1.0 Purpose

The purpose of this procedure is to define the method for the quantitation and/or identification of β-Hydroxy-β-Methyl Butyrate (HMB) and β-Hydroxy Butryrate (BHB) in raw materials and finished product dietary supplements using HPLC and UV/VIS spectrophotometry.

2.0 Scope

This procedure applies to the quantification and identification of HMB, along with its analogous forms, and BHB along with its analogous forms in raw materials and finished products. BHB and HMB are poor chromophores and were measured at 214 nm. Other wavelengths can be used if interferences are present.

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 the QC Laboratory Management and/or Analytical Development to keep this procedure aligned with current practices.

4.0 Definitions

4.1 **UV/VIS** – Ultraviolet and Visible Electromagnetic Spectrums

4.2 **H_3PO_4** – Phosphoric Acid

4.3 **HCl** – Hydrochloric Acid

4.4 **ACN** – Acetonitrile

4.5 **KH_2PO_4** – Monobasic Potassium Phosphate

4.6 **H_2O** –Water

4.7 **BHB** – β-Hydroxy Butryrate; 3-Hydroxybutanoic acid

4.8 **HMB** – β-Hydroxy-β-Methyl Butyrate ; β-hydroxyisovaleric acid

5.0 References

5.1 MV-LAB-18-135, Protocol, β-Hydroxybutyrate and β-Hydroxy-β-Methylbutyrate Determination using HPLC with UV/VIS Spectroscopy

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

6.0 Supplies

6.1 Chemicals: All reagents are HPLC grade or better.

6.1.1 H_2O ($\geq$ 18.2 MΩ·cm)

6.1.2 ACN

6.1.3 H_3PO_4

6.1.4 HCl

6.1.5 KH_2PO_4

6.1.6 BHB reference standard

6.1.7 HMB reference standard

6.2 Glassware

6.2.1 HPLC vials, 12mm x 32mm with screw cap enclosures with septa

6.2.2 Scintillation vials

6.2.3 Mobile phase containers

6.2.4 Volumetric glassware as required by standard and sample preparations

6.3 Disposables

6.3.1 Pipette tips for adjustable pipettes

6.3.2 1.5mL microcentrifuge tubes

6.3.3 16mL test tubes

6.3.4 Disposable plastic luer lock syringe – 3mL, 6mL, or 10mL

6.3.5 Nylon syringe filters, 0.45µm

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 Ultrasonic bath

6.4.4 Vortex

6.4.5 Stir plate

6.4.6 Eppendorf centrifuge

6.4.7 Adjustable pipettes

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

7.1 Buffer – 0.05M KH_2PO_4 (aq)

7.1.1 Transfer 6.803 g of KH_2PO_4 to a 1000-mL mobile phase bottle.

7.1.2 Add 950 mL of H_2O.

7.1.3 Adjust to pH 2.9 ± 0.5 with H_3PO_4.

7.1.4 Transfer to a 1000-mL volumetric flask and dilute to volume with H_2O.

7.2 Mobile Phase A – Buffer and ACN (95:5)

7.2.1 Transfer 50 mL of ACN to a 1000-mL mobile phase bottle.

7.2.2 Add 950 mL of Buffer, and mix well.

7.3 Mobile Phase B – Acetonitrile and H_2O (80:20)

7.3.1 Transfer 800ml of ACN to a 1000-mL mobile phase bottle.

7.3.2 Add 200ml of H_2O, and mix well.

7.4 Diluent– 0.1N HCl

7.4.1 Transfer 900mL of H_2O to a 1000-mL mobile phase bottle.

7.4.2 Add 100mL of 1N HCl. and mix well.

7.5 Standard Preparation

7.5.1 The linear range of the method for each analyte is listed below. All standard and sample preparations must be within the linear range of the method.

7.5.1.1 HMB – 0.2 to 1.6 mg/mL

7.5.1.2 BHB – 0.04 to 1.0 mg/mL

7.5.2 The standard is prepared by weighing no less than the minimum weight of the analytical balance, then bringing up to the final volume in an appropriate volumetric flask using dissolution buffer and sonicating for 10 minutes.

7.5.3 To manage large volumes, the standard can be initially prepared at a higher concentration and further diluted into the linear range using Diluent. **Equilibrate the standard solution to room temperature prior to performing further dilution.** Dilutions can be made using volumetric glassware and/or adjustable 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. Dilutions can be prepared in HPLC vials.

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 above.

7.6.2 For raw materials: weigh no less than 20 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. Dilute to volume with Diluent, and sonicate for 10 min.

7.6.3 For solid and liquid dose finished products: Combine and homogenize no less than ten dosage units. Based on the label claim and 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 linear range. Dilute to volume with Diluent, and sonicate for 10 min.

7.6.4 For chewable gels (gummies), homogenize at least 10 dosage units according to the procedure outlined in D-793 Cryogenic Grinding of Chewable Gels. Quickly weigh a portion of the pooled and homogenized dosages into a beaker. Use several small portions of Diluent to completely transfer the sample into a suitably sized volumetric flask to generate an analyte concentration that is within the validated linear range. Dilute to volume, and sonicate for 10 min.

7.6.5 To manage large volumes, the sample can be initially prepared at a higher concentration and further diluted into the linear range using Diluent. **Equilibrate the sample solution to room temperature prior to performing further dilution.** Dilutions can be made using volumetric glassware and/or adjustable pipettes. Dilutions can be prepared in HPLC vials.

7.6.6 If particulates remain in the final sample preparation, a portion may be centrifuged at 10,000 rpm for 5 min prior to HPLC analysis. Alternatively, the sample may be filtered through a 0.45 µm membrane discarding the first 3 – 4 mL.

8.0 Test Conditions

8.1 Gradient with wash (used for sample injections)

Time	%A	%B
0.00	100	0.0
15.00	80.0	20.0
15.01	0.00	100
20.00	0.00	100
20.01	100	0
32.00	100	0

8.2 Gradient no wash (used for blank and standard injections)

Time	%A	%B
0.00	100	0.0
15.00	80.0	20.0
15.01	100	0
19.00	100	0

8.3 Column – Acclaim C18, 5µm, 120Å, LC column, 250mm x 4.6mm, or equivalent

8.4 Flow Rate – 0.5mL/min

8.5 UV Detection – 214nm

8.6 Injection Volume - 10µL

8.7 Column Temperature – Ambient (not controlled)

8.8 3-D Spectral Range – 200 to 300 nm

8.9 Recommended Sequence

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

8.9.2 Make five injections of the Working Standard.

8.9.3 Make a single injection of each Sample Preparation.

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

8.10 System Suitability

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

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

8.11 Column Wash and Storage

8.11.1 Rinse the column with H_2O / ACN (90/10) at 1 mL/min for at least 15 min.

8.11.2 Rinse the column with H_2O / ACN (50/50) at 1 mL/min for at least 10 min.

8.11.3 Store the column with H_2O / ACN (50/50).

9.0 Calculations

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)

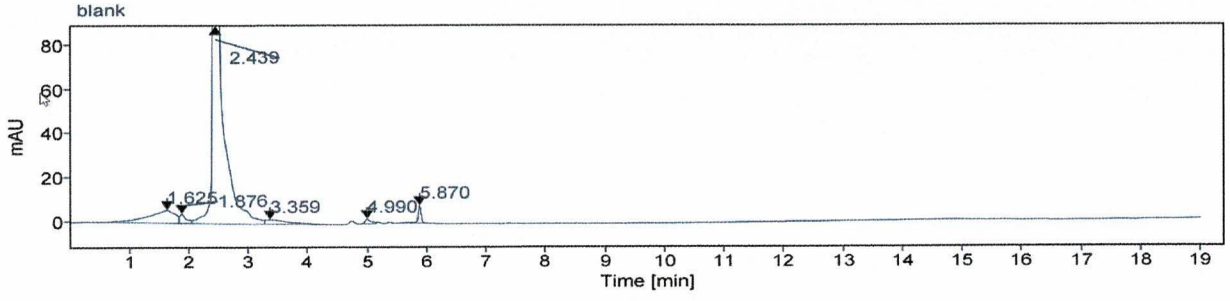
V_{spl} Volume of the sample preparation accounting for dilutions in mL

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.

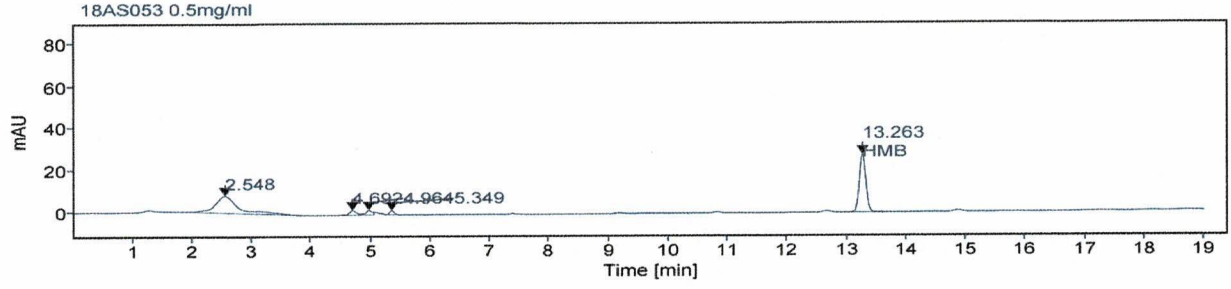
LA Label amount in mg per dose or 1 for raw materials

10.0 Example Chromatography

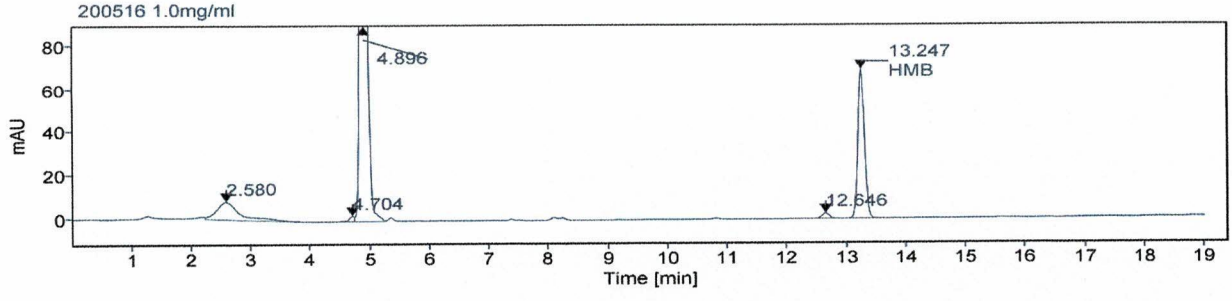
10.1 Blank



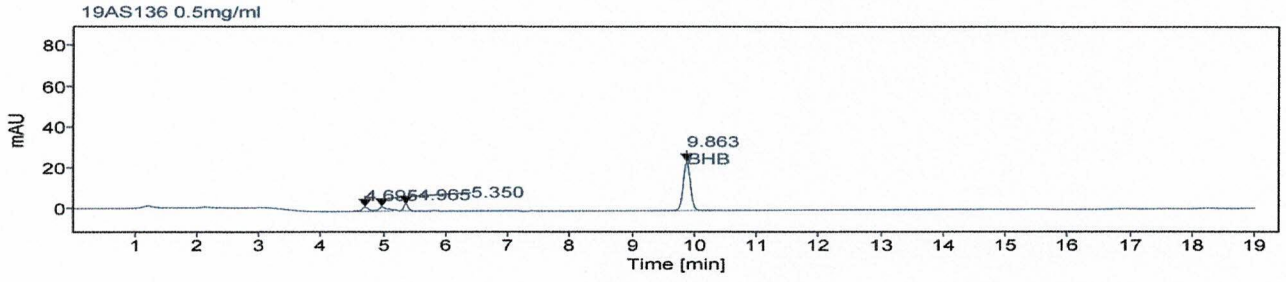
10.2 HMB Standard



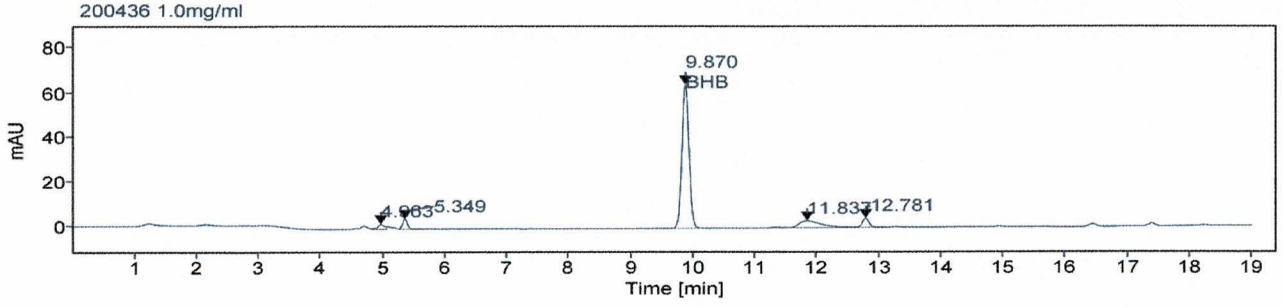
10.3 HMB Sample



10.4 BHB Standard



10.5 BHB Sample



11.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	01/02/19	New	N/A	J. Maignan
1	06/20/22	Update for consistency with current methods, add recommended sequence section, replace requirements with system suitability section, add example chromatography, and remove 229nm as quantitation wavelength.	CC-22-0280	S. Sassman
2	05/15/23	Remove unnecessary information and align with current SOP format, add instruction to follow test details containing product specific sample preparation, add specific sample prep instructions for different dosage forms. Updated logo and format.	CC-23-0232	S. Sassman