1.0 Purpose

The purpose of this procedure is to define the method for the determination of Eicosapentaenoic Acid (EPA), Docosahexaenoic Acid (DHA), and total Omega 3 fatty acids in raw materials and finished products by GC-FID.

2.0 Scope

This procedure applies to the determination of EPA, DHA, and total Omega 3 fatty acids in raw materials and finished products by GC-FID 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/Analytical Development Management 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 **EPA** – Eicosapentaenoic Acid

4.6 **DHA** – Docosahexaenoic Acid

5.0 References

5.1 PRTCL-20-0076, Protocol, Validation of an Analytical Method for the Determination of EPA and DHA by GC-FID

6.0 Supplies

6.1 Chemicals

6.1.1 Fish oil reference standard

6.1.2 Docosahexaenoic acid ethyl ester reference standard

6.1.3 Eicosapentaenoic acid ethyl ester reference standard

6.1.4 Methyl tricosanoate

6.1.5 Docosahexaenoic acid methyl ester

6.1.6 Tetracos-15-enoic acid (nervonic acid) methyl ester

6.1.7 Boron trichloride (12%) in methanol

6.1.8 Butylated hydroxytoluene (2,6-di-tert-butyl-4-methylphenol)

6.1.9 Sodium Hydroxide

6.1.10 Sodium Chloride

6.1.11 Sodium Sulfate Anhydrous

6.1.12 2,2,4-Trimethylpentane

6.1.13 Methanol

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

6.4 Equipment

6.4.1 Agilent 7890 GC with FID detector

6.4.2 Analytical Balance

7.0 GC Conditions

7.1	Column:	Supelcowax 10, 30 m x 0.25 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:	250 °C
7.4	Detector Temp:	270 °C
7.5	Equilibration Time:	0.5 min
7.6	Flow Rate:	1 mL/min
7.7	Run Time:	35.3 min
7.8	Split ratio:	600:1
7.9	Septum purge:	Off
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	0.3 µL
7.14	Injection Type	Standard
7.15	Plunger Speed	Fast
7.16	Wash Solvent	2,2,4-trimethylpentane

7.17 Temperature Ramp (Raw Materials and System Suitability Solution)

Ramp Rate (°C/min)	Temp (°C)	Hold Time (min)
N/A	170	2
3	240	10

8.0 Antioxidant Solution

8.1 Accurately weigh and transfer about 10 mg of butylated hydroxytoluene to a suitable container.

8.2 Add 200 mL of 2,2,4-trimethylpentane

8.3 Mix well to dissolve.

9.0 Internal Standard Solution

9.1 Accurately weigh and transfer 350 mg of methyl tricosanoate to a 50-mL volumetric flask.

9.2 Dissolve in and dilute to volume with *Antioxidant Solution.*

10.0 Sodium Hydroxide Solution

10.1 Transfer 1 g of sodium hydroxide to a suitable container.

10.2 Add 50 mL of methanol, and sonicate to dissolve.

11.0 Saturated Sodium Chloride Solution

11.1 Transfer 20 g of sodium chloride to a suitable container.

11.2 Add 50 mL of H_2O.

11.3 Shake for at least 10 min.

11.4 Allow particulates to settle before use.

12.0 Sample Solution A

Note: Do not use plastic pipets or containers for sample preparation. DHA, EPA and their methyl esters are extremely lipophilic, and will absorb strongly to plastic surfaces.

12.1 According to Table 1, transfer and accurately weigh the required amount of sample to a 10-mL volumetric flask.

12.2 Dissolve in and dilute to volume with *Antioxidant Solution.*

12.3 If the test sample contains EPA and DHA in ethyl ester form, proceed to step 0. If the test sample contains EPA and DHA as triglycerides or the form is unknown, continue with step 12.4 .

12.4 Transfer 2.0 mL of the resulting solution to a 10-mL headspace vial.

12.5 Evaporate the solvent under a gentle stream of nitrogen.

12.6 Add 1.5 mL of *Sodium Hydroxide Solution.*

12.7 Purge the container with argon or nitrogen gas, cap tightly, and mix.

12.8 Heat in a boiling water bath for 7 min, then allow to cool.

12.9 Remove the cap, and add 2.0 mL of boron trichloride in methanol.

12.10 Purge the container with argon or nitrogen gas, cap tightly, and mix.

12.11 Heat in a boiling water batch for 30 min.

12.12 Allow to cool to room temperature, and remove the cap.

12.13 Add 5.0 mL of *Saturated Sodium Chloride Solution.*

12.14 Add 1.0 mL of 2,2,4-trimethylpentane, purge with argon or nitrogen gas, and cap tightly.

12.15 Mix on a vortex mixer or shake vigorously for at least 60 sec.

12.16 Allow the upper layer to become clear, remove the cap, and transfer the upper layer to a small glass vial.

12.17 Add another 1.0 mL of 2,2,4-trimethylpentane to the remaining bottom layer.

12.18 Purge the container with argon or nitrogen gas, and cap tightly.

12.19 Mix on a vortex mixer or shake vigorously for at least 15 sec.

12.20 Allow the upper layer to become clear, remove the cap, and combine the upper layer with the upper layer from the first extraction.

12.21 Add 1.0 mL of H_2O to the combined extracts, purge the container with argon or nitrogen gas, cap, and shake well.

12.22 Allow the layers to separate, remove the cap, and discard the bottom layer.

12.23 Add another 1.0 mL of H_2O to the combined extracts, purge the container with argon or nitrogen gas, cap, and shake well.

12.24 Allow the layers to separate, remove the cap, and discard the bottom layer.

12.25 Add 0.5 g of anhydrous sodium sulfate, and swirl gently for at least 15 seconds.

12.26 Transfer the solution to a 2-mL GC vial for analysis.

Table 1: Sample Weight Required for EPA + DHA Determination

Sum of EPA + DHA	Sample Weight
30% - 50%	0.4 g – 0.5 g
50% - 70%	0.3 g
>70%	0.25 g

13.0 Sample Solution B

13.1 According to Table 1, transfer and accurately weigh the required amount of sample to a 10-mL volumetric flask.

13.2 Dissolve in and dilute to volume with *Internal Standard Solution.*

13.3 If the test sample contains EPA and DHA in ethyl ester form, proceed to step 14.0.

13.4 Proceed as directed in steps 12.4 - 12.25.

14.0 Standard Solution A

14.1 Accurately weigh and transfer about 60 mg of DHA reference standard to a 10-mL volumetric flask.

14.2 Dissolve in and dilute to volume with *Internal Standard Solution.*

14.3 If the test sample contains EPA and DHA in ethyl ester form, proceed to step 15.0.

14.4 If the test sample contains EPA and DHA in triglyceride form, proceed as directed in steps 12.4 - 12.25.

15.0 Standard Solution B

15.1 Accurately weigh and transfer about 90 mg of EPA reference standard to a 10-mL volumetric flask.

15.2 Dissolve in and dilute to volume with *Internal Standard Solution.*

15.3 If the test sample contains EPA and DHA in ethyl ester form, proceed to step **Error! Reference source not found.**.

15.4 If the test sample contains EPA and DHA in triglyceride form, proceed as directed in steps 12.4 - 12.25.

16.0 Standard Solution C

16.1 Accurately weigh and transfer 300 mg of Fish Oil reference standard to a 10-mL volumetric flask.

16.2 Dissolve in and dilute to volume with *Antioxidant Solution*.

16.3 Proceed as directed in steps 12.4 - 12.25.

17.0 System Suitability Solution

Note: This solution is only required if the test sample is in triglyceride form and only if tetracos-15-enoic acid methyl ester is not clearly observed in the chromatogram obtained from *Test Solution 2*.

17.1 Accurately weigh and transfer about 5.0 mg of tetracos-15-enoic acid (nervonic acid) methyl ester to a glass scintillation vial.

17.2 Add 10.0 mL of *Antioxidant Solution*, and swirl to dissolve.

17.3 Transfer about 55 mg of docosahexaenoic acid methyl ester to another glass scintillation vial.

17.4 Using a 20-mL glass pipet, transfer the solution containing nervonic acid methyl ester to the scintillation vial containing docosahexaenoic acid methyl ester.

17.5 Mix to dissolve.

18.0 Recommended Sequence

18.1 Make at least two injections of *Internal Standard Solution*.

18.2 Make one injection of *Sample Solution A*.

18.3 Make one injection of *Sample Solution B*.

18.4 Make one injection of *Standard Solution A*.

18.5 Make one injection of *Standard Solution B*.

18.6 Make one injection of *Standard Solution C*.

18.7 Make one injection of *System Suitability Solution*.

19.0 System Suitability Requirements

19.1 Resolution

Note: Resolution is only required if the test sample is in triglyceride form.

19.1.1 From the injection of *Sample Solution B* or *System Suitability Solution*.

19.1.2 The resolution between the docosahexaenoic acid methyl ester and tetracos-15-enoic acid methyl ester is NLT 1.0.

20.0 Peak Identification

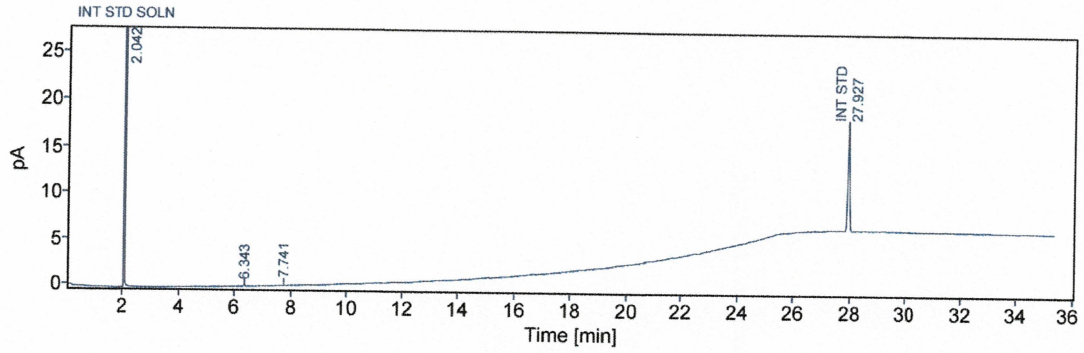
20.1 Approximate retention times for the acid methyl esters are given below. Use the injection of *Standard Solution C*, along with the certificate of analysis for the fish oil reference standard, to identify fatty acid ester peaks.

20.2 Use the injections of *Sample Solution A* and *Sample Solution B* to identify the internal standard peak. It should be the only peak present in *Sample Solution B* that is not present in *Sample Solution A*.

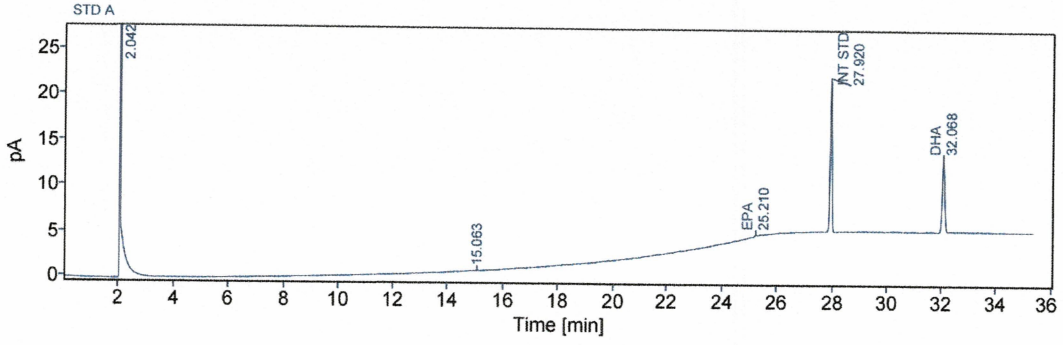
Analyte	Ret Time (min)
C16:0 (Palmitic)	11.91
C18:0 (Stearic)	16.50
C18:3 n-3	19.76
C18:4 n-3	20.55
C20:0 (Arachidic)	21.34
C20:4 n-3	25.36
C20:5 n-3 (EPA)	25.98
C22:0 (Behenic)	26.16
C23:0 (Internal Std)	28.87
C21:5 n-3	28.93
C22:5 n-3	32.21
C24:1 n-9 (Nervonic)	33.19
C22:6 n-3 (DHA)	33.39

21.0 Example Chromatograms

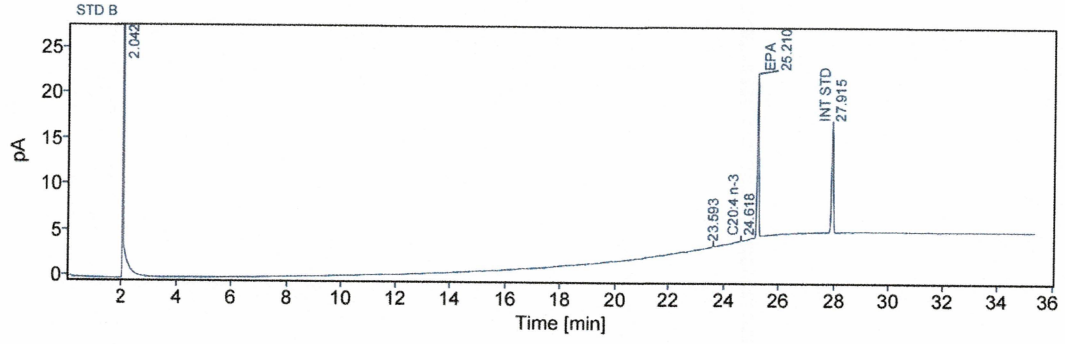
21.1 Internal Standard Solution



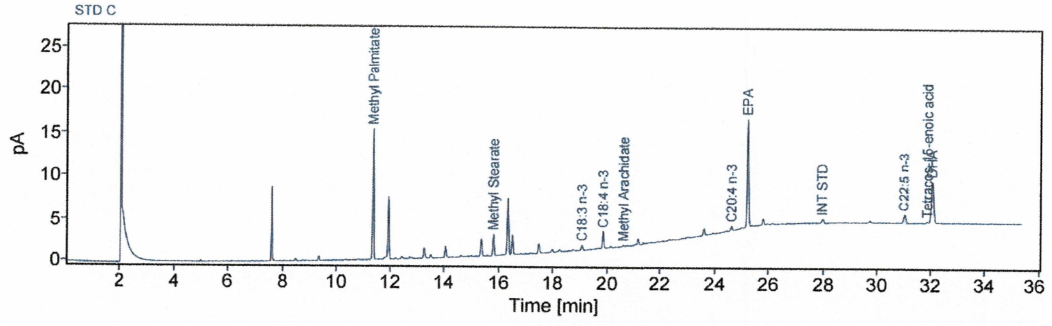
21.2 Standard Solution A



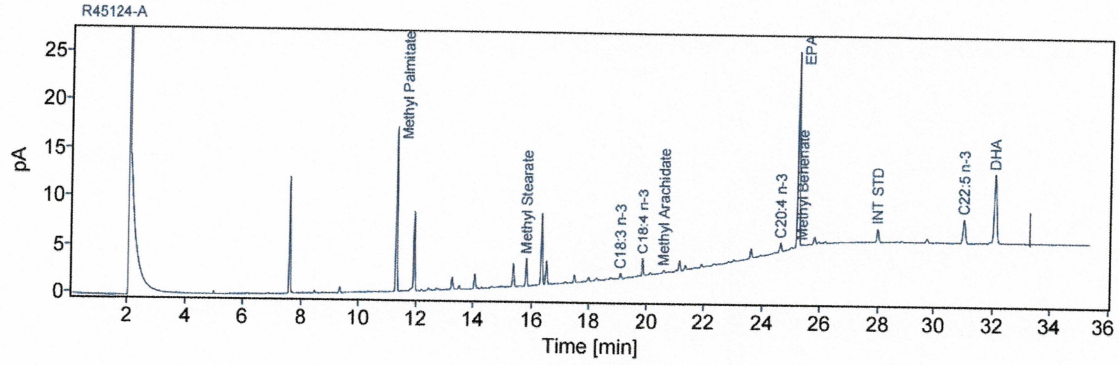
21.3 Standard Solution B



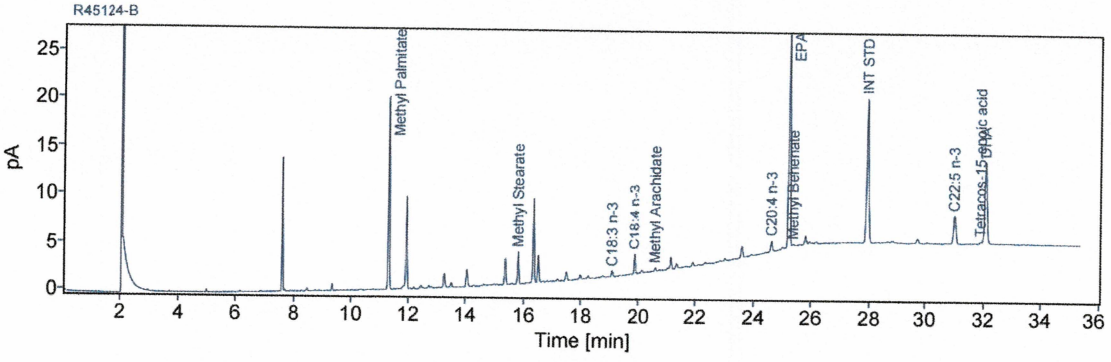
21.4 Standard Solution C



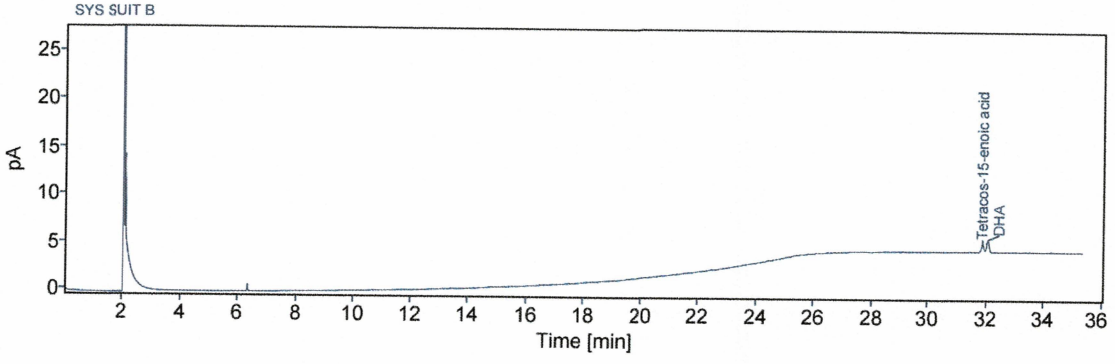
21.5 Sample Solution A



21.6 Sample Solution B



21.7 System Suitability Solution



22.0 Example Calculations

22.1 If the sample contains DHA and EPA in triglyceride form:

$$\text{EPA or DHA (mg)} = \frac{r_{st}}{\mathrm{R_{st}} \times [(r_b \div R_{b)} - (r_a \div R_a)]} \times \frac{Wt_{st} \times P}{Wt_b} \times \mathrm{FW} \times \mathrm{M}$$

r_{st} Peak area of internal standard in Standard Solution A or B

$\mathrm{R_{st}}$ Peak area for EPA or DHA in Standard Solution A or B

r_b Peak area for internal standard in Sample Solution B

R_b Peak area of EPA or DHA in Sample Solution B

r_a Peak area of peak at the ret time of internal standard in Sample SD-730olution A

R_a Peak area of EPA or DHA in Sample Solution A

Wt_{st} Weight of reference standard used to prepare Standard Solution A or B (mg)

P Purity of reference standard from the CoA (% w/w)

Wt_b Weight of sample used to prepare Sample Solution B (mg)

FW Theoretical fill/tablet weight (mg)

M Factor to express as free fatty acids (EPA=0.915 and DHA=0.921)

22.2 If the sample contains EPA and DHA in ethyl ester form, calculate as outlined in section 22.1, but with M = 1.0.

22.3 To calculate Total Omega-3 Fatty Acids

$$Total\ (mg) = EPA + DHA + [A_{n-3} \times (EPA + DHA) \div (A_{EPA} + A_{DHA})]$$

EPA = content of EPA from Section 22.1 or 22.2

DHA = content of DHA from Section 22.1 or 22.2

A_{n-3} = sum of the peak areas for C18:3 n-3, C18:4 n-3, C20:4 n-3, C21:5 n-3, and C22:5 n-3 in the chromatogram of *Sample Solution A*.

A_{EPA} = area of the EPA peak in the chromatogram of *Sample Solution A*.

A_{DHA} = area of the DHA peak in the chromatogram of *Sample Solution A*

23.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	09/17/20	New	N/A	S. Sassman
1	04/08/22	Remove requirement for System Suitability A (theoretical area percentages), update to reflect current lab practices, add example chromatography.	CC-22-0166	S. Sassman