VOLUNTARY MONOGRAPH

Omega-3 DHA
Omega-3 EPA
Omega-3 DHA & EPA

DEFINITION

Omega-3 fatty acids, EPA and DHA, consist of the all cis forms of 5, 8, 11, 14, 17-eicosapentaenoic acid and 4, 7, 10, 13, 16, 19-docosahexaenoic acid, respectively. Omega-3 fatty acid products may be found with DHA as the predominant fatty acid, EPA as the predominant fatty acid or mixtures of DHA and EPA in varying combinations.

The content of omega-3 EPA and DHA is expressed as free fatty acid equivalents on a weight/weight basis, as mg EPA per gram or mg DHA per gram when available as single sources of omega-3. If a mixture of EPA and DHA exist in the product, the total omega-3 EPA and DHA content may be expressed individually and as total mg EPA and DHA per gram.

For other Omega-3 Fatty Acids see Assay method.

Food approved antioxidant may be added to enhance omega-3 EPA, omega-3 DHA, and omega-3 EPA and DHA product stability.

SCOPE

Applicable to omega-3 EPA and DHA fatty acids obtained from fish, plant, or microbial sources. Not applicable to cod liver oil. Omega-3 EPA and DHA may be found esterified as triglycerides, re-esterified as glycerides, or esterified as ethyl esters. Not applicable to free fatty acid product forms. Applicable to bulk oil product and encapsulated oil intended for use as dietary supplements. Not applicable to formulations, specialty delivery systems, and EPA and DHA concentrates >80% wt/wt. The specifications described herein apply throughout the stated lifetime (shelf-life) of the product.

CHARACTERISTICS

Long chain omega-3 EPA and DHA products are generally liquids at ambient temperature. The color varies from pale, light-yellow to orange. The products have a characteristic odor ranging from bland to mild fish-like.

IDENTIFICATION

Examine the chromatograms obtained in the assay for long chain omega-3 EPA and DHA. The presence of EPA and/or DHA based on retention time comparison to authentic reference standards establishes the identity of the product.

TESTS

Acid value. Maximum 3 mg KOH/g; AOCS Official Method Cd 3d-63

Peroxide value. Maximum 5 meq/kg; AOCS Official Method Cd 8-53

Anisidine value. Maximum 20; AOCS Official Method Cd 18-90

TOTOX. Maximum 26 (result of calculation, (2 x PV) + AV)

PCDDs and PCDFs. Maximum 2 pg WHO-PCDD/F-TEQ/g

Dioxin limits include the sum of polychlorinated dibenzo-*para*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) and are expressed in World Health Organization (WHO) toxic equivalents using WHO-toxic equivalent factors (TEFs). This means that analytical results relating to 17 individual dioxin congeners of toxicological concern are expressed in a single quantifiable unit: TCDD toxic equivalent concentration or TEQ.

PCBs. Total PCBs should be expressed on a weight/weight basis and should include IUPAC congeners 28, 52, 101, 118, 138, 153 and 180
Maximum: 0.09 mg/kg

Dioxin- like PCBs Maximum 3 pg WHO –TEQ/g (maximum for Dioxin and Furans remains at 2 pg/g).

Sample preparation and appropriate methods of analysis for PCDDs, PCDFs, and PCBs are described in draft European Commission Directive "laying down the sampling methods and the methods of analysis for the official control of dioxins and the determination of dioxin-like PCBs in foodstuffs". Annex II of this document describes appropriate methods of analysis along with a specific list of PCDD, PCDF and PCB congeners to be included in the calculation. Gas chromatography coupled with high-resolution mass spectrometry has proved to provide required sensitivity and specificity. Identification of congeners should be performed according to EPA Method 1613 revision B: Tetra- through octa-chlorinated dioxins and furans by isotope dilution HRGC/HRMS or similar.

Heavy Metals.

Lead (Pb):	Less than 0.1 mg/kg
Cadmium (Cd):	Less than 0.1 mg/kg
Mercury (Hg):	Less than 0.1 mg/kg
In-organic Arsenic (As):	Less than 0.1 mg/kg

Note: The maximum intake of PCDDs, PCDFs, PCBs, other pesticides, and heavy metals in various parts of the world are based on Maximum Allowable Daily Levels (MADL) instead of absolute amounts in oil; therefore, appropriate consideration should be given to recommended daily serving and intake.

ASSAY

The assay used for quantitative determination of EPA and DHA content in omega-3 products is applicable to triglyceride and ethyl ester product forms with results expressed as mg DHA/g and mg EPA/g after correction to free fatty acid equivalents.

EPA and DHA. *Carry out the operations as rapidly as possible, avoiding exposure to actinic light, oxidizing agents, oxidation catalysts (for example, copper and iron) and air.*

Gas chromatography.

The assay is carried out on the methyl or ethyl esters of all-*cis*-eicosa-5,8,11,14,17-pentaenoic acid (EPA; 20:5 n-3) and all-*cis*-docosa-4,7,10,13,16,19-hexaenoic acid (DHA; 22:6 n-3) in the sample to be examined.

Internal standard. Methyl tricosanoate R.

Test solution (a). Prepare 3 solutions for each sample.

1. Dissolve the sample to be examined according to the table below and about 70.0 mg of the internal standard in a 0.05 g/l solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 ml with the same solution.

Approx. sum EPA + DHA	Amount sample to be weighed
30 – 50 %	0.4 – 0.5 g
50 – 70 %	0.3 g
70 – 80 %	0.25 g

Ethyl esters are now ready for analysis. For triglycerides continue as described in 2.

2. Introduce 2.0 ml of the solution obtained from step 1 into a quartz tube and evaporate the solvent at 40-50°C with a gentle current of *nitrogen R*. Add 1.5 ml of a 20 g/l solution of *sodium hydroxide R* in *methanol R*, cover with *nitrogen R*, cap tightly with a polytetrafluoroethylene-lined cap, mix and heat on a steaming water-bath for 7 min. Allow to cool to 40-50°C.

3. Add 2 ml of *boron trichloride-methanol solution R*, cover with *nitrogen R*, cap tightly, mix and heat on a water-bath for 30 min. Cool to 40-50°C, add 1 ml of *trimethylpentane R*, cap and shake vigorously for at least 30 s. Immediately add 5 ml of a saturated solution of *sodium chloride R*, cover with *nitrogen R*, cap and shake vigorously for at least 15 s. Transfer the upper layer to a separate tube. Shake the methanol layer once more with 1 ml of *trimethylpentane R*. Wash the combined trimethylpentane extracts with 2 quantities, each of 1 ml, of *water R* and dry over *anhydrous sodium sulphate R*.

Test solution (b). (to be prepared at the same time as test solution (a))

Dissolve 0.300 g of the sample to be examined in a 0.05 g/l solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 ml with the same solution. Proceed as described for test solution (a).

Reference solution (a). Prepare 3 individual solutions (to be prepared at the same time as test solution (a))

Dissolve 60.0 mg of *docosahexaenoic acid ethyl ester CRS*, about 70.0 mg of the internal standard and 90.0 mg of *eicosapentaenoic acid ethyl ester CRS* in a 0.05 g/l solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 ml with the same solution. For analysis of ethyl esters the solutions are now ready for analysis. For analysis of triglycerides continue as described in step 2 for preparation of test solution (a).

Reference solution (b).(for system suitability of recovery vs. the theoretical response of the Flame Ionisation Detector (FID))

Introduce 0.3 g of *methyl palmitate R*, 0.3 g of *methyl stearate R*, 0.3 g of *methyl arachidate R* and 0.3 g of *methyl behenate R* into a 10 ml volumetric flask, dissolve in a 0.05 g/l solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 ml with the same solution.

Reference solution (c). (for system suitability of chromatographic resolution)

Introduce a sample containing about 55.0 mg *docosahexaenoic acid methyl ester CRS* and about 5.0 mg of *15-tetracosenoic acid methyl ester CRS* diluted to 10.0 ml of a 0.05 g/l solution of *butylhydroxytoluene R* in *trimethylpentane R*.

Column:[1]

— *material*: fused silica

— *dimensions*: *l* = at least 25 m, Ø = 0.25 mm,

— *stationary phase*: bonded *polyethylene glycol polymer* (film thickness 0.2 µm).

Carrier gas: *hydrogen for chromatography R* or *helium for chromatography*.

Split: 1:200, alternatively splitless with temperature control (samples need to be diluted 1:200 with a 0.05 g/l solution of *butylhydroxytoluene R* in *trimethylpentane R* before injection)

[1] CP-Wax 52CB, 25 m x 0.25 mm I.D. 0.2. µm film thickness, Chrompack cat. no. 7713 or equivalent will be suitable

Temperature:

	Time (min)	Temperature (°C)
Column	0 – 2	170
	2 – 25.7	170 - 240
	25.7 - 28	240
Injection port		250
Detector		270

Detection: flame ionisation.

Injection: twice 1 µl of each solution.

The assay is not valid unless:

— the chromatogram obtained with reference solution (b) gives area per cent compositions increasing in the following order: *methyl palmitate, methyl stearate, methyl arachidate, methyl behenate*; the difference between the percentage area of *methyl palmitate* and that of *methyl behenate* is less than 2 area per cent units,

— the chromatogram obtained with reference solution (c) shows 2 resolved peaks corresponding to *docosahexaenoic acid methyl ester CRS* and *15-tetracosenoic acid methyl ester CRS*, giving a chromatographic resolution of minimum 1.2,

— the chromatogram obtained with test solution (a) shows a resolution of *methyl tricosanoate R* and any *heneicosenoic acid methyl* ester present when compared with the chromatogram obtained with test solution (b) (if not, a correction term has to be used),

— experiments using the method of standard additions to test solution (a) show more than 95 per cent recovery of the added *eicosapentaenoic acid ethyl ester CRS* and *docosahexaenoic acid ethyl ester CRS*, when due consideration has been given to the correction by the internal standard.

Calculate the content of EPA and DHA as mg fatty acid/g oil using the following expression and taking into account the certified value of the reference substances. The results should be rounded to the nearest 10 mg/g based on the method's precision.

$$A_x \times \frac{A_3}{m_3} \times \frac{m_1}{A_1} \times \frac{m_{x,r}}{A_{x,r}} \times \frac{1}{m_2} \times C \times 1000$$

m_1 = mass of the internal standard in test solution (a), in milligrams,

m_2 = mass of the sample in test solution (a), in milligrams,

m_3 = mass of the internal standard in reference solution (a), in milligrams,

$m_{x,r}$ = mass of *eicosapentaenoic acid ethyl ester CRS* or *docosahexaenoic acid ethyl ester CRS* in reference solution (a), in milligrams,

A_x = area of the peak corresponding to *eicosapentaenoic acid ester* or *docosahexaenoic acid ester* in the chromatogram obtained with test solution (a),

$A_{x,r}$ = area of the peak corresponding to *eicosapentaenoic acid ester* or *docosahexaenoic acid ester* in the chromatogram obtained with reference solution (a),

A_1 = area of the peak corresponding to the internal standard in the chromatogram obtained with test solution (a),

A_3 = area of the peak corresponding to the internal standard in the chromatogram obtained with reference solution (a),

C = a conversion factor to fatty acids based on the difference in molecular weight of ethyl esters in the standard and fatty acid

$C_{EPA} = 0.915$

$C_{DHA} = 0.921$

Total omega-3-acids. From the assay for EPA and DHA, calculate the content of the total omega-3-acids using the following expression and identifying the peaks from the chromatograms:

$$EPA + DHA + \frac{A_{\text{n}-3}\,(EPA + DHA)}{A_{\text{EPA}} + A_{\text{DHA}}}$$

EPA = content of EPA obtained from the assay for EPA and DHA,

DHA = content of DHA obtained from the assay for EPA and DHA,

$A_{\text{n-3}}$ = sum of the areas of the peaks 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 chromatogram obtained with test solution (b),

A_{EPA} = area of the peak corresponding to EPA methyl ester in the chromatogram obtained with test solution (b),

A_{DHA} = area of the peak corresponding to DHA methyl ester in the chromatogram obtained with test solution (b).