INDUSTRY ADVISORY:
ACCURATE QUANTIFICATION OF EPA, DHA AND TOTAL OMEGA-3 CONTENT OF OMEGA-3 OILS BY GC-FID

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Purpose

This document provides practical advice towards achieving the most accurate quantification of EPA, DHA and Total Omega-3 by gas chromatography and flame emission detection. This advisory was developed because, in the opinion of expert analytical chemists in the industry, the variability in results observed in the AOCS GOED Nutraceutical Oils Laboratory Proficiency Program (LPP) is too high, indicative of the fact that a portion of laboratories do not know how to properly set up their instrument and carry out the sample preparation and analysis. This advisory is complementary to GOED's Technical Guidance Documents, which provide a description of the GOED Fatty Acid method (equivalent to Ph.Eur. 2.4.29). This advisory can also be a helpful complement to other methods for EPA and DHA quantification but does not constitute any mandatory instructions to GOED members.

All companies that test for EPA, DHA and Total Omega-3 in their own laboratory should participate in the AOCS GOED Nutraceutical Oils LPP. A laboratory that has its GC instrument correctly set up should be able to achieve an accurate value, i.e. within 2% of the LPP consensus value. It is easy to carry out the method perfectly but get the wrong result when the instrument is not correctly set up. In 2021, there were only three laboratories (of 29) that were within ± 5 mg/g from the consensus value for all six test samples.

This Industry Advisory only includes the practical complementary information and tips from analytical chemists whose laboratories have consistently (over a period of multiple consecutive years) scored within 5 mg/g of the EPA/DHA consensus values for the samples provided in the AOCS-GOED Nutraceutical Oils LPP. The aim of this Advisory is for other laboratories to observe an improvement in the accuracy of quantification of EPA and DHA in omega-3-rich oils.

Advice on method execution and instrument setup

The GOED Fatty Acid Method (equivalent to Ph.Eur. 2.4.29) can be set up as either split and splitless method. There are different types of injectors for GCs. This means that one brand of GC can be accurate with split method and another brand of GC can be accurate with splitless method. Experience also shows that injection temperature and changes in makeup flow for the detector can influence accuracy.
Many GCs do not manage to give a correct A% for fatty acids. One would think that the reference solutions (a1) and (a2) would fix this problem, but if the A% of EPA/DHA/internal standard are completely wrong, the calculated mg/g will be wrong. The response factor for EPA and DHA is calculated according to the method, but if this calculated response factor is too far away from the theoretical value, the answer will not be correct. The reason for incorrect response factors would most times come from a GC that discriminates long fatty acids (wrong A%). The discrimination of long fatty acids is not easy to explain and has to do with the differences in boiling points of different fatty acids, the injector type and injector conditions.

An easy way to test if a GC is giving the correct fatty acid composition is to run standard materials GLC 6A and GLC 68D from Nu-Chek Prep Ltd. These samples can be bought as methyl esters and only need dilution with iso octane before analysis.

GLC 6A is a mix of four fatty acids. From the weight % composition one can calculate the theoretical A%. The GOED/PhEur fatty acid method accepts +/- 1.0 A% from theoretical values, but one should act if differences are more than ± 0.5 A%.

GLC 6A

Fatty acid	Weight%	Theoretical A%
C16:0 25	25	24.4
C18:0 25	25	24.8
C20:0 25	25	25.2
C22:0 25	25	25.6

GLC 68D is a mix of 20 fatty acids, including EPA and DHA. From the weight % composition one can calculate theoretical A%. Recovery of EPA and DHA should be within (100 ± 5) %. This means that A% EPA should be within 9.81 – 10.85 A% and A% DHA should be within 11.97 – 13.23 A%. These may seem to be wide limits, but experience shows that no GCs have more than 100 % recovery. GLC 68D can also be used to confirm separation between C24:0, DHA, and C24:1.

GLC 68D

Fatty acid	Weight%	Theoretical A%
C14:0	6,00	5,67
C14:1	1,00	0,95
C16:0	16,00	15,48
C16:1	5,00	4,87
C18:0	8,00	7,89
C18:1 n-9	13,00	12,91
C18:1 n-7	4,00	3,97
C18:2	2,00	2,00
C18:3	2,00	2,01
C20:0	1,00	1,00
C20:1	9,00	9,07
C20:2	1,00	1,01

C20:4 n-6	3,00	3,08
C20:3 n-3	1,00	1,02
C20:5 n-3 (EPA)	10,00	10,33
C22:0	1,00	1,01
C22:1	3,00	3,06
C24:0	1,00	1,03
C22:6 n-3 (DHA)	12,00	12,60
C24:1	1,00	1,03

In the GOED/PhEur fatty acid method there is a calculation of the response factor for EPA and DHA. These response factors should be close to 1.0 for both EPA and DHA. If calculated response factors are below 0.90 or higher than 1.1, one should take action and run GLC 6A and GLC 68D.

Recommended action points if GLC 6A and/or GLC 68D are outside recommended values:

- Try both split and splitless method, and determine which functions better for your injector system for long-chain fatty acid analysis
- Try different injection temperatures (between 200 – 250 °C)
- Try different make up gas flows for FID (10 – 50 ml/min)

Peak resolution:

The GOED/PhEur fatty acid method has limits for resolution between peaks:

- For ethyl esters one needs minimum 1.2 between C23:0 and C21:5 n-3.
- For triglycerides one needs minimum 1.2 between DHA and C24:1.

The type of GC-column and GC temperature program decides the peak separation.

Columns known to work are these two (identical stationary phase):

- CP-Wax 52CB, 25 m x 0.25 mm I.D., 0.2 µm film (Agilent CP7713i).
- SCION -WAX, 25 m x 0.25 mm I.D., 0.2 µm film (SCION SC32780).

According to Ph.Eur. 2.2.46. *CHROMATOGRAPHIC SEPARATION TECHNIQUES* one can adjust the chromatographic conditions, without the need for revalidating the method, to satisfy system suitability criteria. There are of course limits to how much one can change. Here are

some examples:

- Film thickness: – 50 per cent to + 100 per cent (capillary columns).
- Column dimensions: length: ± 70 per cent; internal diameter: ± 50 per cent.
- Flow rate: ± 50 per cent.
- Temperature: ± 10 per cent.

A slower temperature gradient from 170 °C might help to get the peak separation needed. Conditioning of the column for several hours at max temperature (5-24 hours) might also help.

Number of replicates and system suitability:

In the GOED and PhEur method it is stated that you should make two replications of EPA/DHA standard (reference solutions a1 and a2) and sample (test solution a). A few companies make three replicates (weigh out three replicates). Analyzing your active ingredient and three replicates will lower the standard deviation of your result. It is also a good idea to have limits for the difference between lowest and highest replicate (e.g. max 7 mg/g between lowest and highest replicate for EPA and similar for DHA).

A system suitability test (five injections of the same sample preparation (control sample)) can be included. The max accepted relative standard deviation should be 1,1 % (according to PhEur), but normally it should be 0,1 - 0,3 % for both EPA and DHA.

Examples from a company with a well-functioning method:

Results from a 3-year stability study sees max 3-4 mg/g difference for EPA/DHA between day 0 and 36 months (and all time points in between).

Results from validation mg/g EE:

EE-sample	EPA - EE (mg/g)	DHA - EE (mg/g)
Technician 1, day 1		
Mean (n=6 replicates)	301	208
Technician 2, day 2		
Mean (n=3 replicates)	299	206
Technician 3, day 3		
Mean (n=3 replicates)	299	206

There is very little difference between different technicians and different days (even different GCs were used).

Results from validation mg/g TG:

TG-sample	EPA-FA (mg/g)	DHA-FA (mg/g)
Analyst 1, day 1		
Mean (n=6 replicates)	291	193
Analyst 2, day 2		
Mean (n=3 replicates)	289	192
Analyst 3, day 3		
Mean (n=3 replicates)	291	192

As for EE, there is little difference between the mean values for each day. One might get a bit higher variance between different days and analysts with two replicates (instead of three).

Secondary standard:

In pharmaceutical laboratories it is normal procedure to certify a secondary standard.

This is done because primary standards (like EPA-EE and DHA-EE from Nu-Chek Prep Ltd) are very expensive. A typical secondary standard for the analysis of Omega3 would be a capsule of an ethyl ester with a high content of EPA/DHA (like 450 mg/g EPA-EE and 350 mg/g DHA-EE).

The secondary standard is certified by analyzing it many times (at least 10 different days) as a normal sample. The mean value of all replicates (20-30 replicates) is used as the certified values for EPA and DHA in the secondary standard. Relative standard deviation for all replicates should be max 2.0 % for EPA and 2.0 % for DHA.

Note that before making a secondary standard one must be sure that the accuracy and precision of your method is good (always get correct EPA/DHA in the AOCS GOED Nutraceutical Oils LPP).

This advisory was coordinated with GOED's Technical Committee and made possible through the expertise of Geir Frode Olsen (Epax Norway, Pelagia, 2022). We welcome additional expertise by those analytical chemists with proven sustained, multi-year proficiency in the AOCS-GOED Nutraceuticals LPP, to be added in future updates of this Industry Advisory.

September 13, 2022 - Gerard Bannenberg & the GOED Technical Committee