## 1.0 Purpose

The purpose of this procedure is to define the method for the determination of tributyrin in raw materials and finished products by GC-FID.

## 2.0 Scope

This procedure applies to the determination of tributyrin in raw materials and finished products in the QC Laboratory at Ion Labs.

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

## 4.0 Definitions

4.1 **QC** – Quality Control

4.2 **GC** – Gas Chromatography

4.3 **FID** – Flame Ionization Detection

4.4 **CoA** – Certificate of Analysis

4.5 **TBT** – Tributyrin

4.6 **DGM** – Diethylene glycol methyl ether

4.7 **THF** – Tetrahydrofuran

## 5.0 References

5.1 PRTCL-20-0041, Protocol, Validation of an Analytical Method for the Determination of Tributyrin by GC-FID

5.2 RPT-21-0024, Report, D-729 Estimation of Uncertainty

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

## 6.0 Supplies

6.1 Chemicals

6.1.1 TBT Reference Standard (>97%)

6.1.2 DGME (ACS reagent grade or better)

6.1.3 THF (Not stabilized, GC grade or better)

6.2 Compressed Gases (use ultra-high purity gases)

6.2.1 Hydrogen

6.2.2 Helium

6.2.3 Air

6.2.4 Nitrogen

6.3 Supplies and Glassware

6.3.1 1.0-mL gas-tight syringe

6.3.2 Volumetric glassware as required for standard and sample preparation

6.4 Equipment

6.4.1 Agilent 7890 GC with FID detector

6.4.2 Analytical Balance

## 7.0 GC Conditions

7.1 Column: Agilent HP-5, 30 m x 0.32 mm x 0.25 µm or equivalent

7.2 Inlet Liner: Restek, 4.0 mm ID x 6.3 mm OD x 78.5 mm length straight liner with glass wool or equivalent

7.3 Injector Temp: 220 °C

7.4 Detector Temp: 280 °C

7.5 Equilibration Time: 0.5 min

7.6 Flow Rate: 2 mL/min

7.7 Run Time: 11 min

7.8 Split ratio: 10:1

7.9 Septum purge: 3 mL/min

7.10 Air flow: 350 mL/min

7.11 Hydrogen flow: 30 mL/min

7.12 Makeup flow: 30 mL/min (column + makeup = constant)

7.13 Injection Volume: 1 µL

7.14 Injection Type: Standard

7.15 Plunger Speed: Fast

7.16 Wash Solvent: THF

7.17 Temperature Ramp (Raw Materials and System Suitability Solution)

| Ramp Rate (°C/min) | Temp (°C) | Hold Time (min) |
|---|---|---|
| N/A | 50 | 1 |
| 20 | 250 | 0 |

## 8.0 Diluent Preparation

8.1 For 1 L: Mix 1.0 mL of DGME with 1000 mL of THF.

## 9.0 Standard Preparation

9.1 Use the actual purity from the CoA for the reference material in calculations.

9.2 Working Standard: Draw about 0.05 mL of reference standard into a 1-mL gas-tight syringe, and wipe the outside of the needle with a Kimwipe. Place a 25-mL volumetric flask on a balance, and press Tare. Transfer about 25 mg of reference standard (about 5 drops) into the flask, taking care to direct the standard directly to the bottom of the flask. Record the weight of standard added. Dilute to volume with Diluent, and mix well. The standard preparation may be scaled up as desired. Prepare a second standard (Std B) as a standard recovery check.

## 10.0 Sample Preparation

10.1 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.

10.2 The validated linear range of the method is 0.8 – 1.2 mg/mL. The content of the sample preparation must be within the linear range. **Flask size used cannot be less than 50mL.**

10.3 Ensure that the sample is thoroughly homogenized prior to weighing. It is not recommended to use a mortar and pestle to grind the sample because tributyrin is a viscous liquid. For most solid and liquid samples, transfer the entire sample to a container of suitable size so that the sample takes up less than half of the container volume, and homogenize by turning end-over-end for at least one minute. For gummies, homogenize as outlined in D-793 Cryogenic Grinding of Chewable Gels.

10.4 The use of glass pipets is recommended for transfers and dilutions. TBT may absorb to plastics, and THF is not compatible with automatic pipets.

10.5 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. Add Diluent to volume, and sonicate for 10 min.

10.6 For solid or liquid dose finished products: Combine and homogenize no less than ten dosage units. Based on the label claim and fill weight (capsules), serving size (powders) or tablet 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. Add Diluent to volume, and sonicate for 10 min.

10.7 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. Add Diluent to volume, and sonicate for 10 min.

10.8 To manage large volumes, the sample can be initially prepared at a higher concentration and further diluted into the linear range. **Equilibrate the stock sample to room temperature prior to performing further dilution.**

10.9 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.

## 11.0 Recommended Sequence

11.1 Make two injections of Diluent

11.2 Make five injections of Working Standard A

11.3 Make two injections of Working Standard B

11.4 Make a single injection of each Sample Preparation

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

## 12.0 System Suitability Requirements

12.1 Samples and Standards should be prepared and analyzed on the same day.

12.2 No significant (>0.5%) interfering peaks are present in the blank (Diluent) injection.

12.3 The %RSD of the peak area ratio in five consecutive injections of the Working Standard is NMT 2.0%.

12.4 The % recovery of Working Standard A, using Working Standard B is 98-102%.

12.5 The %RSD of the peak area ratio for all injections of Working Standard A is NMT 2%.

## 13.0 Retention Times

13.1 DGME = 4.0 min (elutes as a doublet, use the second peak)

13.2 TBT = 9.9 min

## 14.0 Example Calculations

$$\%\ \text{Assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{Spl_{wt}} \times \frac{FW}{LA} \times 100$$

| | |
|---|---|
| $R_u$ | Sample peak area ratio |
| $R_s$ | Mean Working Standard A peak area ratio (5 injections) |
| $Wt_{std}$ | Weight of reference standard used to prepare Working Standard A (mg) |
| $P$ | Purity of reference standard from the CoA (% w/w) |
| $V_{std}$ | Volume of Working Standard A (mL) |
| $V_{spl}$ | Volume of Sample Solution (mL) |
| $Spl_{wt}$ | Sample weight (mg) |
| FW | Theoretical fill/tablet weight (mg, use 1 for raw materials) |
| LA | Label amount (mg, use 1 for raw materials) |

## 15.0 Reporting Results

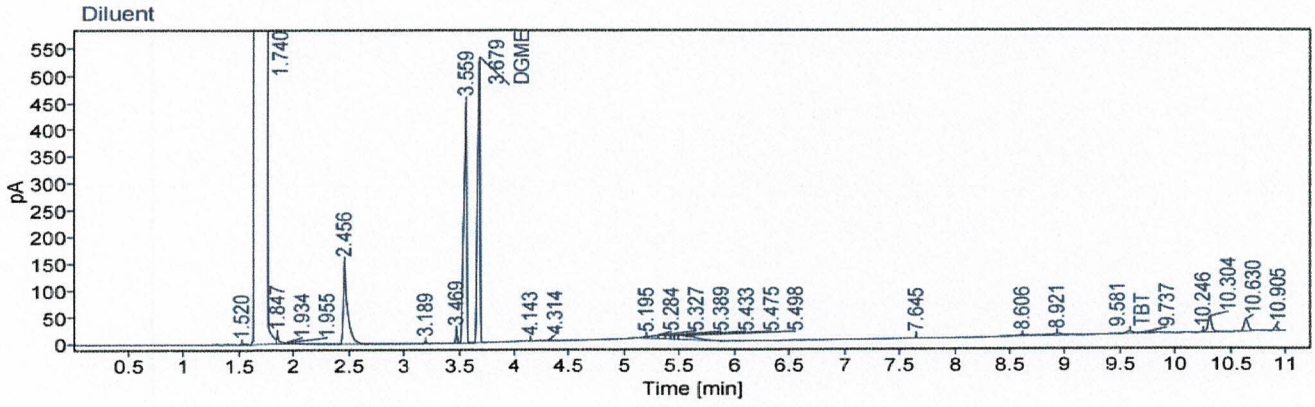
15.1 The expanded uncertainty for Tributyrin is 1.0%, with a coverage factor of 2

15.2 Report results along with the expanded uncertainty and coverage factor in the following format:

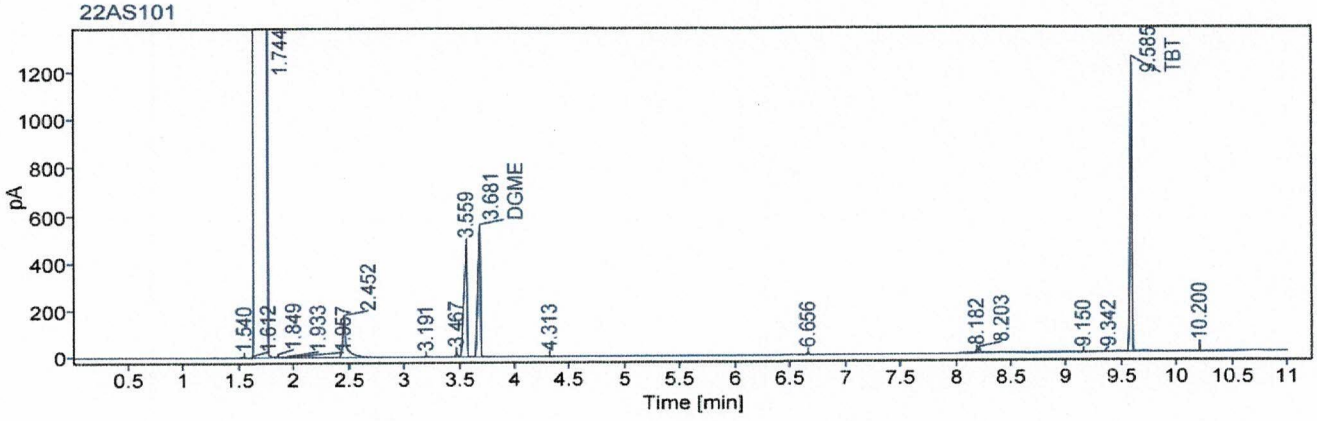
15.2.1 102% of Label Claim, U = ± 1.0% k = 2

# 16.0 Example Chromatography

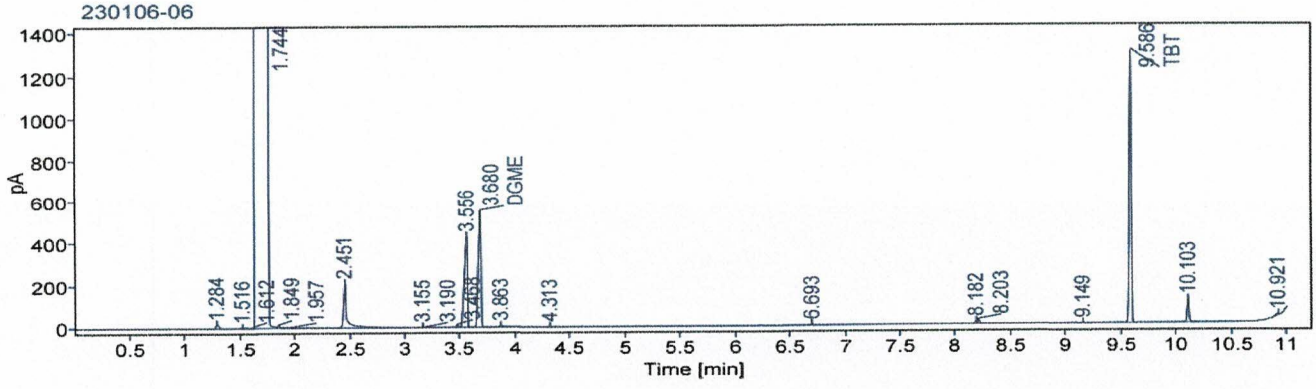
## 16.1 Blank (Internal Standard Solution)




## 16.2 Standard




## 16.3 Finished Product Sample



## 17.0 Revision History

| Revision | Date | Description of Changes | CCR # | By |
|---|---|---|---|---|
| 0 | 05/29/20 | New Document | N/A | S. Sassman |
| 1 | 11/04/21 | Updated to reflect ISO 17025:2017 requirements | CC-21-0407 | J. Sassman |
| 2 | 05/16/23 | Add instruction to refer to test details for product specific sample prep, add specific sample prep for different dosage forms, add example chromatography, add reference to cryogenic grinding SOP for gummies, | CC-23-0227 | S. Sassman |