1.0 Purpose

The purpose of this procedure is to define the method for the quantitation and/or identification of nobiletin 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 nobiletin in raw materials and finished products. Nobiletin is a good chromophore and was measured at 280, other wavelengths can be used if interferences are present.

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

4.2 **KH_2PO_4** – Monobasic Potassium Phosphate

4.3 **H_3PO_4** – Phosphoric Acid

4.4 **ACN** – Acetonitrile

4.5 **H_2O** – Water (≥ 18.2MΩ·cm, 0.22µm filtered)

4.6 **Nobiletin** – 2-(3,4-Dimethoxyphenyl)-5,6,7,8-tetramethoxychromen-4-one

4.7 **QC** – Quality Control

5.0 References

5.1 USP41-NF36

5.2 Analysis of Synephrine in the Peel of Citrus Fruit, Immature Citrus Fruit and Decoctions of Chinese Medicinal Prescriptions Containing these Crude Drugs by Capillary Electrophoresis

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 Nobiletin reference standard

6.2 Glassware

6.2.1 HPLC vials, 12mm x 32mm with screw cap enclosures with septa

6.2.2 Scintillation Vials

6.2.3 Mobile Phase Containers

6.2.4 Volumetric glassware as required by standard and sample preparations

6.3 Disposables

6.3.1 10mL Pipette Tips

6.3.2 1mL Pipette Tips

6.3.3 200µL Pipette Tips

6.3.4 1.5mL centrifuge tubes

6.3.5 16mL Test Tubes

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

6.3.7 Nylon Syringe Filters, 0.2µm or 0.45µm

6.3.8 Weigh paper

6.4 Equipment

6.4.1 Suitable gradient HPLC system consisting of a pump, autosampler, column oven and UV detector with a chromatographic data handling system

6.4.2 Analytical Balance

6.4.3 Ultrasonic bath

6.4.4 Vortex

6.4.5 Stir Plate

6.4.6 Eppendorf Centrifuge

6.4.7 Adjustable Pipette(s)

7.0 Preparation of Mobile Phase, Diluent, 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 Prepared by adding 500mg KH_2PO_4 to a 1L volumetric flask and adding about 950mL H_2O. Adjust pH to 3.5 with H_3PO_4. After pH is adjusted, dilute to 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 6.25 mg of reference standard into a 25-mL volumetric flask.

7.4.2 Dissolve in and dilute to volume using Diluent.

7.4.3 Standard preparation may be scaled up if necessary.

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.

7.5.1 For raw materials: weigh no less than 25 mg into a suitably sized volumetric flask of no less than 25 mL. Dilute to volume using Diluent, and sonicate for 15 minutes.

7.5.2 For solid dose finished products: Combine and homogenize no less than ten dosage units. Based on the label claim and fill weight (for capsules) or tablet weight per dose, weigh no less than 25 mg of the pooled dosages into a suitably sized volumetric flask of no less than 25 mL, dilute to volume using Diluent, and sonicate for 15 minutes.

7.5.3 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. Wipe the outside of the pipet, and rinse the pipet three times with Diluent collecting the rinses in the volumetric flask. Dilute to volume using Diluent.

7.5.4 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 glass beaker. Dissolve the sample in Diluent, transfer the dissolved sample into a suitably sized volumetric flask of no less than 25 mL volume using small portions of Diluent to ensure complete transfer, and dilute to volume with Diluent.

7.5.5 Perform further dilutions as required using Diluent.

7.5.6 Filter an aliquot of the sample solution through a 0.45 µm membrane discarding the first 2 - 3 mL of filtrate before collecting a portion in a vial for analysis.

7.5.6.1 Alternatively, the solution may be centrifuged to remove particulates provided that the final solution is clear.

8.0 Test Conditions

8.1 Gradient

Time	%A	%B
0.00	25	75
6.00	25	75

12.0	90	10
15.0	90	10
15.1	25	75
18.0	25	75

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

8.3 Flow Rate – 1.0mL/min

8.4 UV Detection – 280nm

8.5 Injection Volume – 10µL

8.6 Column Temperature – 25°C

8.7 Recommended 3-D Spectral Range – 200nm to 400nm

8.8 Recommended Sequence

8.8.1 Make at least 2 injections of a Blank (Diluent).

8.8.2 Make five injections of the Working Standard.

8.8.3 Make a single injection of each Sample Preparation.

8.8.4 Make a single injection of the Working Standard after every ten samples and at the end of the run.

8.9 System Suitability

8.9.1 The %RSD of five consecutive injections of the Working Standard is NMT 5.0%.

8.9.2 The %RSD of all standard injections is NMT 5%.

8.10 Recommended Column Wash and Storage

8.10.1 Rinse the column with H_2O / ACN (90/10) at 1 mL/min for at least 15 min.

8.10.2 Rinse the column with H_2O / ACN (50/50) at 1 mL/min for at least 10 min.

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

9.0 Calculations

9.1 Example calculations for determining finished product % label or raw material % purity

9.1.1 $$\% \text{ assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times \frac{SS}{LA} \times 100$$

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)

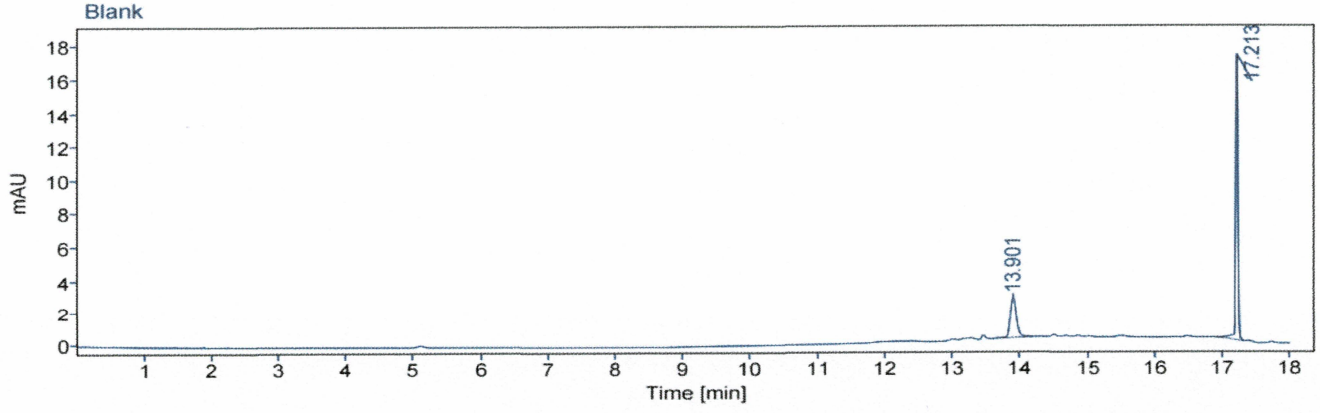
V_{spl} Volume of the sample preparation accounting for dilutions in mL

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.

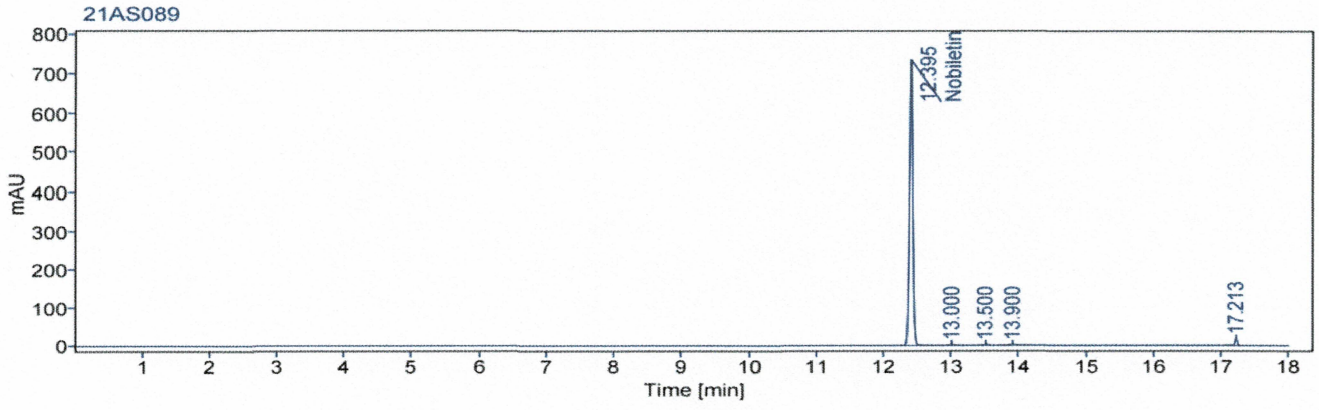
LA Label amount in mg per dose or 1 for raw materials

10.0 Example Chromatography

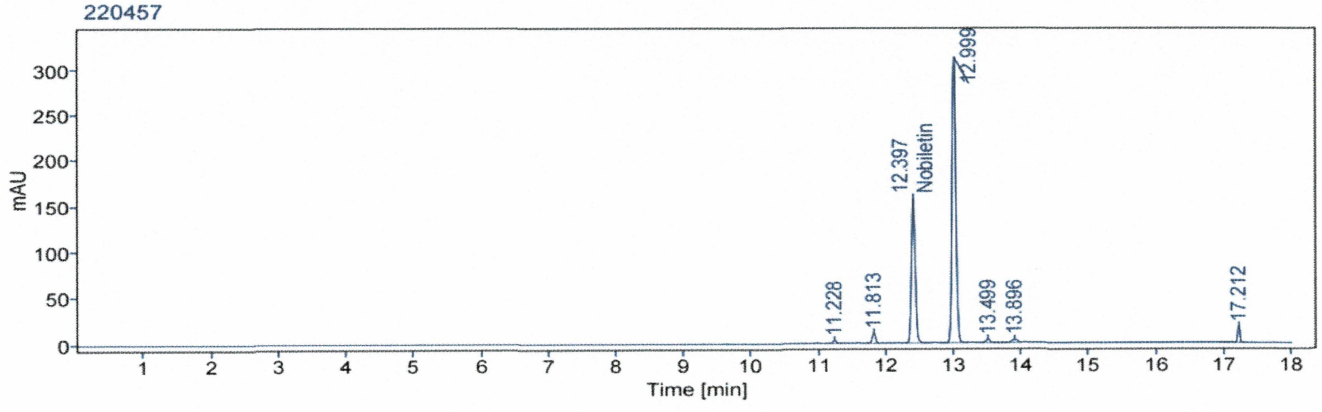
10.1 Blank



10.2 Working Standard



10.3 Sample



11.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	01/02/19	New	N/A	J. Maignan
1	03/20/23	Update for consistency with current laboratory practices, simplify standard preparation, add specific sample prep instructions for various dosage forms, add recommended sequence, add system suitability section, add column wash and storage recommendation, add example chromatography. Update logo and format.	CC-23-0144	S. Sassman