1.0 Purpose

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

2.0 Scope

This procedure applies to the determination of phytosterols in raw materials and finished products in the QC Laboratory.

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-FID** – Gas Chromatography with Flame Ionization Detection

4.3 **PTFE** – Polytetrafluoroethylene

5.0 References

5.1 PRTCL-20-0113, Protocol, Validation of an Analytical Method for the Determination of Phytosterols by GC-FID

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

6.0 Supplies

6.1 Chemicals

6.1.1 Stigmasterol Reference Standard (>97%)

6.1.2 Tetrahydrofuran (ACS reagent grade or better)

6.1.3 5α-Cholestane (ACS reagent 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 Volumetric glassware as required for standard and sample preparation

6.3.2 2-mL GC vials with PTFE lined closures

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	Injection Volume	1 µL
7.4	Oven Temp:	270 °C (isothermal)
7.5	Injector Temp:	300 °C
7.6	Detector Temp:	320 °C
7.7	Equilibration Time:	0.5 min
7.8	Flow Rate:	1.2 mL/min
7.9	Run Time:	20 min
7.10	Split ratio:	50:1
7.11	Septum purge:	Off
7.12	Air flow:	350 mL/min
7.13	Hydrogen flow:	30 mL/min
7.14	Makeup flow:	30 mL/min (column + makeup = constant)
7.15	Injection Type	Standard
7.16	Plunger Speed	Fast
7.17	Wash Solvent	THF

8.0 Internal Standard Solution Preparation

8.1 Accurately weigh and transfer about 50 mg of 5α-cholestane to a suitable container.

8.2 Add 200-mL of tetrahydrofuran.

8.3 Mix until completely dissolved.

9.0 Working Standard Preparation

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

9.2 Accurately weigh and transfer about 25 mg of stigmasterol to a 100-mL volumetric flask.

9.3 Dissolve in and dilute to volume with Internal Standard Solution.

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 of this method.

10.2 The validated linear range of the method is 0.01 – 1.0 mg/mL. The content of the three most abundant phytosterols in the sample preparation must be within the linear range.

10.3 Ensure that the sample is thoroughly homogenized prior to weighing.

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

10.5 For finished products, pool at least 10 dosage units and homogenize. Based on the dosage weight (for powders), fill weight (for capsules) or tablet weight and the label claim, weigh no less than 20 mg of the pooled dosages into a suitably sized volumetric flask to generate concentrations for the three most abundant phytosterols that are within the validated

linear range, add Internal Standard Solution to about 70% of the flask volume and shake for at least 10 minutes. Dilute to volume with Internal Standard Solution, and mix well.

10.6 For raw materials, based on the expected potency, weigh no less than 20 mg of sample into a suitably sized volumetric flask to generate concentrations for the three most abundant phytosterols that are within the validated linear range, add Internal Standard Solution to about 70% of the flask volume and shake for at least 10 minutes. Dilute to volume with Internal Standard Solution, and mix well.

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 no less than 200 mg of the pooled and homogenized dosages into a suitably sized beaker. Add a volume of Internal Standard Solution equivalent to 50% of the desired flask volume, add a stir bar, cover the top of the beaker, and stir until dissolved. Transfer the solution to a volumetric flask of size suitable to generate concentrations for the three most abundant phytosterols that are within the validated linear range. Use several small portions of Internal Standard Solution to rinse any remaining residue from the beaker into the volumetric flask ensuring complete transfer, and dilute to volume using Internal Standard Solution.

10.8 To manage large volumes, the sample can be initially dissolved in a smaller volume and a portion further diluted using Internal Standard Solution to bring the analyte concentration into the linear range.

10.9 If particulates remain in sample preparations, remove them by allowing the particulates to settle, filtration, or centrifugation. For filtration, use a 0.45 µm membrane discarding the first 2-3 mL before collecting a portion for analysis. For centrifugation, spin at 10,000 rpm for 5 min.

11.0 Recommended Sequence

11.1 Make two injections of Internal Standard Solution

11.2 Make five injections of Working Standard A

11.3 Make a single injection of each Sample Preparation

11.4 Make a single injection of Working Standard A after every 10 injections and at the end of the run.

12.0 System Suitability Requirements

12.1 No significant (>0.5%) interfering peaks are present in the injection of Internal Standard Solution.

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

12.3 The %RSD of the peak area ratio in all injections of the Working Standard is NMT 3.0%.

12.4 The Tailing Factor for the stigmasterol peak in the injection of the Working Standard is within the range 0.8 – 1.2.

12.5 The USP resolution between the three most abundant phytosterol peaks and any adjacent peak is NLT 1.0.

13.0 Retention Times

13.1 5α-cholestane 7.12 min

13.2 Cholesterol 11.50 min

13.3 Brassicasterol 12.61 min

13.4 Campesterol 14.20 min

13.5 Campestanol 14.42 min

13.6 Stigmasterol 15.10 min

13.7	Sitosterol	16.91 min
13.8	Sitostanol	17.25 min
13.9	Avenasterol	17.52 min

14.0 Relative Response Factors

14.1	Cholesterol	0.909
14.2	Brassicasterol	1.000
14.3	Campesterol	0.951
14.4	Campestanol	0.925
14.5	Stigmasterol	1.000
14.6	Sitosterol	0.915
14.7	Sitostanol	0.942
14.8	Avenasterol	1.000

15.0 Example Calculations

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

R_u	Sample peak area ratio
R_s	Mean Working Standard A peak area ratio (all injections)
Wt_{std}	Weight of reference standard used to prepare Working Standard (mg)
P	Purity of reference standard from the CoA (% w/w)
V_{std}	Volume of Working Standard (mL)

V_{spl} Volume of Sample Solution (mL)

Spl_{wt} Sample weight (mg)

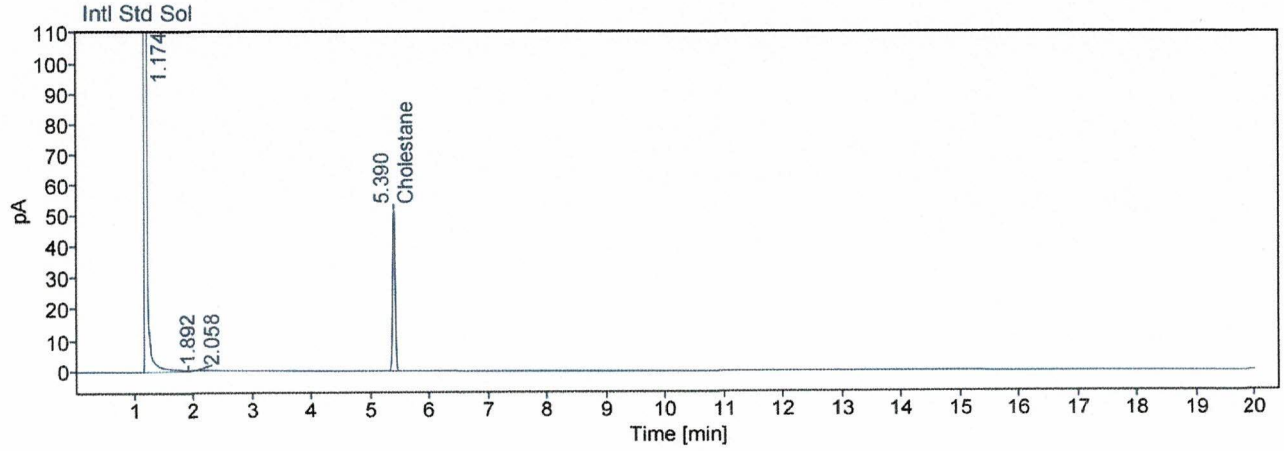
FW Theoretical fill/tablet weight (mg, use 1 for raw materials)

LA Label amount (mg, use 1 for raw materials)

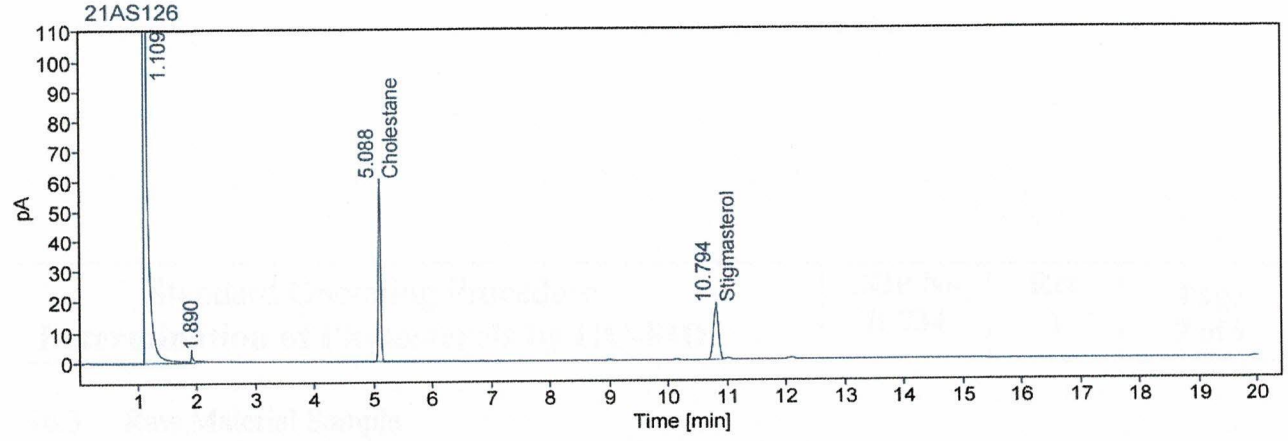
RRF Relative Response Factor

16.0 Example Chromatography

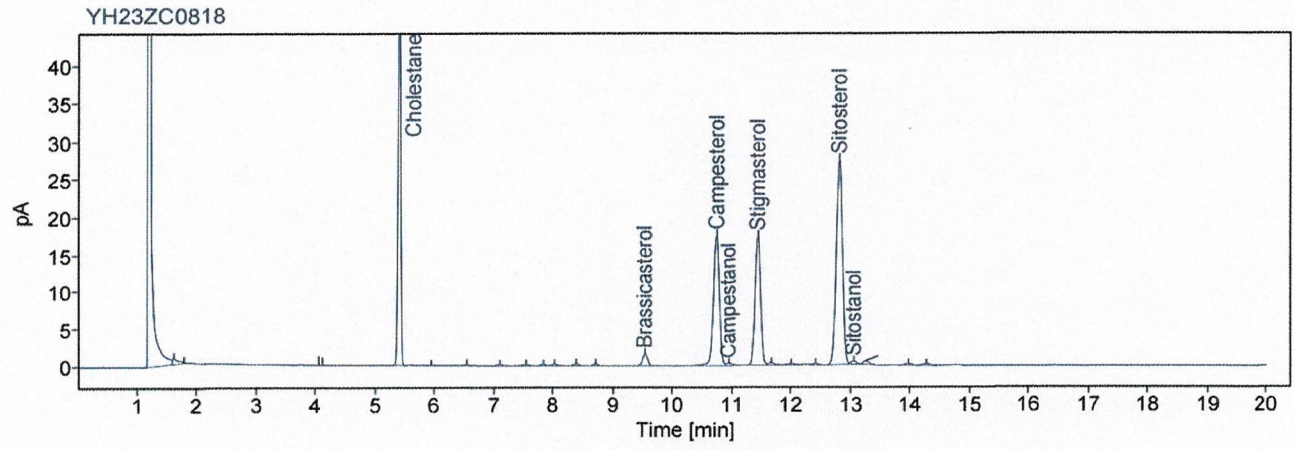
16.1 Blank (Internal Standard Solution)



16.2 Working Standard



16.3 Raw Material Sample



17.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	01/11/21	New	N/A	S. Sassman
1	04/03/24	Add instruction to follow product specific test details if available, add specific sample preparation instructions for gummies, and add example chromatography.	CC-24-0133	S. Sassman