1.0 Purpose

This document describes the analytical procedure for the determination of N-Acetyl Tyrosine in raw materials and finished products.

2.0 Scope

This procedure applies to the identification and quantification of N-Acetyl Tyrosine in raw materials and finished products.

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 the QC Laboratory Management and/or Analytical Development 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 **TFA** – Trifluoroacetic Acid

4.4 **ACN** – Acetonitrile

4.5 **NAT** – N-Acetyl Tyrosine

4.6 **HPLC** – High Performance Liquid Chromatography

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

5.0 References

5.1 MV-LAB-19-123, Protocol, N-Acetyl Tyrosine 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

6.1.3 ACN

6.1.4 TFA

6.1.4 NAT Reference Standard

6.2 Supplies and Glassware

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

6.2.2 Mobile phase containers

6.2.3 Volumetric glassware and/or adjustable pipettes and tips

6.2.4 Weigh paper or funnels

6.2.5 Syringes with 0.45 µm nylon syringe filters

6.2.6 Micro-centrifuge tubes

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 Micro-centrifuge

6.3.4 Sonicator bath

6.3.5 Wrist Action Shaker

7.0 Procedure

7.1 Mobile Phase, Extraction Solvent & Diluent Preparation

7.1.1 Mobile Phase A – 0.1% TFA

7.1.1.1 Combine 1 mL of TFA with 1000 mL of Milli-Q Water. Mix well.

7.1.2 Mobile Phase B – ACN

7.1.3 Extraction Solvent – 50:50 Water / ACN

7.1.3.1 Combine 500ml of ACN with 500 mL of Milli-Q Water. Mix well.

7.1.4 Diluent – 90:10 Water / ACN

7.1.4.1 Combine 100ml of ACN with 900 mL of Milli-Q Water. Mix well.

7.1.5 Preparations may be scaled as necessary

7.2 Standard Prep

7.2.1 Accurately weigh and transfer about 50 mg of NAT reference standard into a 50-mL volumetric flask. Add 25mL of Extraction Solvent. Sonicate for 5min, equilibrate to room temperature, then dilute to volume with Extraction Solvent. This is the Stock Standard.

7.2.2 Transfer 2.5 mL of the Stock Standard to a 50-mL volumetric flask, and dilute to volume using Diluent. This is the 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.

7.3.2 The validated range for the analytical method is 2.3 – 93.0 mcg/mL.

1.1.1 For raw materials: weigh no less than 25 mg into a suitably sized volumetric flask of no less than 25 mL volume to generate an analyte concentration that is

within the validated linearity range. Add Extraction Solvent to ½ of the flask volume, shake mechanically for 15 min, sonicate for 5 min, equilibrate to room temperature, and dilute to volume with Extraction Solvent.

1.1.2 For solid and liquid dose finished products: Combine and homogenize no less than ten dosage units. Based on the label claim and fill weight (capsules), serving size (powders and liquids) or tablet weight per dose, weigh no less than 100 mg of the pooled dosages into a suitably sized volumetric flask of no less than 25 mL to generate an analyte concentration that is within the validated linear range. Add Extraction Solvent to ½ of the flask volume, shake mechanically for 15 min, sonicate for 5 min, equilibrate to room temperature, and dilute to volume with Extraction Solvent.

1.1.3 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 pooled and homogenized dosages into a beaker. Use several small portions of Extraction Solvent to completely transfer the sample into a suitably sized volumetric flask to generate an analyte concentration that is within the validated linear range. Add Extraction Solvent to ½ of the flask volume, shake mechanically for 15 min, sonicate for 5 min, equilibrate to room temperature, and dilute to volume with Extraction Solvent.

1.1.4 To manage large volumes, the standard can be initially prepared at a higher concentration that is within the solubility range of NAT (validated at 0.5 mg/mL) and further diluted into the linear range using Diluent. Dilutions can be made using volumetric glassware and/or adjustable pipettes. Dilutions can be prepared in HPLC vials.

7.3.3 Centrifuge at 10,000 rpm for 5 min prior to HPLC analysis to remove particulates. Alternatively, the sample may be filtered through a 0.45 µm membrane discarding the first 3 – 4 mL.

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: 1 mL/min

7.4.4 Wavelength: 277 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

7.4.8 Mobile Phase Gradient

Time, min	% A	% B
0-3	90	10
3-6	80	20
6-10	90	10

7.5 Recommended Sequence

7.5.1 Make at least 2 injections 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 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

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

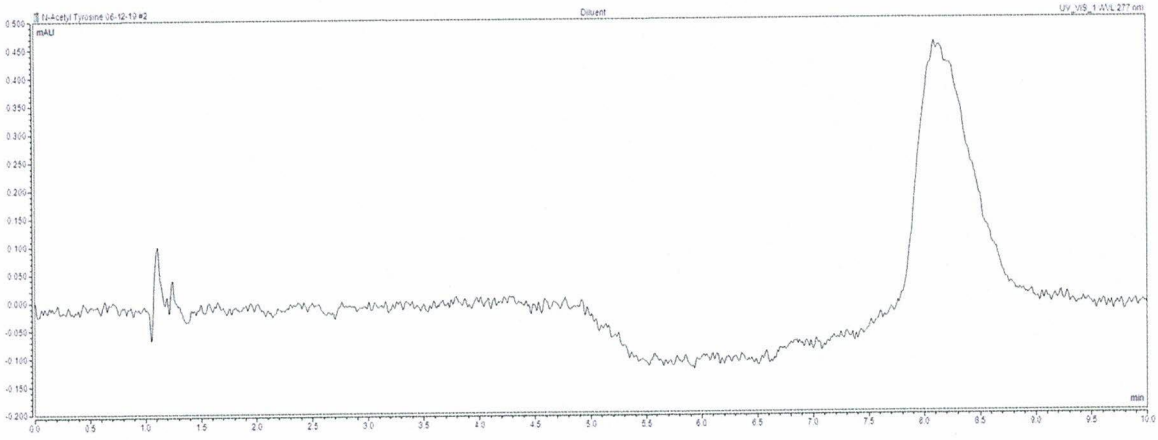
R_u	Sample peak area
R_s	Mean peak area of all standard injections
Wt_{std}	Weight of the reference standard in mg (corr. for water if applicable)
V_{std}	Volume of the standard preparation accounting for dilutions in mL
P	Purity of the reference standard in decimal format
SA	Sample amount in mg
V_{spl}	Volume of the sample preparation accounting for dilutions in mL
SS	Serving size: Weight of a single dosage unit or 1 for raw materials.
LA	Label amount in mg of analyte or 1 for raw materials

7.8 Column Wash and Storage

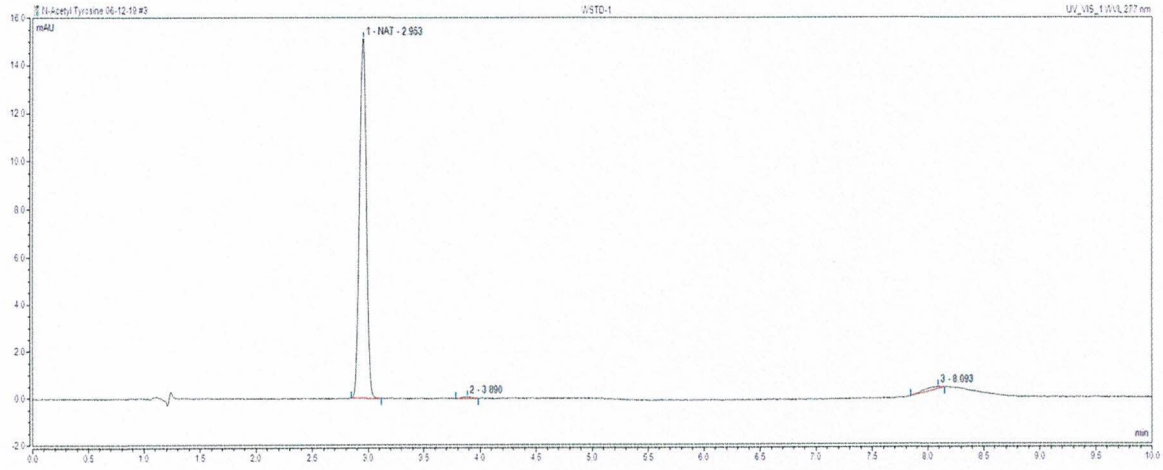
7.8.1 Wash and store the column in 50:50 ACN / Milli-Q Water.

8.0 Example Chromatograms

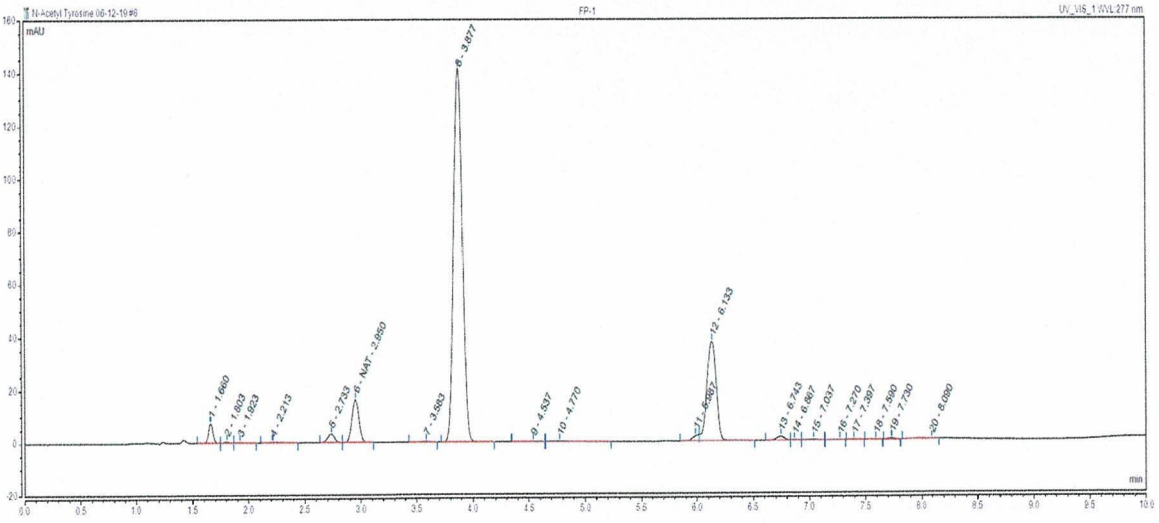
8.1 Typical Diluent Chromatogram



8.2 Typical Working Standard Chromatogram



8.3 Typical Sample Chromatogram



9.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	06/26/19	New	N/A	C. Perry
1	06/22/22	Updated logo and format.	CC-22-0285	K. Burris
2	06/06/23	Minor edits for clarity, add instruction to follow product specific sample preparation in test details, add specific sample prep instructions for different dosage forms,	CC-23-0267	S. Sassman