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Omega-3-Acid Ethyl Esters

DEFINITION

Omega-3-Acid Ethyl Esters is a mixture of ethyl esters, principally the ethyl esters of eicosapentaenoic acid (EPAee) (C20:5 n−3, EE) and docosahexaenoic acid (DHAee) (C22:6 n−3, EE). It may also contain ethyl esters of alpha-linolenic acid (C18:3 n−3, EE), moroctic acid (C18:4 n−3, EE), eicosatetraenoic acid (C20:4 n−3, EE), heneicosapentaenoic acid (C21:5 n−3, EE), and docosapentaenoic acid (C22:5 n−3, EE). Tocopherol may be added as an antioxidant.

IDENTIFICATION

• **A.** The retention times of the principal peaks in *Test solution 4* correspond to those of eicosapentaenoic acid ethyl ester and docosahexaenoic acid ethyl ester in *Standard solution 1b* and *Standard solution 1a*, as obtained in the *Assay*.

• **B.** It meets the acceptance criteria in *Table 1* of the *Assay*.

ASSAY

• **Content of EPAee, DHAee, and Total Omega-3-Acid Ethyl Esters**

(See *Fats and Fixed Oils ⟨401⟩, Procedures, Omega-3 Fatty Acids Determination and Profile*.)

Test solution 3, Test solution 4, Standard solution 1a, Standard solution 1b, System suitability solution 1, Chromatographic system, and **System suitability:** Proceed as directed in the chapter.

Analysis

Samples: *Test solution 3*, *Test solution 4*, *Standard solution 1a*, and *Standard solution 1b*

Calculate the content of EPAee and DHAee in the portion of Omega-3-Acid Ethyl Esters taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U)$$

R_U = peak area ratio of EPAee or DHAee to the internal standard from *Test solution 3*

R_S = peak area ratio of the EPAee to the internal standard from *Standard solution 1b* or DHAee to the internal standard from *Standard solution 1a*

W_S = weight of DHAee taken for preparing *Standard solution 1a* or weight of EPAee taken for preparing *Standard solution 1b* (mg)

W_U = weight of sample taken for preparing *Test solution 3* (g)

Calculate the content of total omega-3-acid ethyl esters in the portion of Omega-3-Acid Ethyl Esters taken:

$$\text{Result} = r_{FAn\text{-}3ee}[(EPAee + DHAee)/(r_{EPAee} + r_{DHAee})] + EPAee + DHAee$$

$r_{FAn\text{-}3ee}$ = sum of the peak areas of alpha-linolenic acid ethyl ester (C18:3 n−3, EE), moroctic acid ethyl ester (C18:4 n−3, EE), eicosatetraenoic acid ethyl ester (C20:4 n−3, EE), heneicosapentaenoic acid ethyl ester (C21:5 n−3, EE), and docosapentaenoic acid ethyl ester (C22:5 n−3, EE) in *Test solution 4*

$EPAee$ = content of EPAee (mg/g)

$DHAee$ = content of DHAee (mg/g)

r_{EPAee} = peak area of EPAee in *Test solution 4*

r_{DHAee} = peak area of DHAee in *Test solution 4*

Acceptance criteria: It conforms to the acceptance criteria in *Table 1*. Articles labeled as Omega-3-Acid Ethyl Esters type A meet *Acceptance Criteria II*.

Table 1

		Acceptance Criteria I		Acceptance Criteria II (for articles labeled as Omega-3-Acid Ethyl Esters type A)	
Name	**Relative Retention Time**	**NLT**	**NMT**	**NLT**	**NMT**
C18:3 n−3, EE[a]	0.585	—	—	—	—
C18:4 n−3, EE[b]	0.608	—	—	—	—
C20:4 n−3, EE[c]	0.777	—	—	—	—
C20:5 n−3, EE (EPAee)[d]	0.796	430 mg/g	495 mg/g	365 mg/g	435 mg/g
C21:5 n−3, EE[e]	0.889	—	—	—	—
C22:5 n−3, EE[f]	0.977	—	—	—	—
C22:6 n−3, EE (DHAee)[g]	1.000	347 mg/g	403 mg/g	290 mg/g	360 mg/g
EPAee + DHAee	—	800 mg/g	880 mg/g	700 mg/g	749 mg/g
Total omega-3-acid ethyl esters	—	90% (w/w)	—	78% (w/w)	—

[a] Alpha-linolenic acid ethyl ester.

[b] Moroctic acid ethyl ester.

[c] Eicosatetraenoic acid ethyl ester.

[d] Eicosapentaenoic acid ethyl ester.

[e] Heneicosapentaenoic acid ethyl ester.

[f] Docosapentaenoic acid ethyl ester (clupanodonic acid ethyl ester).

[g] Docosahexaenoic acid ethyl ester.

IMPURITIES

- **Fats and Fixed Oils ⟨401⟩, *Procedures, Trace Metals*:** NMT 0.1 ppm each of lead (Pb), cadmium (Cd), arsenic (As), and mercury (Hg)
- **Cholesterol**

Internal standard stock solution: 3 mg/mL of 5α-cholestane in *n*-heptane. [Note—Prepare fresh before use.]

Internal standard solution: 0.3 mg/mL of 5α-cholestane in *n*-heptane. [Note—Prepare fresh before use.]

Standard stock solution: 3.0 mg/mL of cholesterol in *n*-heptane. [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*-heptane 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% 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 four 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 µL of ethyl acetate, and mix well. Transfer 200 µL 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*-heptane. [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-Acid Ethyl Esters to a 15-mL centrifuge tube. Add 1.0 mL of the *Internal standard solution*. Prepare as directed in the *Standard solution* beginning with "Evaporate to dryness".

Chromatographic system

(See *Chromatography ⟨621⟩, System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m capillary; coated with a 0.25-µm film of G27 phase

Temperatures

Injection port: 320°

Detector: 300°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
170	0	170	1
170	4	320	1.5

Carrier gas: Helium

Flow rate: 1.3 mL/min

Injection volume: 1 µL

Injection type: Splitless injection system

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 1.2 between alpha tocopherol and cholesterol

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the content of total cholesterol in the portion of Omega-3-Acid Ethyl Esters taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U)$$

R_U = peak area ratio of the cholesterol peak to the internal standard from the *Sample solution*

R_S = peak area ratio of the cholesterol peak to the internal standard from the *Standard solution*

W_S = weight of cholesterol in the *Standard solution* (mg)

W_U = weight of Omega-3-Acid Ethyl Esters in the *Sample solution* (g)

Acceptance criteria: NMT 3.0 mg/g

- **Oligomers**

Mobile phase: Tetrahydrofuran

System suitability solution: Monodocosahexaenoin, didocosahexaenoin, and tridocosahexaenoin[1] in *Mobile phase*, with concentrations of about 0.5, 0.3, and 0.2 mg/mL, respectively.

Sample solution 1: 5.0 mg/mL of Omega-3-Acid Ethyl Esters in tetrahydrofuran

Sample solution 2: [Note—Use *Sample solution 2* where the results of this test using *Sample solution 1* exceed the *Acceptance criteria* due to the presence of monoglycerides.] Weigh 50 mg of Omega-3-Acid Ethyl Esters into a quartz tube, add 1.5 mL of a 20-g/L solution of sodium hydroxide in methanol, cover with nitrogen, cap tightly with a polytef-lined cap, mix, and heat on a water bath for 7 min. Allow to cool. Add 2.0 mL of boron trichloride–methanol solution, cover with nitrogen, cap tightly, mix, and heat on a water bath for 30 min. Cool to 40°–50°, add 1 mL of isooctane, cap, and shake vigorously for NLT 30 s. Immediately add 5 mL of saturated sodium chloride solution, cover with nitrogen, cap, and shake thoroughly for NLT 15 s. Transfer the upper layer to a separate tube. Shake the methanol layer with 1 mL of isooctane. Wash the combined isooctane extracts with two quantities, each of 1 mL of water. Carefully evaporate the solvent under a stream of nitrogen, and then add 10.0 mL of tetrahydrofuran to the residue. Add a small amount of anhydrous sodium sulfate, and filter.

Chromatographic system

(See *Chromatography ⟨621⟩, System Suitability*.)

Mode: LC

Detector: Differential refractometer

Columns: Three in concatenated series, 7.8-mm × 30-cm; 7-µm packing L21, with pore sizes in the range of 5–50 nm, arranged with decreasing pore size from the injection port to the detector to fulfill the system suitability requirements

Flow rate: 0.8 mL/min

Injection volume: 40 µL

System suitability

Sample: *System suitability solution*

[Note—The elution order is tridocosahexaenoin, didocosahexaenoin, and monodocosahexaenoin.]

Suitability requirements

Resolution: NLT 2.0 between monodocosahexaenoin and didocosahexaenoin; NLT 1.0 between didocosahexaenoin and tridocosahexaenoin

Analysis

Samples: *Sample solution 1* and *Sample solution 2*

Measure the areas of the major peaks.

Calculate the percentage of oligomers in the portion of Omega-3-Acid Ethyl Esters taken to prepare *Sample solution 1*:

$$\text{Result} = (r_I/r_T) \times 100$$

r_I = sum of the peak areas with a retention time less than that of the ethyl esters peaks

r_T = sum of all the peak areas

Calculate the percentage of oligomers in the portion of Omega-3-Acid Ethyl Esters taken to prepare *Sample solution 2*:

$$\text{Result} = (r_I/r_T) \times 100$$

r_I = sum of all the peak areas with a retention time less than that of the methyl esters peaks

r_T = sum of all the peak areas

Acceptance criteria: NMT 1.0% of oligomers

Change to read:

• **Limit of Dioxins, Furans, and Polychlorinated Biphenyls (PCBs):** Determine the content of polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) ▲using the current revision of▲ (USP 1-Dec-2022) method No. 1613 ▲▲ (USP 1-Dec-2022) of the Environmental Protection Agency. Determine the content of polychlorinated biphenyls (PCBs) ▲using the current revision of▲ (USP 1-Dec-2022) method No. 1668 ▲▲ (USP 1-Dec-2022) of the Environmental Protection Agency.

Acceptance criteria: The sum of PCDDs and PCDFs is NMT 1 pg/g of WHO toxic equivalents. The sum of PCBs (IUPAC congeners PCB-28, PCB-52, PCB-101, PCB-118, PCB-138, PCB-153, and PCB-180) is NMT 0.5 ppm.

• **Limit of Total Unidentified Fatty Acid Ethyl Esters**

[Note—This test is not required for articles labeled as Omega-3-Acid Ethyl Esters type A.]

From the chromatogram obtained with *Test solution 4* in the *Assay* for *Content of EPAee, DHAee, and Total Omega-3-Acid Ethyl Esters*, determine the peak area of the largest single unidentified peak with a relative retention time different from those in *Table 3*.

Table 3

Identified Ethyl Ester	Relative Retention Time
Phytanic acid	0.416
C16:3 n−4	0.431
C16:4 n−1	0.468
C18:3 n−6	0.557
C18:3 n−4	0.574
C18:3 n−3	0.585
C18:4 n−3	0.608
C18:4 n−1	0.618
Furan acid 5	0.691
C19:5	0.710
C20:3 n−6	0.720
C20:4 n−6	0.736
Furan acid 7	0.744
C20:4 n−3	0.777
Furan acid 8	0.783
EPA	0.796
Furan acid 9	0.867
C21:5 n−3	0.889
C22:4	0.917
Furan acid 10	0.922
C22:5 n−6	0.939
Furan acid 11	0.963
C22:5 n−3	0.977
DHA	1.000

Calculate the content of unidentified fatty acid ethyl esters in area percentage:

$$\text{Result} = 100 - (100 \times \Sigma\, Aiee/r_T)$$

Aiee = peak area of each identified ethyl ester in *Table 3*

r_T = sum of all the peak areas except solvents and butylated hydroxytoluene

Acceptance criteria: The area of the largest single unidentified peak is NMT 0.5% of the total area. The total area of unidentified peaks as calculated above is NMT 2%.

• **Limit of Non-Omega-3-Acid Ethyl Esters**

[Note—This test is only required for articles labeled as Omega-3-Acid Ethyl Esters type A.]

From the chromatogram obtained with *Test solution 4* in the *Assay* for *Content of EPAee, DHAee, and Total Omega-3-Acid Ethyl Esters*, calculate the amounts of C18:1 n−9 ethyl ester and C20:4 n−6 ethyl ester in the portion of Omega-3-Acid Ethyl Esters taken:

$$\text{Result} = (Aiee/r_T) \times 100$$

Aiee = peak area of C18:1 n−9 ethyl ester or C20:4 n−6 ethyl ester

r_T = sum of all the peak areas except solvents and BHT

Acceptance criteria

C18:1 n−9 ethyl ester: NMT 6.0%

C20:4 n−6 ethyl ester: NMT 4.0%

SPECIFIC TESTS

• **Fats and Fixed Oils ⟨401⟩, *Procedures, Acid Value*:** NMT 2.0

• **Fats and Fixed Oils ⟨401⟩, *Procedures, Anisidine Value*:** NMT 15

• **Fats and Fixed Oils ⟨401⟩, *Procedures, Peroxide Value*:** NMT 10.0

• **Absorbance**

Sample solution: Transfer 300 mg, accurately weighed, to a 50-mL volumetric flask. Dissolve in and dilute immediately with isooctane to volume. Pipet 2.0 mL into a 50-mL volumetric flask, and dilute with isooctane to volume.

Acceptance criteria: NMT 0.55, determined at 233 nm, with isooctane being used as the blank

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight, light-resistant containers under a nitrogen atmosphere. Store at controlled room temperature.

• **Labeling:** The label states the content of DHA ethyl ester and EPA ethyl ester in mg/g, the sum of the EPA and DHA ethyl esters contents in mg/g, and the content of the total omega-3-acid ethyl esters in weight percentage (w/w). It also states the name of any added antioxidant. Articles which meet *Acceptance Criteria II* of the *Assay* and the *Limit of Non-Omega-3-Acid Ethyl Esters* are labeled as Omega-3-Acid Ethyl Esters type A.

• **USP Reference Standards ⟨11⟩**

USP Alpha Tocopherol RS

[1] Suitable grades of monodocosahexaenoin, didocosahexaenoin, and tridocosahexaenoin may be obtained from Nu-Chek Prep.

Auxiliary Information - Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
OMEGA-3-ACIDS ETHYL ESTERS	Fatkhulla K Tadjimukhamedov Associate Scientific Liaison	NBDS2020 Non-botanical Dietary Supplements
REFERENCE STANDARD SUPPORT	RS Technical Services RSTECH@usp.org	NBDS2020 Non-botanical Dietary Supplements

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