	<b>Standard Operating Procedure</b> <b>Determination of Chrysin &amp; 3,3-Diindolylmethane by HPLC/UV</b>		<b>SOP Number</b> <b>D-774</b>	<b>Revision</b> <b>1</b>
			<b>Effective Date</b> <i>06/29/22</i>	<b>Page</b> <b>Page 1 of 7</b>
<b>Written by/ Date</b> <i>KBurns 06/22/22</i>		<b>Reviewed by/ Date</b> <i>SAS 06/22/22</i>		<b>Approved by/ Date</b> <i>SS 06/22/22</i>
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## 1.0 Purpose

This document describes the analytical procedure for the determination of Chrysin and 3,3-Diindolylmethane in raw materials and finished products.

## 2.0 Scope

This procedure applies to the identification and quantification of Chrysin and 3,3-Diindolylmethane in raw materials and finished products. This method was validated under Protocol MV-LAB-19-124.

## 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 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 **H<sub>3</sub>PO<sub>4</sub>** – Phosphoric Acid
- 4.4 **MeOH** – Methanol
- 4.5 **CHSN** – Chrysin
- 4.6 **DiIM** – 3,3-Diindolylmethane

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4.7 **HPLC** – High Performance Liquid Chromatography

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

## **5.0 References**

5.1 MV-LAB-19-124, Protocol, Chrysin & 3,3-Diindolylmethane Determination Using HPLC with UV/Vis Spectroscopy

## **6.0 Reagents, Supplies, Glassware, and Equipment**

6.1 Chemicals – All reagents are ACS grade or better

6.1.1 Milli-Q Water

6.1.2 MeOH

6.1.3 H<sub>3</sub>PO<sub>4</sub>

6.1.4 CHSN Reference Standard

6.1.5 DiIM Reference Standard

6.2 Supplies and Glassware

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

6.2.2 Volumetric glassware and/or adjustable pipettes and tips

6.2.3 Weigh paper or funnels

6.2.4 10ml Syringes with 17mm x 0.45u Nylon Syringe Filters

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 Sonicator bath

6.3.4 Wrist Action Shaker

## 7.0 Procedure

### 7.1 Mobile Phase, Extraction Solvent & Diluent Preparation

#### 7.1.1 Mobile Phase A – 0.1% H<sub>3</sub>PO<sub>4</sub>

7.1.1.1 Combine 1 mL of H<sub>3</sub>PO<sub>4</sub> with 1000 mL of Milli-Q Water. Mix well.

#### 7.1.2 Mobile Phase B - MeOH

#### 7.1.3 Extraction Solvent – MeOH

#### 7.1.4 Diluent – MeOH

7.1.5 Preparations may be scaled as necessary

### 7.2 Standard Prep

7.2.1 Accurately weigh and transfer about 25 mg of CHSN and DiIM reference standards into a 50-mL volumetric flask. Add 35mL of Extraction Solvent. Sonicate for 5min, cool, then QS to volume with Extraction Solvent.

7.2.2 Dilute 1:50 w/ Diluent for a Working Standard concentration of 10 µg/ml.

### 7.3 Sample Preparation

7.3.1 The validated range for the analytical method is 12.8 – 255.4 ng CHSN and 13.0 – 259.4 ng DiIM on column.

7.3.2 For finished products, at least 10 dosage units are pooled and ground by mortar and pestle. Based on the fill weight (for capsules) or tablet weight per dose and the label claim, weigh no less than 716 mg of the pooled dosages into a suitably sized volumetric flask to generate an analyte concentration that is within the validated linearity range for the analyte being tested.

7.3.3 Prepare raw materials like standards. (However, be sure to consult the specification for expected potency, as raw material samples may not be 100%.)

7.3.4 Samples can be extracted in Extraction Solvent at any volume starting from 25mL. The volume chosen must be in the solubility range of CHSN & DiIM (validated at 0.01 mg/ml). To manage large volumes, the sample can be initially

dissolved in a smaller volume that is within the solubility range and a portion further diluted to bring the analyte concentration into the linear range.

7.3.5 Fill the flask to about 70% of the calculated volume with Extraction Solvent and shake mechanically for 10 minutes. Sonicate for 5 minutes, cool to room temperature and bring up to volume with Extraction Solvent. Filter a portion for use in subsequent dilutions.

7.3.6 Perform further dilutions as required (if applicable) using Diluent.

7.3.7 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.

#### 7.4 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: 0.9 mL/min

7.4.4 Wavelength: 284 nm

7.4.5 Injection Volume: 5 µL

7.4.6 Run Time: 10 minutes.

7.4.7 3-D Spectral Range (for Identification) - 200nm to 400nm

7.4.8 Mobile Phase Gradient - Isocratic

#### 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.

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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

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

$R_u$  Sample peak area

$R_s$  Mean (n=5) standard peak area

$Wt_{std}$  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 (solids) or mL (liquids)

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

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

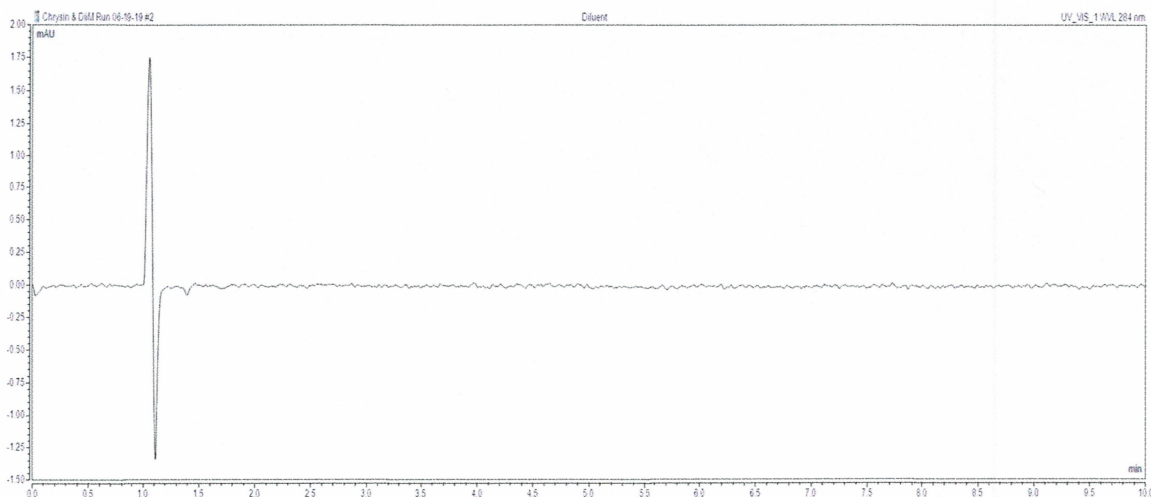
$LA$  Label amount in mg of analyte (use 1 for raw materials)

7.8 Column Wash and Storage

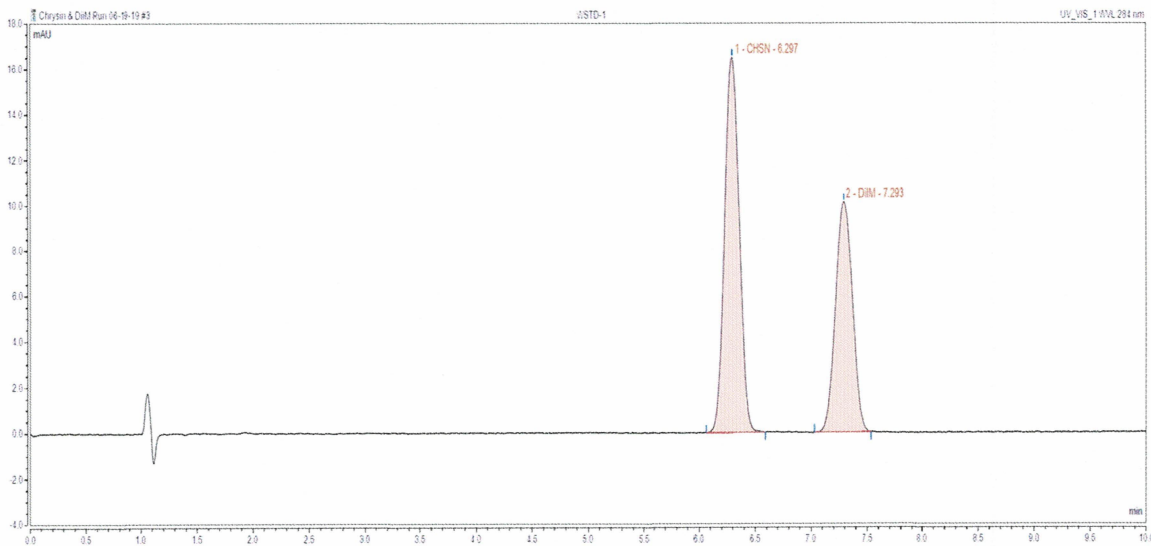
7.8.1 Wash and store the column in 75:25 MeOH / Milli-Q Water.

## 8.0 Chromatograms

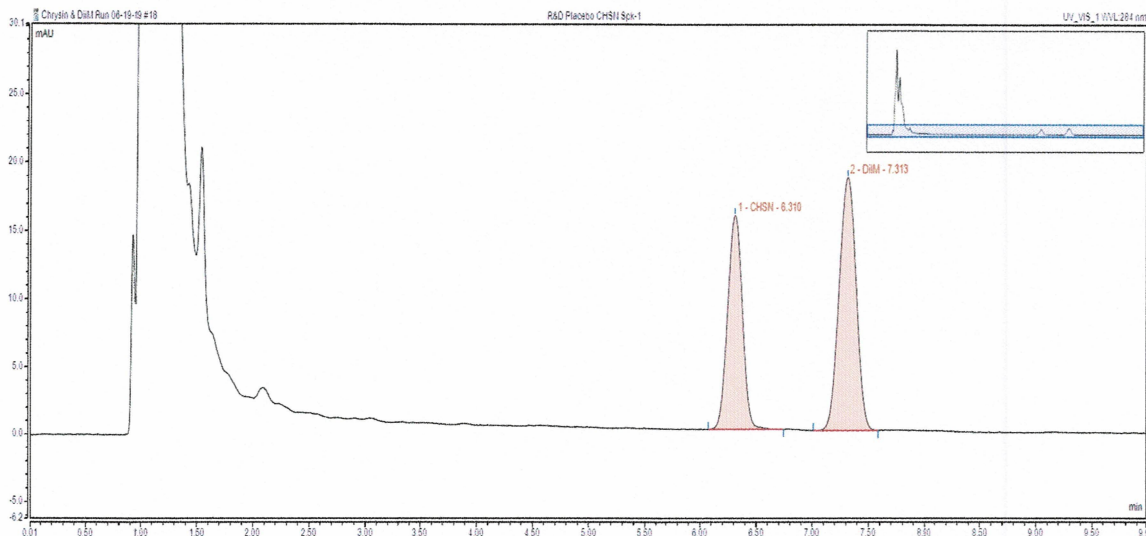
### 8.1 Typical Diluent Chromatogram



### 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-22-0287	K. Burris