

Omega-3 Free Fatty Acids

DEFINITION
Omega-3 Free Fatty Acids is a mixture of omega-3 acids in their free form. It consists mainly of alpha-linolenic acid (C18:3 n-3), moroctic acid (C18:4 n-3), eicosatetraenoic acid (C20:4 n-3), eicosapentaenoic acid (EPA) (C20:5 n-3), heneicosapentaenoic acid (C21:5 n-3), docosapentaenoic acid (C22:5 n-3), and docosahexaenoic acid (DHA) (C22:6 n-3). It contains NLT 80% (w/w) of total omega-3 free fatty acids. It contains NLT 45% (w/w) and NMT 65% (w/w) of EPA, and NLT 10% (w/w) and NMT 30% (w/w) of DHA. Suitable antioxidants in appropriate concentrations may be added.

IDENTIFICATION

- A.** The retention times of the peaks of EPA methyl ester and DHA methyl ester of the *Sample solution* correspond to those of the respective compounds of the *Standard solution*, as obtained in the test for *Content of EPA, DHA, and Total Omega-3 Free Fatty Acids*.
- B.** It meets the requirements of the test for *Content of EPA, DHA, and Total Omega-3 Free Fatty Acids*.

COMPOSITION

- CONTENT OF EPA, DHA, AND TOTAL OMEGA-3 FREE FATTY ACIDS**

System suitability solution 1: Transfer 250 mg of [USP Omega-3 Free Fatty Acids RS](#) and 70 mg of [USP Methyl Tricosanoate RS](#) (internal standard) to a 100-mL flask suitable for heating under reflux. Add 8 mL of a 2% (w/v) solution of [sodium hydroxide](#) in [methanol](#) to the flask. Heat the mixture under reflux while stirring until the sample is dissolved. Add through the reflux condenser 10 mL of [14% boron trichloride–methanol solution](#). Continue to reflux the solution for NLT 2 min, and then cool to room temperature in a water bath. Add 10 mL of [n-hexane](#) and then 20–30 mL of 10% (w/v) [sodium chloride](#) solution. Cap the flask and shake vigorously for NLT 30 s. Allow the layers to separate and dry the upper layer over [anhydrous sodium sulfate](#) before analysis.

System suitability solution 2: Transfer 55 mg of DHA methyl ester and about 5 mg of tetracos-15-enoic acid methyl ester, accurately weighed, to a 10-mL volumetric flask. Dissolve in and dilute with [n-hexane](#) to volume.

Standard solution: Transfer 70 mg of [USP Methyl Tricosanoate RS](#) (internal standard), 60.0 mg of [USP Docosahexaenoic Acid RS](#), and 90.0 mg of [USP Eicosapentaenoic Acid RS](#) to a 100-mL flask suitable for heating under reflux. Prepare as directed for *System suitability solution 1* starting with “Add 8 mL”.

Sample solution: Prepare as directed for *System suitability solution 1* except replace the [USP Omega-3 Free Fatty Acids RS](#) with Omega-3 Free Fatty Acids.

Chromatographic system
(See [Chromatography \(621\)](#), [System Suitability](#).)

Mode: GC
Detector: Flame ionization
Column: 0.25-mm × 25-m; coated with a 0.20-µm film of phase G16
Temperatures
Injection port: 250°
Detector: 270°
Column: See [Table 1](#).

Table 1

Initial Temperature (°)	Hold Time at 170° (min)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
170	2	3	240	2.5

Carrier gas: Helium

Flow rate: 1 mL/min
Injection volume: 1 µL
Injection type: Split; split ratio, 200:1

System suitability

Samples: *System suitability solution 1*, *System suitability solution 2*, and *Standard solution*
[NOTE—The relative retention times for DHA methyl ester and tetracos-15-enoic acid methyl ester from *System suitability solution 2* are 1.0 and about 1.01, respectively. The relative retention times for EPA methyl ester, methyl tricosanoate (internal standard), and DHA methyl ester from the *Standard solution* are 1.0, about 1.2, and about 1.3, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram of *System suitability solution 1* is similar to the reference chromatogram provided with the lot of [USP Omega-3 Free Fatty Acids RS](#) being used.
Resolution: NLT 1.2 between DHA methyl ester and tetracos-15-enoic acid methyl ester, *System suitability solution 2*
Relative standard deviation: NMT 2.0% for peak response ratios of the EPA methyl ester and DHA methyl ester to the internal standard, *Standard solution*

Analysis

Samples: *System suitability solution 1*, *Standard solution*, and *Sample solution*
Identify the relevant fatty acid methyl esters (see [Table 2](#)) in the chromatogram of the *Sample solution* by comparing the peaks with those in the chromatogram of *System suitability solution 1* and the reference chromatogram provided with the lot of [USP Omega-3 Free Fatty Acids RS](#) being used.
Separately calculate the percentage of EPA and DHA, as free fatty acids, in the portion of Omega-3 Free Fatty Acids taken:

Result = (R_U/R_S) × (W_S/W_U) × 100

- R_U = peak response ratio of the EPA methyl ester or DHA methyl ester to the internal standard from the *Sample solution*
- R_S = peak response ratio of the EPA methyl ester or DHA methyl ester to the internal standard from the *Standard solution*
- W_S = weight of [USP Eicosapentaenoic Acid RS](#) or [USP Docosahexaenoic Acid RS](#) taken to prepare the *Standard solution* (mg)
- W_U = weight of Omega-3 Free Fatty Acids taken to prepare the *Sample solution* (mg)

Calculate the percentage of the total omega-3 free fatty acids in the portion of Omega-3 Free Fatty Acids taken:

Result = EPA + DHA + [A_{n-3} × (EPA + DHA)/(A_{EPA} + A_{DHA})]

- EPA = content of EPA, as free fatty acid (%)
- DHA = content of DHA, as free fatty acid (%)
- A =
- A_{n-3} = sum of the peak areas corresponding to C18:3 n-3, C18:4 n-3, C20:4 n-3, C21:5 n-3, and C22:5 n-3 methyl esters in the *Sample solution*
- A_{EPA} = peak area corresponding to the EPA methyl ester in the *Sample solution*
- A_{DHA} = peak area corresponding to the DHA methyl ester in the *Sample solution*

Acceptance criteria: See [Table 2](#).

Table 2

Name	Relative Retention Time	Acceptance Criteria, NLT (% w/v)	Acceptance Criteria, NMT (% w/v)
C18:3 n-3, FFA ^a	0.564	—	—
C18:4 n-3, FFA ^b	0.592	—	—
C20:4 n-3, FFA ^c	0.770	—	—

Name	Relative Retention Time	Acceptance Criteria, NLT (% w/v)	Acceptance Criteria, NMT (% w/v)
C20:5 n-3, FFA (EPA) ^d	0.793	45	65
C21:5 n-3, FFA ^e	0.887	—	—
C22:5 n-3, FFA ^f	0.973	—	—
C22:6 n-3, FFA (DHA) ^g	1.000	10	30
Total omega-3 FFA	—	80	—

^a Alpha-linolenic free fatty acid.

^b Moroctic free fatty acid.

^c Eicosatetraenoic free fatty acid.

^d Eicosapentaenoic free fatty acid.

^e Heneicosapentaenoic free fatty acid.

^f Docosapentaenoic free fatty acid (clupanodonic free fatty acid).

^g Docosahexaenoic free fatty acid.

IMPURITIES

• CHOLESTEROL

Internal standard stock solution: 3 mg/mL of 5 α -cholestane in [n-hexane](#). [NOTE—Prepare fresh before use.]

Internal standard solution: 0.3 mg/mL of 5 α -cholestane in [n-hexane](#). [NOTE—Prepare fresh before use.]

Standard stock solution: 3.0 mg/mL of [USP Cholesterol RS](#) in [n-hexane](#). [NOTE—This solution is stable for 6 months stored in a freezer.]

Transfer 1.0 mL of this solution to a 10.0-mL volumetric flask. Dilute with [n-hexane](#) to volume. [NOTE—Prepare this solution fresh daily.]

Standard solution: Transfer 1.0 mL each of the *Standard stock solution* and the *Internal standard solution* to a 15-mL centrifuge tube.

Evaporate to dryness at about 50° with a gentle stream of nitrogen. Add 0.5 mL of 50% (w/v) [potassium hydroxide](#) and 3 mL of [alcohol](#), fill the tube with nitrogen, and cap. Heat the sample at 100° for 60 min using a heating block. Cool for about 10 min. Add 6 mL of [water](#) to the tube, and shake for 1 min. Extract the solution 4 times with 2.5-mL portions of [ethyl ether](#), using a vortex mixer or suitable shaker for 1 min for each extraction. Transfer and combine the extracts into a large centrifuge tube, and wash with 5 mL of [water](#), mixing completely with gentle inversion. Remove the water phase, and add 5 mL of 0.5 M [potassium hydroxide](#) to the ether phase, mixing carefully to avoid an emulsion. Remove the potassium hydroxide, and add another 5 mL of [water](#), mixing carefully. Transfer the ether phase to a small centrifuge tube. [NOTE—If an emulsion has occurred, a small amount of [sodium chloride](#) may be added to obtain a separation of the phases.] Evaporate the ether phase to dryness under a stream of nitrogen with careful heating. Dissolve the sample in 600 μ L of [ethyl acetate](#), and mix well.

Transfer 200 μ L of this solution to a sample vial, and dilute with [ethyl acetate](#) to about 2 mL.

Alpha tocopherol stock solution: 1.5–2.0 mg/mL of [USP Alpha Tocopherol RS](#) in [n-hexane](#). [NOTE—This solution is stable for 12 months stored in a freezer.]

System suitability solution: Mix 1.0 mL of the *Standard stock solution*, 1.0 mL of the *Internal standard stock solution*, and 2.0 mL of the *Alpha tocopherol stock solution* in a 50-mL volumetric flask. Evaporate to dryness with the aid of heat, and dilute with [ethyl acetate](#) to volume.

Dilute 1.0 mL of this solution with [ethyl acetate](#) to 10.0 mL. [NOTE—This solution is stable for 6 months stored in a freezer.]

Sample solution: Transfer 100 mg of Omega-3 Free Fatty Acids to a 15-mL centrifuge tube. Add 1.0 mL of the *Internal standard solution*.

Prepare as directed for the *Standard solution* beginning with “Evaporate to dryness”.

Chromatographic system

(See [Chromatography \(621\)](#), [System Suitability](#).)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm $\times$ 30-m capillary; coated with a 0.25- μ m film of phase G27

Temperatures

Injection port: 320°

Detector: 300°