Written by/ Date	Reviewed by/ Date	Approved by/ Date
ANL 01/14/25	AJS 01/20/25	Rec 01/21/25
Title: Analytical Development Scientist	**Title: QC Lab Manager**	**Title: QA/QC Director**

1.0 Purpose

The purpose of this procedure is to define the method for the determination of methylcobalamin and cyanocobalamin by LC-MS.

2.0 Scope

This procedure applies to the determination of methylcobalamin and cyanocobalamin in raw materials and finished products by LC-MS in the QC laboratory at Ion 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 ensure that this 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 **LC-MS** – Liquid Chromatography – Mass Spectrometry

4.2 **QC** – Quality control

4.3 **ACN** – Acetonitrile

4.4 **H_2O** – Deionized water

5.0 References

5.1 PRTCL-21-0058, Protocol, Validation of an Analytical Method for the Determination of Methylcobalamin, Cyanocobalamin, and Hydroxycobalamin

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

6.0 Supplies

6.1 Chemicals

6.1.1 Methylcobalamin and/or cyanocobalamin reference standards

6.1.2 ACN (LC-MS grade)

6.1.3 Methanol (HPLC grade)

6.1.4 Ammonium acetate (LC-MS grade)

6.1.5 Ammonium acetate (ACS reagent grade)

6.1.6 Acetic acid (LC-MS grade)

6.1.7 Acetic acid (ACS reagent grade)

6.1.8 H_2O ($\geq$ 18 MΩ·cm)

6.2 Glassware

6.2.1 Volumetric glassware as required for standard and sample preparations

6.2.2 HPLC vials, 2mL with screw-cap enclosures and septa

6.3 Equipment

6.3.1 Suitable gradient HPLC system consisting of a pump, autosampler, and column oven coupled with Agilent Ultivo mass spectrometer using MassHunter software for instrument control and data processing.

6.3.2 Analytical balance

6.3.3 Wrist action shaker

6.3.4 Sonicator

6.3.5 Adjustable pipette and tips

7.0 Procedure

7.1 Mobile Phase A (15 mM ammonium acetate + 5 mM acetic acid + 10% ACN)

7.1.1 Transfer 0.58 g of ammonium acetate (LC-MS grade) to a 500-mL bottle.

7.1.2 Add 50 mL of ACN.

7.1.3 Add 0.143 mL of acetic acid.

7.1.4 Add 450 mL of H_2O, and mix well.

7.2 Mobile Phase B (15 mM ammonium acetate + 5 mM acetic acid + 46% ACN)

7.2.1 Transfer 0.58 g of ammonium acetate (LC-MS grade) to a 500-mL bottle.

7.2.2 Add 270 mL of H_2O.

7.2.3 Add 0.143 mL of acetic acid.

7.2.4 Add 230 mL of ACN, and mix well.

7.3 Diluent (30 mM ammonium acetate + 10 mM acetic acid + 10% methanol)

7.3.1 **Equilibrate to room temperature before using Diluent.**

7.3.2 Transfer 2.31 g of ammonium acetate (ACS reagent grade) to a 1000-mL bottle.

7.3.3 Add 100 mL of methanol.

7.3.4 Add 0.57 mL of acetic acid.

7.3.5 Add 900 mL of H_2O, and mix well.

7.4 Extraction Solvent (16.5 mM ammonium acetate + 5.5 mM acetic acid + 45% methanol)

7.4.1 **Equilibrate to room temperature before using Extraction Solvent.**

7.4.2 Transfer 550 mL of *Diluent* to a 1000-mL bottle.

7.4.3 Add 450 mL of methanol, and mix well.

7.5 Stock Standard (250 mcg/mL)

7.5.1 **The target analytes are susceptible to degradation when exposed to light and/or heat. Standards and samples must be freshly prepared on same day as testing to ensure accuracy and reliability of results. Previously prepared composites are not acceptable. Standards and samples should be prepared in low-actinic glassware and refrigerated immediately after preparation.**

7.5.2 Include only those analytes being quantified in the *Stock Standard.*

7.5.3 Accurately weigh and transfer about 25 mg of each reference standard into a 100-mL low-actinic volumetric flask.

7.5.4 Dissolve in and dilute to volume with H_2O.

7.6 Intermediate Standard (2 mcg/mL)

7.6.1 Transfer 2.0 mL of *Stock Standard* to a 250-mL low-actinic volumetric flask.

7.6.2 Dilute to volume with H_2O.

7.7 Working Standard (24 ng/mL)

7.7.1 Transfer 3.0 mL of *Intermediate Standard* to a 250-mL low-actinic volumetric flask.

7.7.2 Dilute to volume with *Diluent.*

7.7.3 The *Working Standard* may be stored at 4°C in the dark for the specified time depending on the target analyte:

7.7.3.1 Methylcobalamin: 2 days

7.7.3.2 Cyanocobalamin: 3 days

7.8 Stock Sample Preparation (40 mg/mL)

7.8.1 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.

7.8.2 Homogenize the sample prior to weighing:

7.8.2.1 For tablets, combine at least 10 dosage units and grind in a mortar and pestle.

7.8.2.2 For capsules, remove the fill material from at least 10 dosage units and grind in a mortar and pestle.

7.8.2.3 For powders and liquids, turn end-over-end in a container with sufficient head space to allow thorough mixing.

7.8.3 For chewable gels (gummies), homogenize as outlined in D-793.

7.8.4 The default sample weight is 4 g. Finished products with large dosage units and/or very small amount of target analyte may require larger sample size. For raw materials, the sample size may be decreased with a minimum recommended weight of 50 mg.

7.8.5 Accurately weigh and transfer about 4 g of sample to a 100-mL low-actinic volumetric flask.

7.8.6 Add about 65 mL of *Extraction Solvent*.

7.8.7 Sonicate for 2 minutes, accurately timed, with occasional shaking.

7.8.8 Shake for 10 minutes, accurately timed.

7.8.9 Dilute to volume with *Extraction Solvent*.

7.9 Working Sample Preparation

7.9.1 The target concentration is the concentration of the *Working Standard*. Use the label claim and fill weight (for finished products) or theoretical percent assay (for raw materials) to calculate the dilution of the *Stock Sample* required to reach the target concentration. The final sample concentration must be within the range 15 ng/mL – 35 ng/mL.

7.9.2 Prepare the *Working Sample* in low actinic glassware.

7.9.3 Dilute the *Stock Sample* to the required target concentration using *Diluent*. Multiple dilutions may be required.

7.9.4 **Do not centrifuge.** If particulates remain in the final sample, filter through a 0.45 µm PVDF or nylon syringe filter discarding a portion before collecting the sample for analysis.

7.10 Instrument Method Parameters

7.10.1 A vial with Diluent must be placed in position P1-A1.

7.10.2 A vial with Working Standard must be placed in position P1-A2.

7.10.3 Three separate Instrument Methods must be created with identical parameters except for the *Sampler Pretreatment* section as outlined below.

7.10.4 Column: Agilent Eclipse Plus C18 RRHD, 1.8 µm, 2.1 mm x 50 mm

7.10.5 Sampler

7.10.5.1 Injection Volume: N/A (method uses injector program instead).

7.10.5.2 Sample Thermostat: 12°C (control in Instrument Status pane)

7.10.5.3 Enable Needle Wash: Selected

7.10.5.4 Mode: Flush Port

7.10.5.5 Time: 6 sec

7.10.6 Sampler Pretreatment

7.10.6.1 No Spike

7.10.6.1.1 Draw 8.00 µL from location "P1-A-1" with 100 µL/min using default offset.

7.10.6.1.2 Draw 4.00 µL from sample with 100 µL/min using default offset.

7.10.6.1.3 Wash needle in flushport for 3 sec.

7.10.6.1.4 Mix 12 µL from air with 200 µL/min for 5 times.

7.10.6.2 Low Spike

7.10.6.2.1 Draw 4.00 µL from location "P1-A-1" with 100 µL/min using default offset.

7.10.6.2.2 Draw 4.00 µL from sample with 100 µL/min using default offset.

7.10.6.2.3 Wash needle in flushport for 3 sec.

7.10.6.2.4 Draw 4.00 µL from location "P1-A-2" with 100 µL/min using default offset.

7.10.6.2.5 Wash needle in flushport for 3 sec.

7.10.6.2.6 Mix 12 µL from air with 200 µL/min for 5 times.

7.10.6.3 High Spike

7.10.6.3.1 Draw 4.00 µL from sample with 100 µL/min using default offset.

7.10.6.3.2 Wash needle in flushport for 3 sec.

7.10.6.3.3 Draw 8.00 µL from location "P1-A-2" with 100 µL/min using default offset.

7.10.6.3.4 Wash needle in flushport for 3 sec.

7.10.6.3.5 Mix 12 µL from air with 200 µL/min for 5 times.

7.10.7 Binary Pump

7.10.7.1 Flow Rate: 0.35 mL/min

7.10.7.2 Gradient

Time (min)	%A	%B
0.00	100	0
3.00	25	75
3.01	100	0

5.00	100	0

7.10.7.3 Stoptime: 5 min

7.10.8 Column Oven

7.10.8.1 Temperature: 35 °C

7.10.9 QQQ

7.10.9.1 Acquisition

7.10.9.1.1 Scan Type: MRM

7.10.9.1.2 MS1 Resolution: Unit

7.10.9.1.3 MS2 Resolution: Unit

7.10.9.1.4 Polarity: Positive

7.10.9.1.5 Dwell Time: 400 ms

7.10.9.1.6 Acquisition Parameters

Analyte	ISTD	Type	Precursor (m/z)	Product (m/z)	Fragmentor (V)	CE (V)
cyanocobalamin	no	quantifier	678.6	147.1	160	45
		qualifier	678.6	358.8	160	21
methylcobalamin	no	quantifier	673.2	147.1	140	45
		qualifier	673.2	358.9	140	25

7.10.9.2 Source Parameters

7.10.9.2.1 Ion Source: AJS ESI

7.10.9.2.2 Gas Temperature: 290 °C

7.10.9.2.3 Gas Flow: 5.0 L/min

7.10.9.2.4 Nebulizer Pressure: 25 psi

7.10.9.2.5 Sheath Gas Temperature: 300 °C

7.10.9.2.6 Sheath Gas Flow: 8.0 L/min

7.10.9.2.7 Capillary Voltage (Positive Setpoint): 4000 V

7.10.9.2.8 Nozzle Voltage (Positive Setpoint): 0 V

7.11 Recommended Sequence

7.11.1 Allow the instrument to equilibrate under initial conditions for at least 15 minutes. The sample thermostat must be within 2°C of the setpoint before starting the run.

7.11.2 Make at least 3 injections of *Diluent* using the method for *No Spike*.

7.11.3 Make 5 injections of the *Working Standard* using the method for *No Spike*.

7.11.4 Make a single injection of the *Working Sample* using the method *No Spike*.

7.11.5 Make a single injection of the *Working Sample* using the method for *Low Spike*.

7.11.6 Make a single injection of the *Working Sample* using the method for *High Spike*.

7.11.7 Repeat steps 7.11.4 – 7.11.6 for each sample to be analyzed.

7.12 System Suitability Requirements

7.12.1 The %RSD for five consecutive injections of the *Working Standard* is NMT 5.0%. The RSD is evaluated for each sample set.

7.12.2 The coefficient of determination (R^2) for each standard addition series is NLT 0.990. The coefficient of determination is evaluated for each sample tested.

7.12.3 The quantifier/qualifier ratio for each unspiked sample injection is within ± 20% of the average quantifier/qualifier ratio for the five working standard injections.

7.12.4 No significant (>0.5%) interference is present in the diluent injection.

7.13 Column Wash and Storage

7.13.1 Store the column in Mobile Phase.

8.0 Example calculation

8.1 The calculation may be performed automatically by chromatographic software.

8.2 Plot the data obtained from the unspiked and spiked samples with spike concentration on the x-axis and peak area on the y-axis.

8.3 Perform linear regression of the data to obtain the equation y = mx + b.

8.4 Calculate the amount of target analyte in the sample:

$$\text{Amino Acid (mg)} = \frac{-b \times DF \times FW}{10^6\, {}^{ng}/_{mg} \times m \times SW}$$

b y-intercept of the linear regression

DF Dilution factor in mL

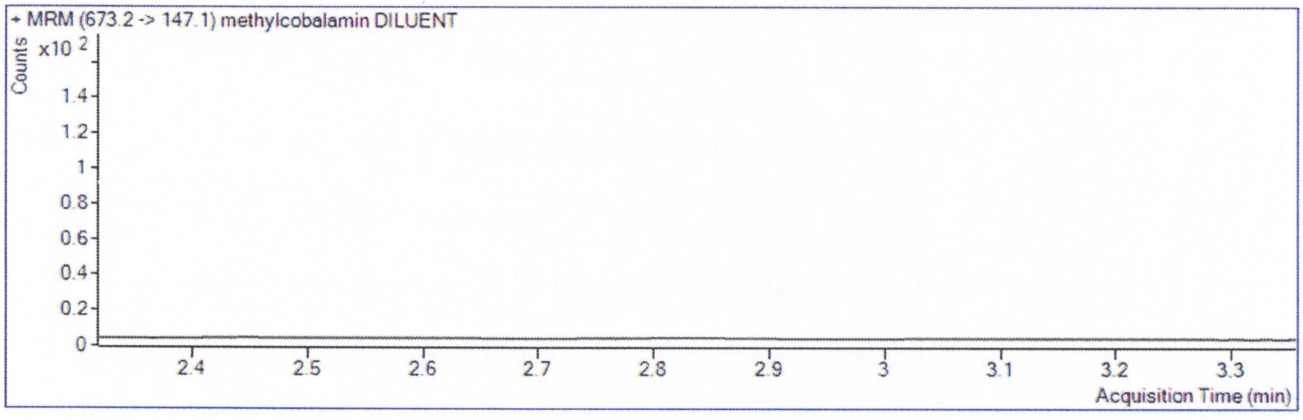
FW Unit dose weight (in g for finished products) or 100 (raw materials)

SW Sample weight in g

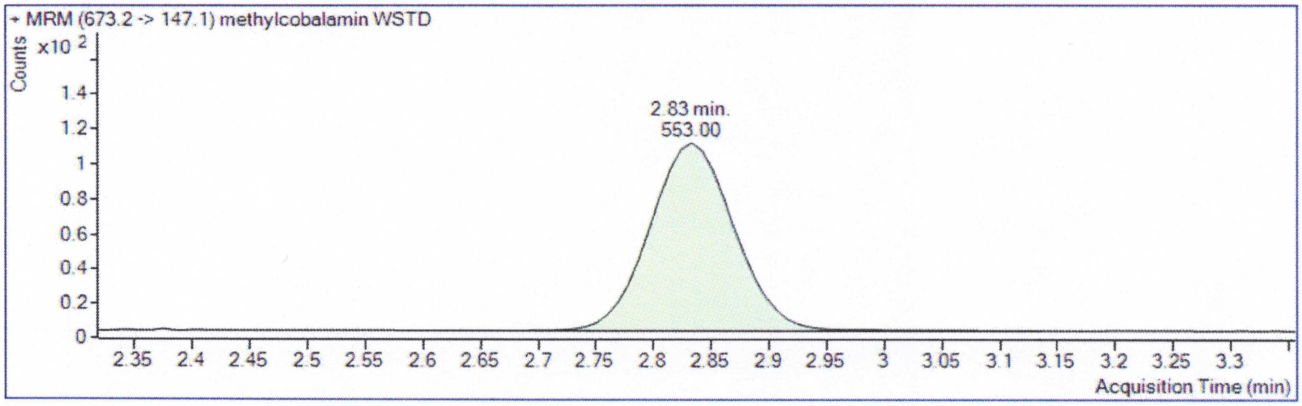
m slope of the linear regression (mL/ng)

9.0 Example Chromatography

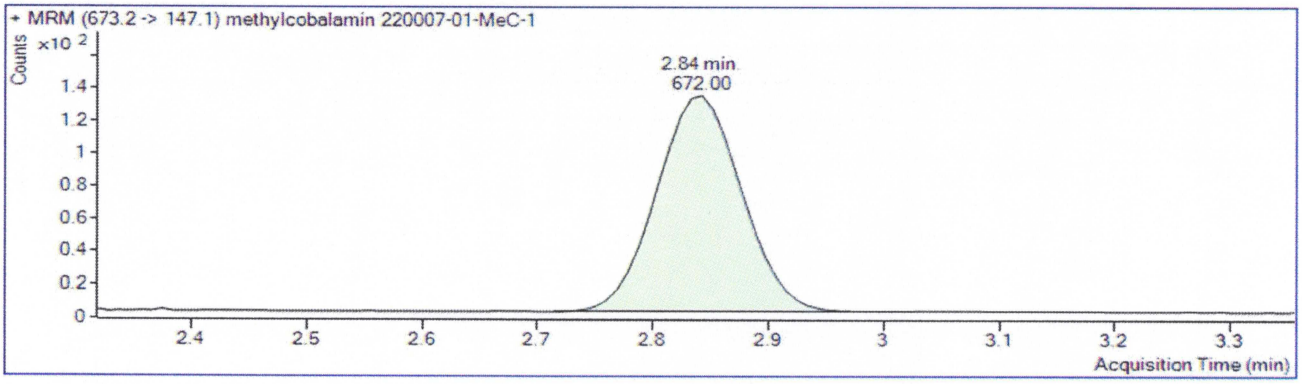
9.1 Methylcobalamin Blank



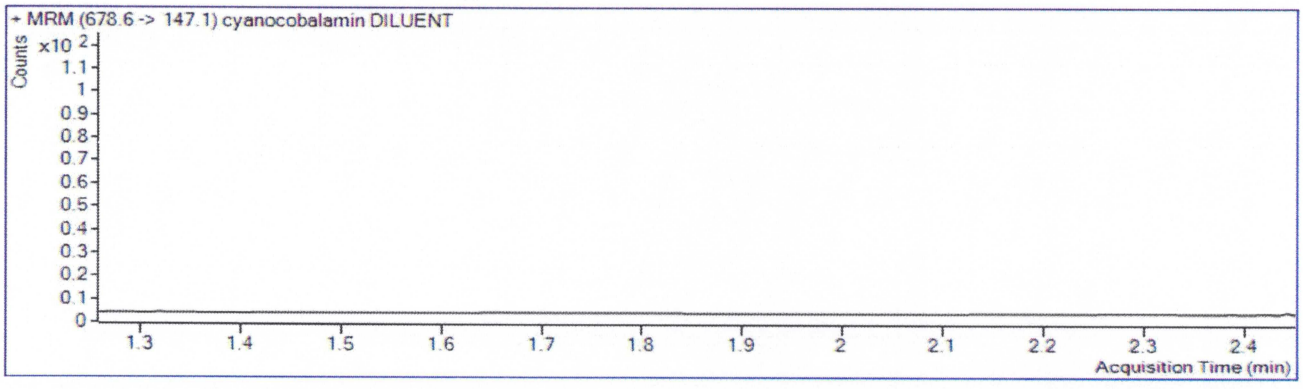
9.2 Methylcobalamin Standard



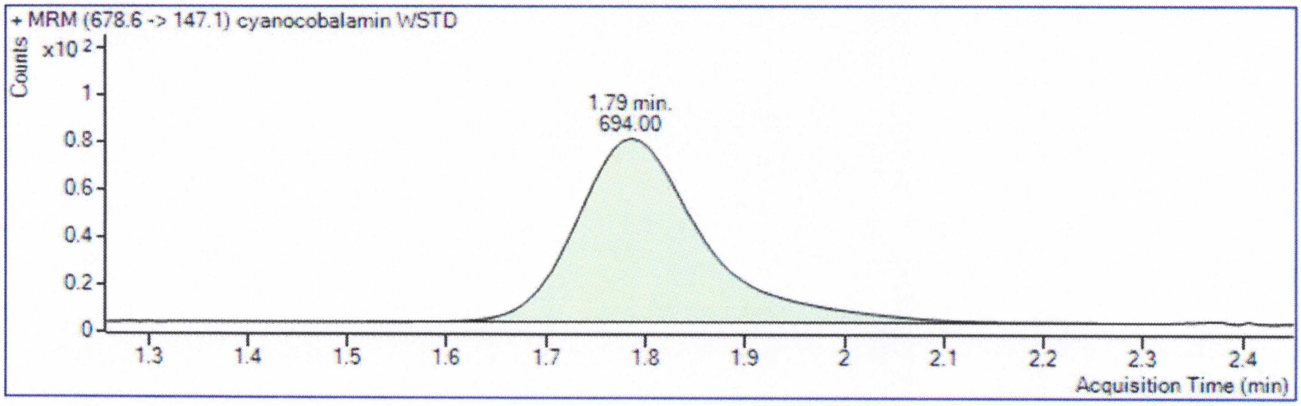
9.3 Methylcobalamin Sample



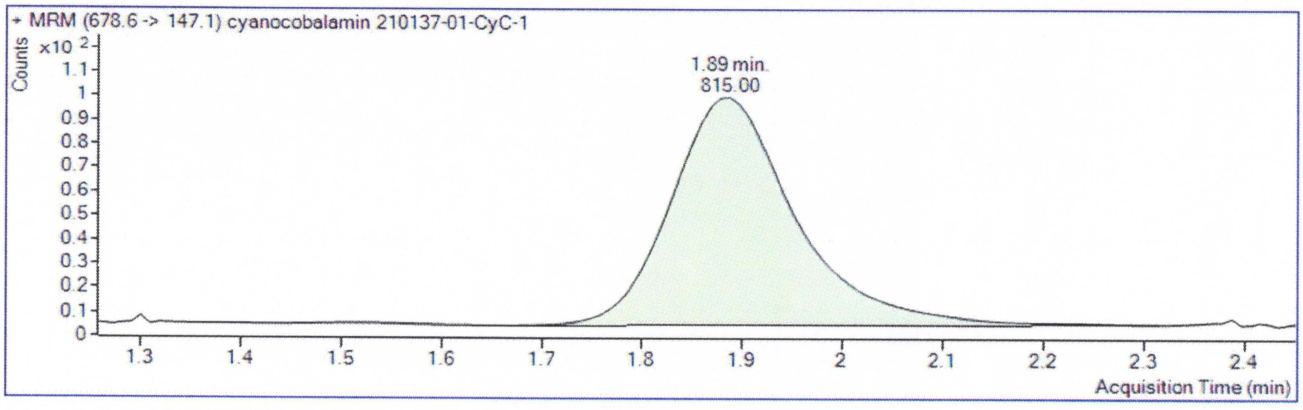
9.4 Cyanocobalamin Blank



9.5 Cyanocobalamin Standard



9.6 Cyanocobalamin Sample



10.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	05/16/22	New	N/A	S. Sassman
1	04/11/23	Add direction to follow product specific test details listed in the product profile, add option to modify the default sample weight, add instruction for homogenization of different dosage forms, add example chromatography.	CC-23-0191	S. Sassman
2	01/14/25	INV-25-0001 and CAPA-25-0002 were iniated to address the initial low LC-MS result for B12 being due to the use of an aged composite this SOP is updated with new suitability of sample composite requirements to prevent recurrence.	CC-25-0013	Ashton Lukes