GOED

SUMMARY REPORT:

Evaluation of Method Suitability for Quantification of EPA, DHA and Total Omega-3 in Omega-3 Oils by ISO 12966

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Executive Summary

There are many analytical methods for quantification of EPA, DHA and Total Omega-3 in omega-3 EPA/DHA oils, several of which are recommended in the GOED Guidance Documents. Other methods are occasionally evaluated to determine their suitability. International Standard method ISO 12966-4 was evaluated by a Working Group (WG) of eight members, all of whom are members of GOED's Technical Committee. The evaluation showed that this method lacks a clearly defined and formalized procedure for quantifying EPA and DHA, increasing the likelihood of obtaining inaccurate results for fish oil/omega-3 products. In addition, there are specific aspects of the method that are incorrect for omega-3 oils. The WG concluded that this method is not suitable for the accurate quantification of EPA, DHA and Total-Omega-3 in omega-3 oils.

Background

While GOED recommends several methods for the quantification of EPA, DHA and Total Omega-3 in omega-3 EPA/DHA oils (see GOED Guidance Documents), various academic and third-party testing laboratories around the world are using a variety of other methods. In some cases, labs are successfully delivering accurate results but in other cases the results are neither accurate nor precise, and concerns exist over the suitability of the methods used. GOED occasionally evaluates methods to understand the suitability of such for the quantification of EPA/DHA in omega-3 oils.

In the present report the results of the evaluation of International Standard method ISO 12966-4 (latest known version "2015-06-01 First Edition") are summarized. This work was done by a Working Group (WG) of eight specialists, all members of GOED's Technical Committee and the report is now available on GOED's website.

The evaluations used a predefined set of criteria (see below). The possible applicability of the method for oils with specific levels of omega-3 EPA/DHA and different lipid classes, or a limited applicability to specific types of omega-3 oils, was also evaluated. Method adaptations and modifications that laboratories might make to the original method were outside the scope of this evaluation. The associated sample preparation methods ISO 12966-2 (*Preparation of Methyl Esters of Fatty Acids*) and ISO 12966-3 (*Preparation of Methyl Esters using Trimethyl Sulfonium (TMSH)*) were made available to the evaluators if needed. A summary of the Working Group evaluations of different aspects of method suitability for this method is provided below.

International Standard Method ISO 12966-4 (2015) – "Animal and Vegetable Fats and Oils – Gas Chromatography of Fatty Acid methyl Esters – Part 4: determination by Capillary Gas Chromatography"

- **Stated requirement or possibility for expression in mg/g (and not in area % only).** Section 10.2.1 states "*The results are expressed in grams per 100 g with one decimal place for values.*" However, the method focuses on determining results (for approximate compositions) as area %, and the calculation of g/100 g directly from the area % value. The method states that "*For most fats and oil, the area fraction of the fatty acid methyl esters is equal to the area fraction of triacylglycerols in grams per 100 g.*" This is not correct for long chain unsaturated fatty acids, and its assumption will give inaccurate results for any oil that contains these fatty acids, including fish

and other marine oils.

While section 10.2.1 states that it can be assumed that the area fraction of TG in g/100 g is equal to the area fraction of the FAME for most fats and oils, it refers to section 10.2.2 for situations where this is not the case. While the WG has considerable experience in lipid analytics and can recognize that section 10.2.1 would not be correct for the accurate quantification of EPA and DHA in omega-3 oils, others who have less technical background may not be aware of this, and there is no specific guidance provided in the method that can be used to determine which section needs to be followed for omega-3 oils.

Section 10.2.1 also states that "*According to the method AOCS Ce 1h-05, the factors for conversion of FAMEs to TAG equivalents are between 0.9114 (C8:0) and 0.9965 (C24:1) and are therefore negligible.*" This statement is inaccurate for omega-3 products. While the factors are correct, they are not negligible.

Section 10.2.2 of the method refers to the use of correction factors and an internal standard only for certain cases, such as when fatty acids with less than 16 carbons are present. This section does not include calculations with an internal standard but uses correction factors and certified reference materials. EPA and DHA are not mentioned in this section.

Section 10.2.3 of the method refers to the use of internal standards for cases when not all fatty acids are quantified, or when it is necessary to determine the absolute amount of a fatty acid in a sample. One might assume that for the quantification of EPA or DHA in an omega-3, this section would be applicable. In these cases, the method focuses on calculated mass fractions in g/100 g, expressed as fatty acid methyl ester. These calculations differ from those found in methods designed specifically for the quantification of EPA and DHA, for example, the GOED method. In addition, the GOED method specifies that results should be expressed as mg/g of triglyceride or ethyl ester, depending on the form they are being used. Expression as mg/g of fatty acid methyl esters is not a useful measurement.

In general, it is easy to view that analysts may directly follow the method of section 10.2.1 and determine EPA and DHA content from area % calculations as part of the total fatty acid profile determination. There is no specific guidance for how to quantify EPA or DHA in ISO 12966-4, but theoretically section 10.2.3 should be followed.

- **Use of internal standard (IS).** Yes. Section 4.2 states, "*For the quantification of (the) fatty acids, in grams per 100 g, the use of a FAME as an internal standard (IS) is necessary.*" The method goes on to suggest a variety of odd-chain FAME that can be used as IS, and allows flexibility for the use of an IS. In both section 4.2 and 10.2.3, several different IS's are mentioned (C11:0, C17:0, C19:0, C21:0, C23:0) and 10.2.3 (C15:0, C17:0. C19:0, C21:0) that can be used depending on the type of fat, and after determining the natural content of any IS of choice. Section 8 implies that an IS is not always required, which creates confusion about when to use an IS. If following section 10.2.3 for the determination of the absolute amount of a fatty acid, the method states that you can use internal standards, this being a special case. As a note, the calculations provided in this method are not the same as those in the GOED method.
- **Use of external reference standard.** Section 4.1 references the use of "Reference fatty acid methyl esters (FAMEs)" and/or reference fats/oils with known composition. What is specified is more of a "reference" standard than a true external standard. Section 4.1 refers to "external standards," but the purpose described in this point is for use of peak identification rather than

quantification of individual fatty acids. It specifies that reference pictures of pure FAMEs, oils with known FA compositions, certified reference materials or individual FAME standards can be used to identify FA, but it does not specify that these are mandatory. The method states that one can use external standards in special cases, that the use of standards depends on the fatty acid composition, and that this possibility is recommended for some oils without clarifying which. The method allows a lot of flexibility. The purpose of correction factors is further specified in section 10.2.2., but this does not seem to be the applicable approach for omega-3 oils.

- **The EPA/DHA concentration in the EPA/DHA reference standard.** There is no reference standard for EPA/DHA mentioned in this method, and it cannot be deduced for which EPA and DHA concentration range the method is suitable.

- **Use of empirical response factors.** The method does not explicitly request the use of empirical response factors; however, section 10.2.2 specifies the use of "specific correction factors" (*Fi*) for individual FAME in special cases (for example when fatty acids with < 16 carbons are present) and when demanded by the client. These specific correction factors should be determined for each instrument through the use of certified reference materials, if the flame- ionization response peak area is not equal to the mass fraction. It is not clear when the approach specified in section 10.2.2 ought to be used or when it should not be used.

  Section 10.2.2 also refers to theoretical FID correction factors (mentioned in Annex A) for informational purposes only. They are not the correction factors used in the calculation formula.

  Section 10.2.3 refers to the use of a correction factor for the internal standard used (F_{IS}). The use of *Fi* together with F_{IS} seems to function as empirical response factors.

- **Molecular weight conversion factors/instructions provided?** Section 10.2.1 refers to AOCS Ce 1h-05 regarding the conversion of FAME to TAG and states, "*According to the method AOCS Ce 1h-05, the factors for conversion of FAMEs to TAG equivalents are between 0.9114 (C8:0) and 0.9965 (C24:1) and are therefore negligible.*" This statement is questionable, especially for long chain fatty acids, and incorrect for omega-3 fatty acids. While the factors are correct, they are not negligible. The method states, "*For most fats and oil, the area fraction of the fatty acid methyl esters is equal to the area fraction of triacyglycerols in grams per 100 g.*" This is not correct for any fish oil or marine oil containing EPA and/or DHA.

- **Repeatability data available?** No repeatability data is available for EPA, DHA or fish oil. Section 11 ("*Precision*") and Annex D provide extensive interlaboratory data on repeatability (r) and reproducibility (R) for many fatty acids for ten different fats and oil types by 13 different laboratories. EPA and DHA are not among the listed fatty acids (and possibly only expected to be present in very low amounts in lard, one the fats tested).

- **Repeatability data support method suitability?** No, not for omega-3. There is no data available for fatty acids over C20:4. The repeatability data set did not include any marine/algal oils. Long chain fatty acid repeatability was not validated, and EPA, DHA or DPA are not included in the precision data (section 11) from the interlaboratory trial because they were not present in the oils tested. The longest PUFA's included in the data set are 20:2, 20:3 and 20:4, all of which were present at very low levels.

- **Reproducibility data available?** No reproducibility data is available for EPA, DHA or fish oil. Section 11 ("*Precision*") and Annex D provide extensive interlaboratory data on repeatability (r) and

reproducibility (R) for many fatty acids for ten different plant oils by 13 different laboratories. Six fish oils analyzed by 21 laboratories.

- **Reproducibility data support method suitability?** No, not for omega-3. There is no data available for fatty acids over C20:4. The reproducibility data set did not include any marine/algal oils. Long chain fatty acid reproducibility was not validated, and EPA, DHA or DPA are not included in the precision data (section 11) from the interlaboratory trial because they were not present in the oils tested. The longest PUFA's included in the data set are 20:2, 20:3 and 20:4, all of which were present at very low levels.
- **Method validated for (omega-3) oil matrix?** The method has not been specifically validated for fish oil, an omega-3 oil matrix, or oils containing omega-3 with >18 carbons. No marine/algal oils were included in the method repeatability testing. The method does state that "*ISO 12966-4 is applicable to "crude, refined, partially hydrogenated, or fully hydrogenated fats, oils, and fatty acids derived from animal and vegetable sources.*" Annex B.3, Figure B.3 shows an example chromatogram of fish oil.
- **Is the method applicable or restricted to a certain EPA/DHA range?** No firm conclusion can be made about the method being appropriate for a certain concentration range, given the absence of specific validation or mention for EPA or DHA. It is assumed that the method can handle low to high levels as long as the peaks are well- resolved, which is achievable as shown in Fig. B.3, but no data is provided in the method to support a specific range of EPA/DHA.
- **Is the method applicable or restricted to a certain class of lipids?** The method is stated to be suitable for crude, refined, partially hydrogenated, hydrogenated fats, oils and fatty acids derived from animal and vegetable sources, implying general applicability to triglyceride oils. It is stated to not be applicable for dairy fats, oils containing high CLA, and oxidized or polymerized oils. This suggests the method is not suitable for oils with short chain fatty acids. There is no specific mention of the method being inappropriate for other lipid classes, such as oils rich in polar lipids. While the method does not exclude suitability for fish oils, EPA and DHA are not included in the validation set.

General comments:

Strictly taken, ISO 12966-4 has not been shown to be suitable for the testing of fish oils, because EPA and DHA are not included in the repeatability/reproducibility results, and fish oil is not one of the matrices included in the validation. The method is a very general one for different vegetable and animal fats and oil types, and while also omega-3 oils are likely in its scope, as illustrated by a sample chromatogram for fish oil, a considerable amount of work and evaluation time is needed to adapt it to the needs of correct EPA and DHA quantification. The Working Group is of the opinion that much experience and fine tuning to identify the right standards and chromatographic conditions is needed to achieve a method that would be suitable for EPA/DHA omega-3 oils. In any case, several aspects of the method description are very general and do not clearly teach what approach to take for omega-3 oils. Also, several assumptions are made that are wrong for accurate quantification of EPA and DHA, and are expected to result in unnecessary inter-laboratory variability:

1) For the quantification of individual fatty acids, such as EPA and DHA, it is understood that section 10.2.3 should be followed since it specifies the use of an IS. Without explicit instructions provided alongside the use of this method on how to analyze EPA and DHA in omega-3 oils, it is easy to see that analysts may follow section 10.12.1 instead to quantify EPA and DHA as part of a fatty acid profiling approach and introduce errors related to the lack of use of an IS, faulty assumptions of a fixed relation between area % and weight percent, and incorrect assumptions that conversion factors are negligible.

2) A suitable IS for the quantification of EPA and DHA in omega-3 oils is not defined in this method. The choice of IS for the quantification of any specific fatty acid is left open to the analyst and also requires the determination of an instrument-specific "specific correction factor," a term likely meaning an empirical response factor. While there is mention of using external reference standards, these are not defined, leaving it open for which concentration range the method is valid (recommended use of nearly pure certified reference standards vs certified fatty acid mixtures with low concentrations of EPA and DHA, with often only containing only one of the two). It is not entirely clear from the method when to use external and internal standards, and the calculations are not the same as used in pharmacopeial methods for EPA and DHA amounts in fish oils and omega-3 concentrates.

Conclusion:

While International Standard method ISO 12966-4 may be suitable for the quantification of EPA and DHA in omega-3 oils, it lacks a clearly defined and formalized procedure for quantifying EPA and DHA, leaving open too many choices, increasing the likelihood of obtaining inaccurate results for fish oil/omega-3 products and a larger than desired interlaboratory variability.

There are specific aspects of the method that are incorrect for omega-3 oils, such as the assumption of a fixed relation between area % and weight percent, and negligibility of conversion factors.

Evaluation of the two methods, shown in tabular form with indications of support for views by number (n) of WG members:

	AOAC Official Method 996.06 (2020 revision) – "Fat (Total, Saturated, and Unsaturated in Foods"
Stated requirement or possibility for expression in mg/g (and not in area % only).	Yes (n = 5) / No (n=3)
Use of internal standard.	Yes (n=7) - Flexibility in the use of IS, not required in all method sections
Use of external reference standard	Yes (n=5) / No (n=1) - Appears more a reference standard for peak identification, not mandatory use
The EPA/DHA concentration in the EPA/DHA reference standard	Not defined (n=7)
Use of empirical response factors	Yes (n = 3) / No (n=5) – So called Correction Factors determined in some case, not specified for EPA/DHA
Molecular weight conversion factors/instructions provided?	Yes (n=2) / No (n=5) – Not specified for EPA/DHA explicitly, indicated for specific cases. Assumption that correction factors are negligible is incorrect
Repeatability data available?	Yes (n=5) / No (n=2) – Not available for fish oil/EPA, DHA
Repeatability data support method suitability?	Yes (n=2) / No (n=4) / n.a. (n=1) – Not available for fish oil/EPA, DHA
Reproducibility data available?	Yes (n=2) / No (n=5) – Not available for fish oil/EPA, DHA
Reproducibility data support method suitability?	Yes (n=1) / No (n=5) / n.a. (n=1) – Not available for fish oil/EPA, DHA
Method validated for (omega-3) oil matrix?	Yes (n=2) / No (n=5) – Incomplete validation, chromatogram for fish oil available
Is the method applicable or restricted to a certain EPA/DHA range?	Yes (n=2) / No (n=5) – No information available whether a restriction is relevant or applicable
Is the method applicable or restricted to a certain class of lipids?	Yes (n=6) / No (n=1) – Method states scope for which oils & fats it is applicable, and for which it is not

Note – Since the information in the method is not always clear regarding its applicability to omega-3 oils, negative answers by evaluators frequently meant "not applicable", or the reverse was true that a factor was applicable in general terms but unclear whether it was applicable to omega-3 oils

References

ISO 12966-4 International Standard "Animal and vegetable fats and oils – Gas chromatography of fatty acid methyl esters – Part 4: Determination by capillary gas chromatography (latest known version "2015-06-01 First Edition")

ISO 12966-2 "*Preparation of Methyl Esters of Fatty Acids*"

ISO 12966-3 "*Preparation of Methyl Esters using Trimethyl Sulfonium (TMSH)*"

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