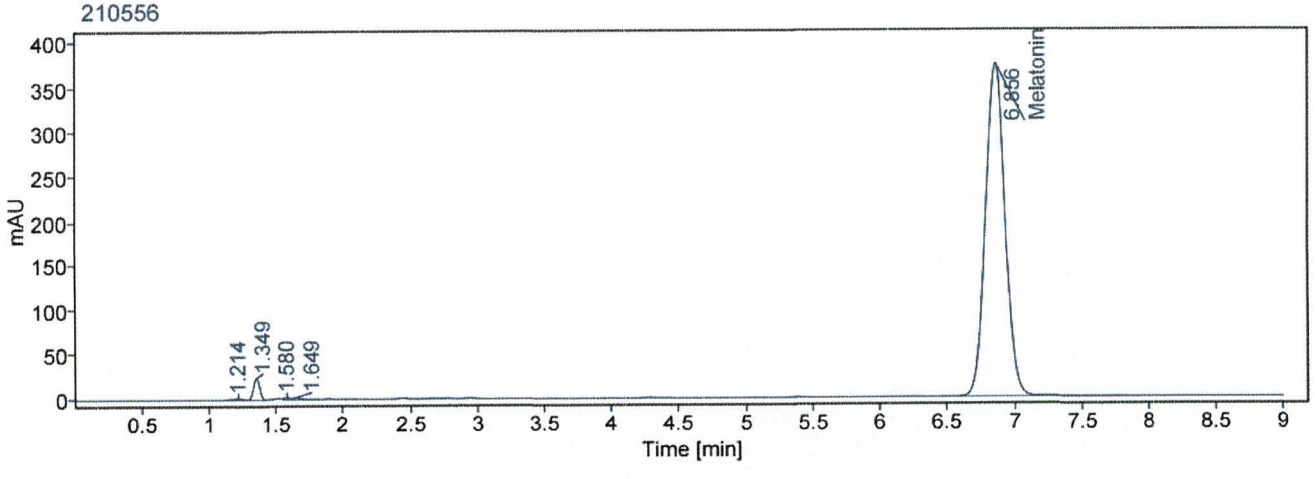
LA Label amount in mg per dose or 1 for raw materials

11.0 Example Chromatography

11.1 Standard



11.2 Sample



12.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	01/02/19	New procedure.	N/A	J. Maignan
1	02/03/22	Added additional detail regarding Sustained Release Melatonin	CC-22-0057	J. Sassman
2	03/09/22	Update to reflect current Ion Labs practices. Update for consistency with current method format.	CC-22-0107	S. Sassman
3	04/11/23	Update for consistency with current methods, add instruction to follow product specific sample preparation from the test details section of product profile, make standard preparation fixed concentration.	CC-23-0183	S. Sassman