1.0 Purpose

The purpose of this procedure is to describe a method for the quantitative analysis and identification of baicalin, hesperidin and naringin in raw materials and finished products.

2.0 Scope

This procedure applies to the identification and quantification of Naringin, Hesperidin and Baicalin in raw materials and finished products.

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 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 to keep this procedure aligned with current practices.

4.0 Definitions

4.1 **ACN** – Acetonitrile

4.2 **H_3PO_4** –Phosphoric Acid

4.3 **MeOH** -Methanol

① wrong date SAS 04/08/24

4.4 **QC** – Quality Control

5.0 References

5.1 MV-LAB-18-062, Protocol, Baicalin Determination using HPLC with UV/VIS Spectroscopy

5.2 PRTCL-20-0096, Protocol, Verification of an Analytical Method for the Determination of Naringin by HPLC-UV

5.3 RPT-20-0050, Report, Verification of an Analytical Method for the Determination of Naringin by HPLC-UV

5.4 MV-LAB-18-064, Protocol, Hesperidin Determination Using HPLC with UV/Vis Spectroscopy

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

6.0 Reagents, Supplies, Glassware and Equipment

6.1 Reagents: all reagents are HPLC grade or better.

6.1.1 Millipore deionized water

6.1.2 ACN

6.1.3 Phosphoric Acid (H_3PO_4)

6.1.4 Baicalin Reference Standard

6.1.5 Hesperidin Reference Standard

6.1.6 Naringin Reference Standard

6.1.7 Methanol (MeOH)

6.2 Supplies and Glassware

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

6.2.2 1L mobile phase container

6.2.3 50mL, 100mL, 250mL, and 500mL volumetric flasks

6.2.4 50mL and 100mL beakers

6.2.5 200uL, 1mL, and 10mL pipette tips

6.2.6 1.5mL and 2.0mL micro centrifuge tubes

6.2.7 10mL plastic luer lock syringe

6.2.8 0.2 or 0.45µm 25mm Nylon syringe filters

6.2.9 22mL screw cap vials (scintillation)

6.2.10 Weigh boats

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 Vortex

6.3.4 Stir Plate

6.3.5 Wrist action shaker

6.3.6 Micro-centrifuge

6.3.7 200uL, 1mL and 10mL pipettes

7.0 Procedure

7.1 Mobile Phase and Diluent Preparation

7.1.1 Mobile Phase A (0.1% H_3PO_4 in H_2O) - is prepared by adding 1mL of H_3PO_4 to 1000mL of Millipore water and mixing thoroughly.

7.1.2 Mobile Phase B (ACN) - Acetonitrile

7.1.3 Extraction solvent/Diluent - Methanol

7.1.4 Preparations may be scaled as necessary

7.2 Standard Preparation

7.2.1 Accurately weigh and transfer about 25 mg of reference standard into a 100 mL volumetric flask. Add ~50 mL of Diluent and sonicate for five minutes.

7.2.2 Equilibrate to room temperature, dilute to volume with Diluent, and mix well – this is the reference standard Stock. Dilute the standard Stock as appropriate to fall within the respective validated linear range.

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 linear ranges are as follows:

7.3.2.1 0.01 – 0.1 mg/mL Baicalin w/ 20µL injection.

7.3.2.2 0.01 – 0.1 mg/mL Hesperidin w/ 20µL injection.

7.3.2.3 0.01 – 0.04 mg/mL Naringin w/ 5µL injection.

Note: When measuring Baicalin in skullcap root, ~10 drops of DMSO should be added to the flask to assure complete extraction of the analyte from the bulk powder.

7.3.3 Ensure that the Stock Sample is prepared at a concentration of no more than 0.25 mg/mL of the target analyte.

7.3.4 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. Fill the flask to about 50% of the chosen volume with Diluent and shake mechanically for 15 minutes. Sonicate for 5 minutes, cool to ambient then QS to volume with Diluent.

7.3.5 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. Fill the flask to about 50% of the chosen volume with Diluent and shake mechanically for 15 minutes. Sonicate for 5 minutes, cool to ambient then QS to volume with Diluent.

7.3.6 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 no less than 200 mg of the pooled and homogenized dosages into a suitably sized beaker. Add a volume of Diluent equivalent to 50% of the desired flask volume, add a stir bar, and stir until dissolved. Transfer the solution to a volumetric flask of size suitable to generate an analyte concentration that is

within the validated linear range. Use several small portions of Diluent to rinse any remaining residue from the beaker into the volumetric flask ensuring complete transfer, and dilute to volume using Diluent.

7.3.7 To manage large volumes, the sample can be initially dissolved in a smaller volume and a portion further diluted using Diluent to bring the analyte concentration into the linear range. Dilutions can be made using volumetric glassware and/or adjustable pipettes. Dilutions can be prepared in HPLC vials.

7.3.8 Filter through a 0.45 µm membrane discarding the first 3 – 4 mL before collecting a portion for analysis, or centrifuge an aliquot at 10,000 rpm for 5 min to remove particulates.

7.4 HPLC Parameters

7.4.1 Column: Phenomenex Kinetex XB-C18, 4.6 x 250mm, 5µm (Or Equivalent)

7.4.2 Column Temperature: 45°C

7.4.3 Flow rate: 1.0 mL/min

7.4.4 Mobile Phase: Gradient

7.4.4.1

Time	%A	%B
0.0	80	20
10.0	80	20
14.1	63	37
25.0	63	37
25.1	80	20

30.0 80 20

7.4.5 Wavelength: Baicalin & Hesperidin – 270 nm, Naringin – 285 nm

7.4.6 Injection Volume: Baicalin & Hesperidin – 20 µL, Naringin - 5 µL

7.4.7 Run Time: 30 minutes

7.4.8 Recommended 3-D Spectral Range (for Identification) - 210nm to 400nm

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 the 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 % LC or raw material % assay:

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

R_u Sample peak area

R_s Mean (n=all) standard peak area

Wt_{std} Weight of the 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 g

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

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

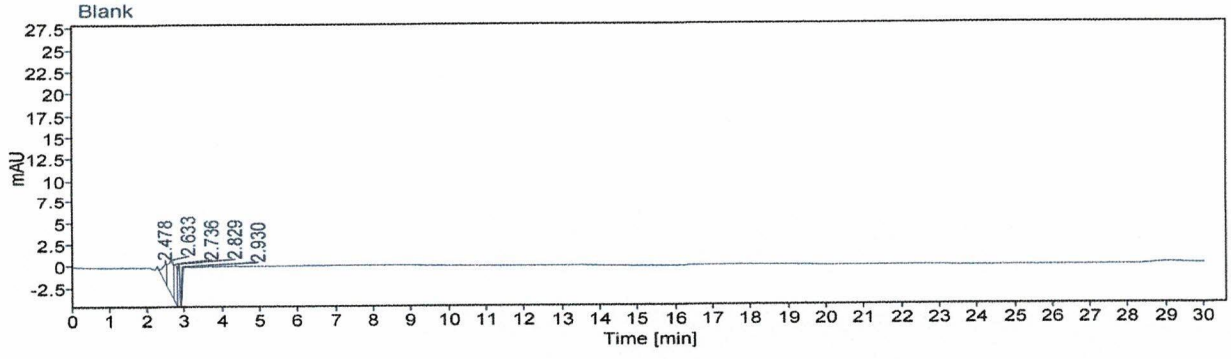
LA Label amount of analyte in mg. (Use 1 for raw materials.)

7.8 System Wash, Column Wash and Column Storage

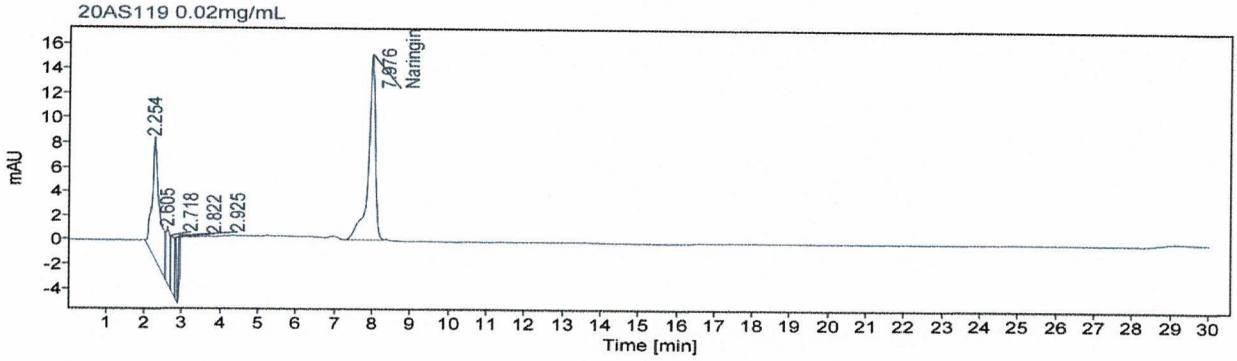
7.8.1 Wash and store the column in 50:50 ACN / Water.

7.9 Example Chromatography

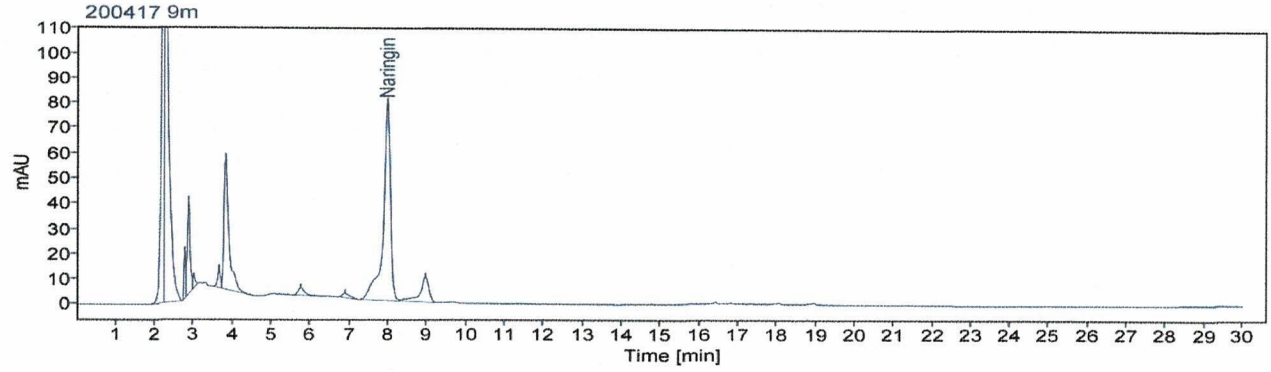
7.9.1 Blank



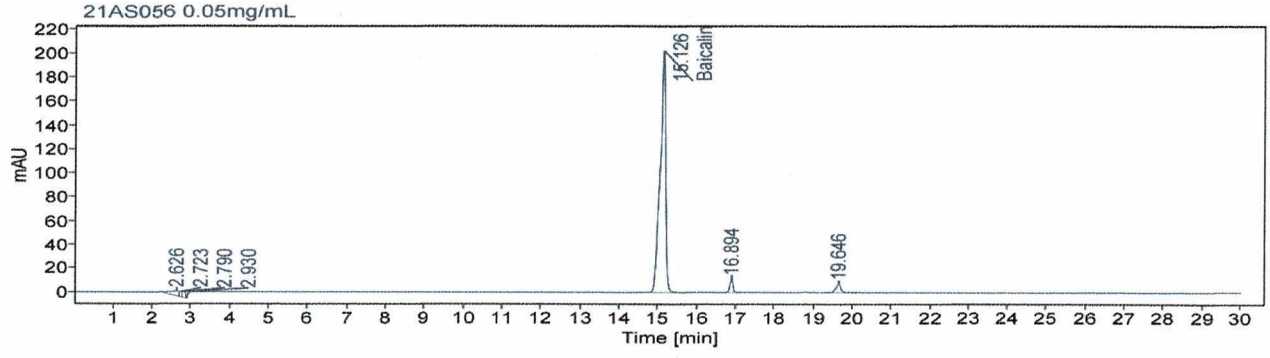
7.9.2 Naringin Working Standard



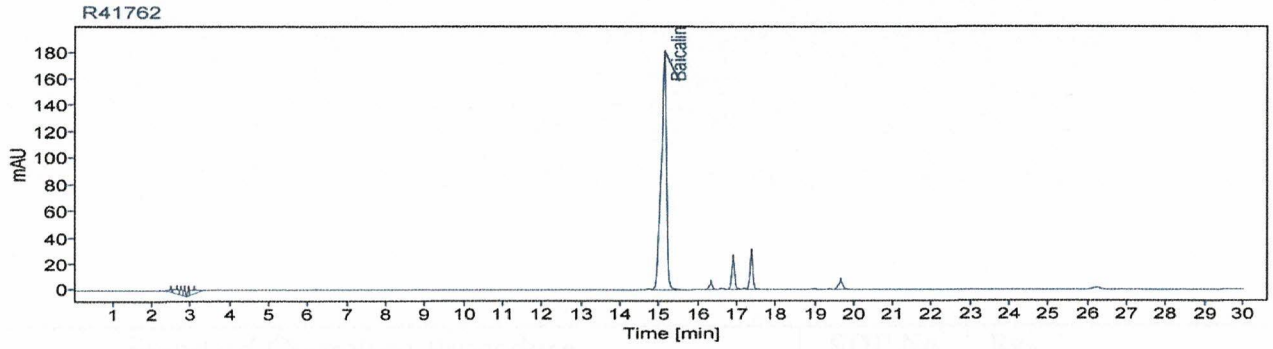
7.9.3 Naringin Sample



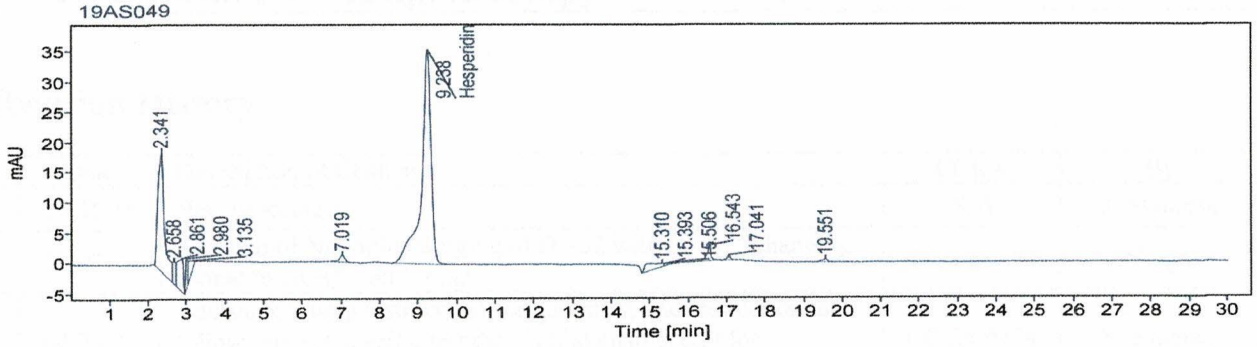
7.9.4 Baicalin Working Standard



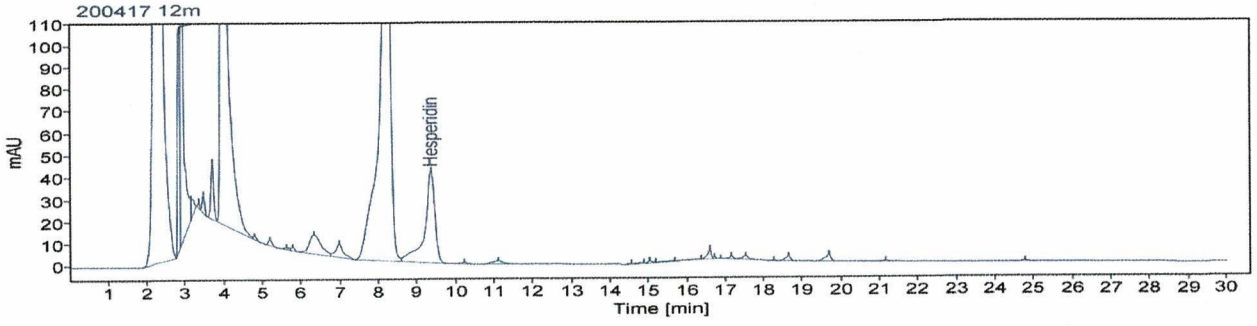
7.9.5 Baicalin Sample



7.9.6 Hesperidin Working Standard



7.9.7 Hesperidin Sample



8.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	05/15/18	New procedure.	N/A	J. Maignan
1	10/27/20	Addition of Naringin, merging of D-752 with D-751. Changing format to current lab format	CC-20-0763	J.Maignan
2	04/03/24	Add sample prep instructions for gummies, add instruction to follow product specific test details if available, edit for consistency with current methods.	CC-24-0134	S. Sassman