1.0 Purpose

The purpose of this procedure is to define the method for the quantification and identification of Cynaropicrin in raw materials and finished products by HPLC using UV-Vis Spectroscopy.

2.0 Scope

This procedure applies to the identification and quantification of Cynaropicrin, using Cynaroside and/or Chlorogenic Acid as a standard in raw materials and finished products by HPLC/UV.

3.0 Responsibility

3.1 It is the responsibility of QC 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 Personnel to keep this procedure aligned with current practices.

4.0 Definitions

4.1 **HPLC/UV** – High Pressure Liquid Chromatography with Ultraviolet Detection

4.2 **QC** – Quality Control

4.3 **CofA** – Certificate of Analysis

4.4 **CNP** – Cynaropicrin

4.5 **CNS** – Cynaroside

4.6 **CGA** – Chlorogenic Acid

4.7 **ACN** – Acetonitrile

4.8 **MeOH** – Methanol

4.9 **TFA** – Trifluoroacetic Acid

4.10 **H_2O** – Deionized water

5.0 References

5.1 MV-LAB-19-033, Protocol, Cynaroside Determination using HPLC with UV/VIS Spectroscopy

6.0 Supplies

6.1 Chemicals: All reagents are HPLC grade or better

6.1.1 CGA Reference Standard

6.1.2 ACN

6.1.3 MeOH

6.1.4 TFA

6.2 Glassware

6.2.1 Volumetric glassware as required for standard and sample preparations

6.3 Disposables (as required for standard and sample preparations)

6.3.1 10mL Pipette Tips

6.3.2 1mL Pipette Tips

6.3.3 200µL Pipette Tips

6.3.4 Microcentrifuge tubes

6.3.5 16mL Test Tubes

6.3.6 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 Centrifuge

6.4.4 Adjustable Pipette

7.0 Procedure

7.1 Mobile Phase Preparation

7.1.1 Mobile Phase A

7.1.1.1 Combine 1000 mL of H_2O with 0.1 mL of TFA

7.1.2 Mobile Phase B

7.1.2.1 Combine 500 mL of ACN with 0.05 mL of TFA

7.1.3 Diluent

7.1.3.1 Combine 250 mL of H_2O with 250 mL of MeOH

7.2 Stock Standard Preparation

7.2.1 Accurately weigh and transfer about 50 mg of CGA reference standard to a 100-mL volumetric flask.

7.2.2 Dissolve in and dilute to volume with Diluent.

7.3 Working Standard Preparation

7.3.1 Transfer 3.0 mL of the Stock Standard to a 25-mL volumetric flask.

7.3.2 Dilute to volume with Diluent.

7.3.3 The standard preparation may be scaled as required.

7.4 Sample Preparation

7.4.1 The linear range of the method is 10 – 100 µg/mL with a 10 µL injection volume. The sample concentration must be within the linear range of the method:

7.4.2 For raw materials: weigh no less than 30 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. Dissolve in and dilute to volume with diluent. Perform further dilutions as required using diluent.

7.4.3 For solid dose finished products: record the weight of ten dosage units, and calculate the average weight of a single dosage unit. Based on the label claim and fill weight (for capsules) or tablet weight per dose, weigh no less than 50 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 linearity range. Dilute the sample to 2/3 of the flask volume with diluent and swirl to dissolve. Shaking or sonication can also be used to assist dissolution. After the sample is dissolved, equilibrate the sample to room temperature (if sonicated), and dilute to volume using diluent. Perform further dilutions as required using diluent.

7.4.4 If particulates remain in the final sample preparation, a portion may be centrifuged at 10,000 rpm for 200 seconds prior to HPLC analysis.

7.4.5 For finished products or raw materials being analyzed for the first time using this method, an in process validation is required to demonstrate spectral purity, baseline separation of peaks, and extraction efficiency as a part of system suitability before data can be reported using this method.

7.5 HPLC Parameters

7.5.1 Column: Phenomenex Luna C18(2), 5µm, 4.6 mm x 250 mm

7.5.2 Column Temperature: 30°C

7.5.3 Flow rate: 1.0 mL/min

7.5.4 Wavelength: 205 nm

7.5.5 Injection Volume: 10 µL

7.5.6 Run Time: 60 min for samples, 25 min for standards

7.5.7 Spectral Range (for Identification)- 210 nm to 360 nm

7.5.8 Gradient Profile:

Time	%MPA	%MPB
0	95	5
4	95	5
21	78	22
26	78	22
44	65	35
49	20	80
50	95	5
60	95	5

7.6 Recommended Sequence

7.6.1 Make at least 2 injections of the diluent.

7.6.2 Make five (5) injections of Standard Solution.

7.6.3 Make a single injection of each Sample Preparation.

7.6.4 Make a single injection of the Standard Solution after every ten (10) sample injections or at the end of a run.

7.7 System Suitability Requirements

7.7.1 The %RSD of the first five (5) standard injections is NMT 5.0%.

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

7.7.3 If present, any interference in the diluent should be subtracted out of the sample and standard peak areas.

7.8 Retention Time (RT), Relative Retention Time (RRT), Relative Response Factor (RRF)

7.8.1 CGA: RT = 18.6 min, RRT = 1.00, RRF = 1.00

7.8.2 CNS: RT = 26.8 min, RRT = 1.44, RRF = 0.336

7.8.3 CNP: RT = 40.9 min, RRT = 2.20, RRF = 0.582

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

7.9.1 $$\%\ \text{assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SW} \times \frac{AW}{LA} \times RRF \times 100$$

R_u Sample peak area

R_s Mean standard peak area

Wt_{std} Weight of reference standard in mg (correct for moisture if required)

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

P Purity of the reference standard in decimal format

SW Sample weight in mg

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

AW Average weight of a single tablet or capsule in mg (use 1 for raw materials)

LA Label amount in mg per dose (use 1 for raw materials)

RRF Relative Response Factor from Section 7.8.

7.10 Column Wash and Storage

7.10.1 Rinse and store the column with H_2O / ACN (50/50)

8.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	06/26/19	New	N/A	S. Sassman
1	07/21/22	Scheduled review: updated logo and format.	CC-22-0289	K. Burris