1.0 Purpose

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

2.0 Scope

This procedure applies to the quantification and identification of cholecalciferol in raw materials and finished products in the QC laboratory at Ion Nutritional Labs.

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 current with latest Ion Nutritional Labs practices.

4.0 Definitions

4.1 **UV/VIS –** Ultraviolet/visible light

4.2 **ACN** – Acetonitrile

4.3 **EtOH** – Ethanol

4.4 **CofA** – Certificate of Analysis

4.5 **Cholecalciferol Reference Standard** – also known as Vitamin D or Vitamin D_3

4.6 **IPA –** Isopropyl Alcohol

4.7 **MeOH** – Methanol

4.8 **DMSO** – Dimethylsulfoxide

4.9 **HCl** – Hydrochloric Acid

4.10 **QC** – Quality Control

4.11 **HPLC** – High Performance Liquid Chromatography

5.0 References

5.1 MV-LAB-13-008, Protocol, Cholecalciferol Determination by HPLC

5.2 NCR-23-0008, Product Specific Method Optimization for Vitamin D in SGM00379 (Notebook 200 Pg 105-106, 111-113)

5.3 PRTCL-24-0035, Supplemental Validation of D-721 for Determination of Cholecalciferol in Chewable Gels by HPLC-UV

6.0 Reagents, Supplies, Glassware and Equipment

6.1 Reagents: all reagents are ACS grade or better

6.1.1 ACN

6.1.2 DMSO

6.1.3 MeOH

6.1.4 Ethanol denatured with 5% IPA and 5% MeOH

6.1.5 Cholecalciferol, traceable standard

6.1.6 2.7N HCl

6.1.7 Deionized Water ($\geq$ 18.2 MΩ·cm)

6.1.8 IPA

6.2 Supplies and Glassware

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

6.2.2 Mobile phase containers

6.2.3 Volumetric flasks

6.2.4 Pipette tips

6.2.5 Plastic luer-lock syringes

6.2.6 0.45µm 25mm PTFE syringe filters

6.2.7 Erlenmeyer flasks

6.2.8 Microcentrifuge tubes

6.2.9 Weigh Paper and/or 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 Acclaim 120 C18, 5µm, 120Å, LC column, 250mm X 4.6mm, or equivalent

6.3.3 Analytical Balance

6.3.4 Micro Analytical Balance

6.3.5 Wrist Action Shaker

6.3.6 Sonicator

6.3.7 Vortex Mixer

6.3.8 Stir Plate

6.3.9 Adjustable Pipette(s)

6.3.10 Microcentrifuge

7.0 Procedure

7.1 Mobile Phase A – 100% ACN

7.2 Mobile Phase B – Ethanol, denatured with 5% IPA and 5% MeOH

7.3 Extraction Solvent / Diluent – Mobile Phase B

7.3.1 Note: Microencapsulated D_3 requires a pre-extraction in H_2O.

7.3.2 Note: Use 1:1 MeOH/DMSO when analyzing gummies.

7.4 Standard Preparation

7.4.1 Use the actual purity from the CofA for cholecalciferol in your calculations.

7.4.2 All Standards are prepared by weighing no less than the minimum weight of the analytical balance.

7.4.3 Accurately weigh and transfer the reference standard into a suitably sized volumetric flask.

7.4.4 Add Diluent to two thirds final volume and place on the wrist action shaker for 15 minutes before bringing up to final volume using Diluent.

7.4.5 Dilutions are prepared using diluent. Dilutions can be made using volumetric glassware or using 10mL, 1mL and 200uL variable pipettes. Specific standard concentrations will approximate the concentration expected to be found in the product being tested based on the sample dilution and calculated from the label. Final dilutions may be prepared directly in HPLC vials.

7.4.6 Alternative standard preparations are acceptable as long as the preparations are within the linear range of this method, which is listed below.

7.5 Sample Preparation

7.5.1 Specific sample testing details are provided in each product 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.

7.5.2 The linear range of the method is listed below. Samples must be prepared within this range.

7.5.2.1 Agilent 1260 with 60 mm flow cell: 0.025 µg/mL – 10 µg/mL.

7.5.2.2 All other instruments: 0.5 µg/mL – 10 µg/mL.

7.5.3 10 or more dosage units can be pooled and ground by mortar and pestle as necessary. (When analyzing gummies, prepare as a cryogrind as per D-793 and extract / dilute using 1:1 DMSO/MeOH as described below in 7.5.10.)

7.5.4 Based on the label claim & dosage form weight, weigh a portion of the pooled dosages to generate an analyte concentration that is within the validated linearity and solubility range for the analyte being tested.

7.5.5 Sample pretreatment to remove encapsulation from encapsulated forms of cholecalciferol:

7.5.5.1 Many solid or powder forms of cholecalciferol are micro encapsulated and require a pretreatment to release the vitamin from the substrate before preparing the sample. The primary method to remove the encapsulation matrix is to pretreat the powdered sample with a volume of H_2O equal to 10% of the flask volume and vortex making sure all water is either absorbed onto the powder or all the powder comes into contact with the water.

7.5.5.2 Other extraction methods may be required based on the encapsulation chemistry.

7.5.6 Sample pretreatment for Gel caps containing cholecalciferol:

7.5.6.1 Add a volume of 2.7N HCL equal to 5% - 10% of the flask volume, and shake on a wrist-action shaker for 30 minutes.

7.5.7 After any necessary pre-treatment steps, add diluent equal to about 1/3 the volume of the flask and shake on the wrist action shaker for 15 minutes.

7.5.8 Next, further dilute to 2/3 the final volume using diluent and mix again on the wrist action shaker for an additional 15 minutes before diluting to final volume.

7.5.9 Samples can be dissolved in diluent at any volume starting from 10mL. 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.

7.5.10 Example for ~3g gummy containing 50µg D_3:

7.5.10.1 Transfer ~3g cryogrind to 125ml Erlenmeyer flask. Add stir bar and ~35ml of 1:1 DMSO/MeOH then stir on a stir plate for 30 minutes (protected from light).

7.5.10.2 Quantitatively transfer the slurry to a 50ml (low actinic) volumetric flask. QS to volume and sonicate for 10 minutes. Filter as described below using a PTFE syringe filter.

7.5.11 The final sample must be filtered or centrifuged before analyzing by HPLC.

7.5.11.1 For filtration, using the final large scale diluted sample, withdraw up to 10 mL using a 10 mL plastic syringe. Filter though a 0.45 µm nylon, PVDF or PTFE membrane, discarding at least 0.5 mL of sample before collecting a portion for analysis. From the collected sample dilute as needed then add 1mL to an HPLC vial for analysis.

7.5.11.2 For centrifugation, using the final large scale diluted sample, fill an even number of 1.5 or 2.0 mL microcentrifuge tubes and pellet insoluble matter for 5 minutes at 10,000 rpm.

7.6 Test Conditions

7.6.1 Gradient- Isocratic

Time	%A	%B
0.00	50	50
10.00	50	50

7.6.2 Column- Acclaim 120 C18, 5µm, 120Å, 250mm X 4.6mm or equivalent.

7.6.3 Flow Rate- 1.0 mL/min

7.6.4 UV Detection- 265 nm

7.6.5 Injection Vol.- 20 µL

7.6.6 Temperature- 35°C

7.6.7 Run Time – at least 10 minutes

7.6.8 Retention Time – about 8.5 minutes

7.6.9 Recommended 3-D Spectral Range- 210nm to 320nm

7.7 Recommended Sequence

7.7.1 Make at least 2 injections of the diluent.

7.7.2 Make five (5) injections of Standard Solution.

7.7.3 Make a single injection of each Sample Preparation.

7.7.4 Make a single injection of the Standard Solution after every six (6) samples and at the end of the run.

7.8 System Suitability Requirements

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

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

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

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

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: Average Weight of a single dosage unit in mg for tablets, capsules, and gummies. Single serving from the theoretical formula in mL for liquids or mg for powders, or 1 for raw materials.

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

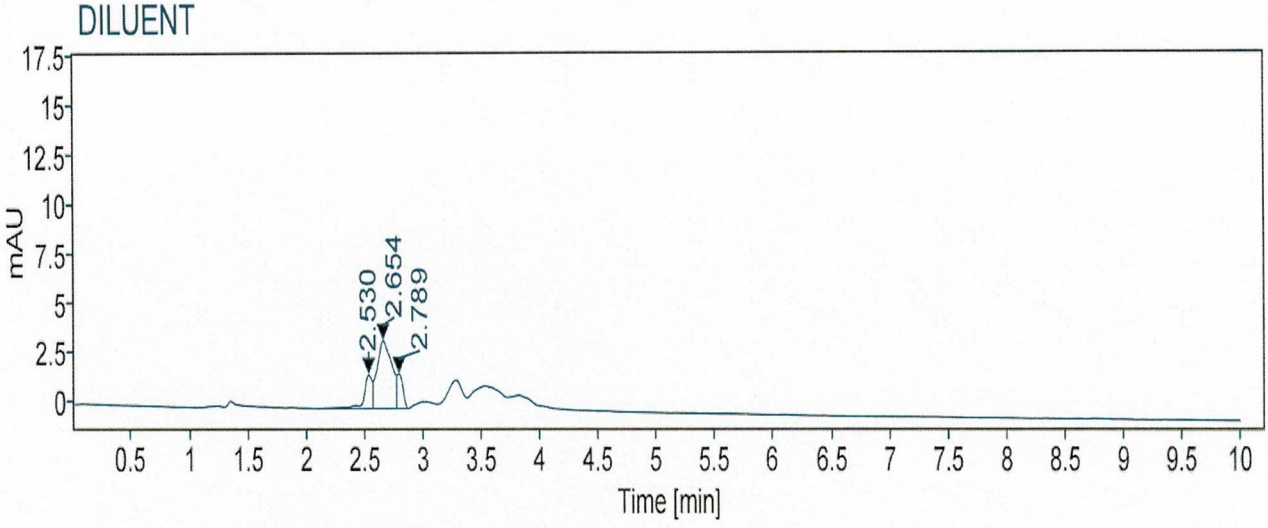
7.10 Column Wash and Storage

7.10.1 Column rinse is not required.

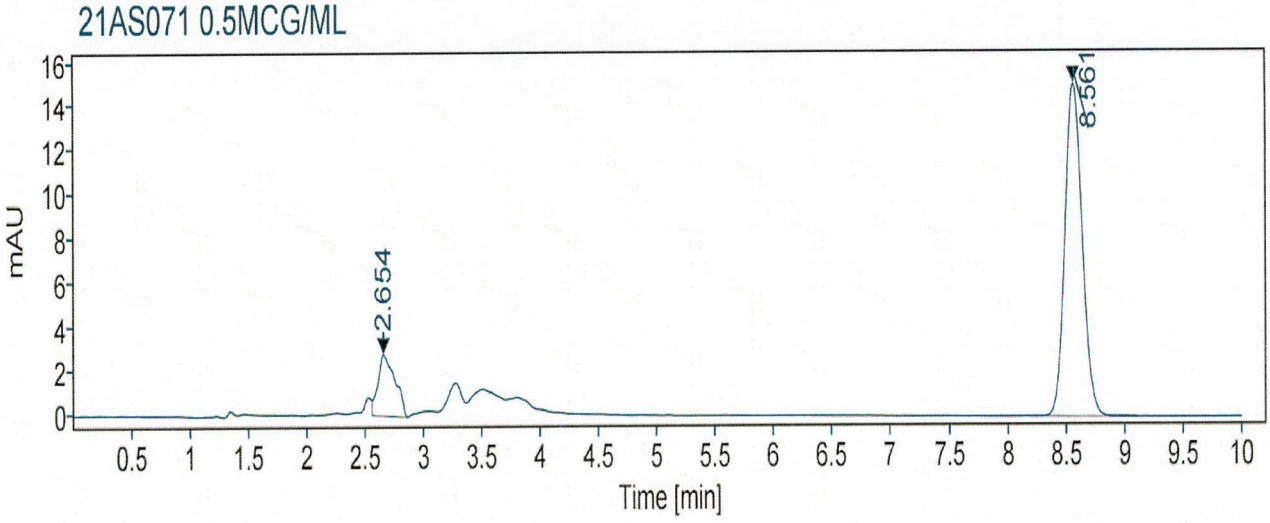
7.10.2 Store the column with Mobile Phase A/B (50/50).

7.11 Example Chromatography

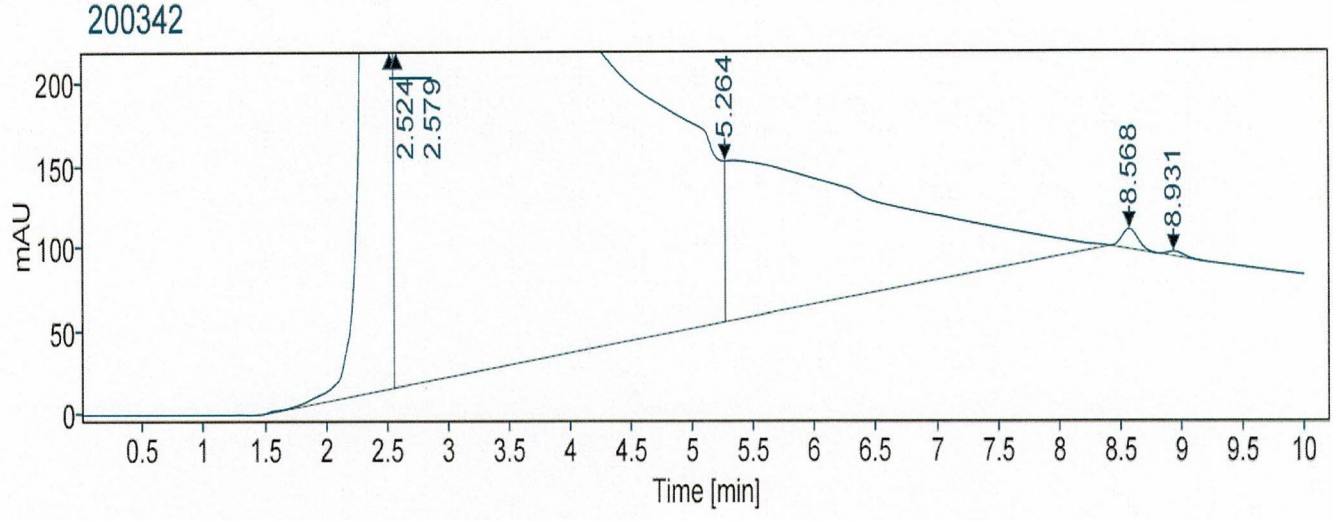
7.11.1 Blank (Diluent)



7.11.2 Working Standard



7.11.3 Sample



8.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	01/20/12	New	-	-
1	05/24/13	Revalidated method, changed mobile phase to ACN:ETOH 50:50, changed calculations to % label, added information on extraction and release conditions	13-405	B. Johns
2	09/09/15	Biennial review: Updated SOP format. Updated HPLC method format.	15-0864	B. Johns
3	02/04/19	Scheduled review: Changed Responsibilities, Added to sample preparation to make it easier to follow, Updated stability requirements.	19-0120	J. Maignan
4	04/11/22	Update to reflect current practices, simplify standard and sample preparation, add recommended sequence section, replace requirements section with system suitability, update example calculation for consistency with current methods, narrow the range for spectral match, add example chromatography.	CC-22-0170	S. Sassman
5	12/20/22	Added test details section. Minor edits.	CC-22-0475	J. Sassman
6	03/09/23	Change section 7.6.7 from 10 minutes to "at least 10 minutes".	CC-23-0115	J. Sassman
7	09/08/23	Extend lower linearity range when using 60mm flow cell, clarify amount of water to use for encapsulated forms of vitamin D.	CC-23-0452	S. Sassman
8	05/15/24	Added details for analysis of finished product gummies.	CC-24-0174	C. Perry