1.0 Purpose

The purpose of this procedure is to describe a method for the quantitative analysis and identification of diindoylmethane in raw materials and finished products using HPLC and UV/VIS spectrophotometry.

2.0 Scope

This procedure applies to the quantification and identification of diindoylmethane in the QC laboratory at Ion Labs. Diindoylmethane was quantified at 280 nm. Other wavelengths can be used with justification 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 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 Ion Labs practices.

4.0 Definitions

4.1 **ACN** – Acetonitrile

4.2 **QC** – Quality Control

4.3 **CofA** – Certificate of Analysis

4.4 **H_2O** – Water

5.0 References

5.1 MV-LAB-18-066, Protocol, Diindoylmethane Determination using HPLC with UV/VIS Spectroscopy

6.0 Reagents, Supplies, Glassware and Equipment

6.1 Reagents: all reagents are HPLC grade or better.

6.1.1 H_2O ($\geq$ 18.2 MΩ·cm)

6.1.2 ACN

6.1.3 Diindoylmethane reference standard

6.2 Glassware

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

6.2.2 Mobile Phase Bottles

6.2.3 Volumetric Flasks

6.2.4 Volumetric Pipets

6.3 Disposables

6.3.1 Pipet Tips

6.3.2 Microcentrifuge Tubes

6.3.3 Plastic Luer Lock Syringe

6.3.4 0.2 or 0.45µm Nylon syringe filters

6.3.5 Weigh Paper or Weigh Boats

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 Eppendorf Centrifuge

6.4.6 Adjustable Pipets

7.0 Preparation of Mobile Phase, Diluent, Standards, and Samples

7.1 Mobile Phase A – H_2O

7.2 Mobile Phase B – ACN

7.3 Diluent – Methanol

7.4 Stock Standard Preparation

7.4.1 Accurately weigh and transfer about 25 mg of reference standard into a 100-mL volumetric flask.

7.4.2 Dissolve in and dilute to volume using Diluent.

7.5 Dissolve in and dilute to volume using Diluent.Working Standard Preparation

7.5.1 Transfer 5.0 mL of Stock Standard into a 50-mL volumetric flask.

7.5.2 Dilute to volume using Diluent.

7.5.3 Alternate standard preparations are acceptable provided that the working standard concentration is within the linear range listed below.

7.6 Sample Preparation

7.6.1 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.

7.6.2 The linear range of the method is 0.01mg/mL to 0.1mg/mL. All standard and sample preparations must be within the linear range of the method.

7.6.3 For finished products, at least 10 dosage units are pooled and ground by mortar and pestle as necessary.

7.6.4 Based on the label claim and fill or tablet weight per dose for finished products or expected potency for raw materials, weigh a portion of the sample into a suitably-sized volumetric flask to generate an analyte concentration that is within the validated linear range for the analyte being tested.

7.6.5 Samples can be dissolved in Diluent at any volume. Suspend the sample in 2/3 flask volume of Diluent and shake mechanically for 20 minutes. Dilute to volume using Diluent. 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 of measurement. The final diluted sample must be filtered or centrifuged before analyzing by HPLC.

7.6.6 For filtration, using the final diluted sample withdraw up to 10mL using a 10mL plastic syringe then filter and discard at least 0.5mL of filtrate before collecting a portion for analysis. From the collected sample dilute as needed then add sample to an HPLC vial for analysis.

7.6.7 For centrifugation using the final diluted sample, fill an even number of 1.5mL or 2.0mL micro centrifuge tubes and pellet out insoluble matter for 5 minutes at 6000rpm.

8.0 Test Conditions

8.1 Gradient-multistep

Time	%A	%B
0.00	98	2
1.00	98	2
2.00	80	20
3.00	80	20
4.00	69	31
6.00	69	31
7.00	55	45
8.00	45	55
10.0	45	55
11.0	35	65

11.1	98	2
15.0	98	2

8.1.1 Column- Symmetry300 C18, 3.5µ, 4.6 X 75mm

8.1.2 Flow Rate- 1.0mL/min

8.1.3 UV detection- 280nm

8.1.4 Injection volume- 20uL

8.1.5 Column Temperature- 45°C

8.1.6 Recommended 3-D Spectral Range- 200nm to 350nm

8.2 Sequence

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

8.2.2 Make five injections of the Working Standard.

8.2.3 Make a single injection of each Sample Preparation.

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

8.3 System Suitability

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

8.3.2 The %RSD of all Working Standard injections is NMT 5%.

8.4 Column Wash and Storage

8.4.1 Rinse the column 100% ACN at 1 mL/min for at least 10 min.

8.4.2 Store the column with 100% ACN.

9.0 Example Calculation

9.1 $$\% \text{ assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times \frac{SS}{LA} \times 100$$

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)

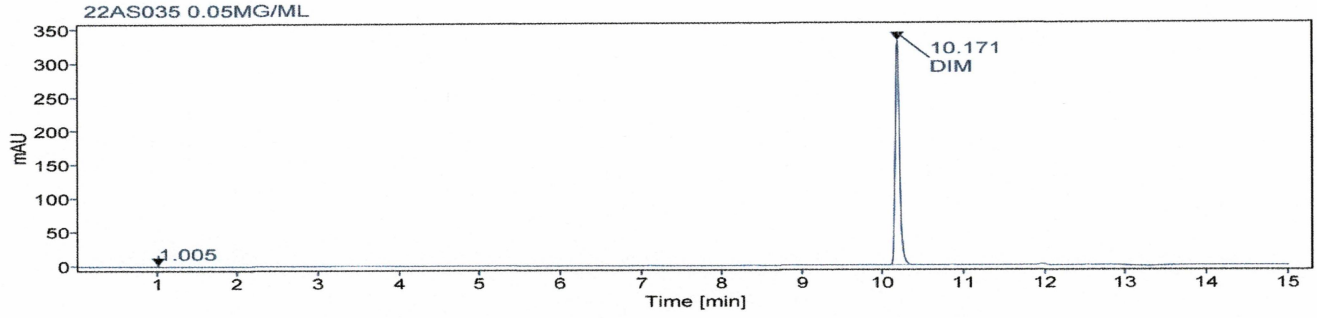
V_{spl} Volume of the sample preparation accounting for dilutions in mL

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.

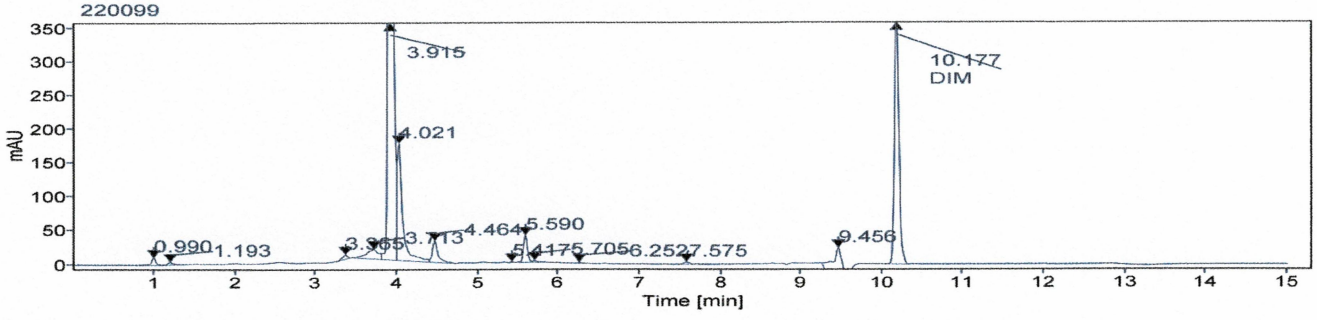
9.2 LA Label amount in mg per dose or 1 for raw materialsExample Chromatography

10.0 Example Chromatography

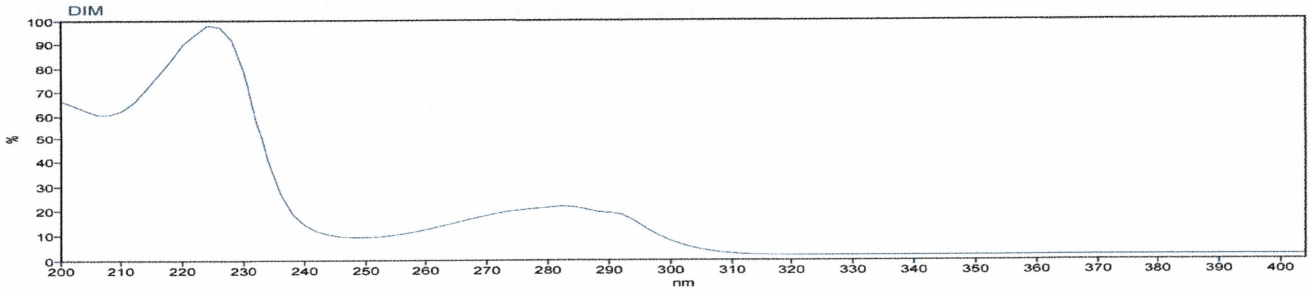
10.1 Standard Injection



10.2 FP Sample Injection



10.3 Example Spectrum for DIM



11.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	06/06/18	New procedure.	N/A	J. Maignan
1	08/24/21	Minor clarifications. Added example chromatography and additional details to test method.	CC-21-0331	J. Sassman
2	09/07/22	Update for consistency with current methods and lab practices, add recommended sequence section, replace requirements with system suitability section, add column wash and storage, make example chromatography easier to read.	CC-22-0364	S. Sassman
3	02/23/23	Simplify standard preparation, add instruction to check the product profile for test details, remove language requiring in-process validation for new products.	CC-23-0096	S. Sassman