Report of the WHO expert consultation on the WHO protocol for measuring trans-fatty acids in foods held virtually on 27 and 30 June 2022
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Background

In May 2018, the World Health Organization (WHO) called for the global elimination of industrially produced trans-fatty acids (TFA) by 2023 as a priority target of the WHO Thirteenth General Programme of Work. In support of implementing this work, WHO published in 2020 a comprehensive protocol for measuring and monitoring industrially produced TFA, intended to serve as a reference method that is globally applicable for surveillance and monitoring of TFA content for different types of foods (hereinafter referred to as “WHO reference protocol”). The WHO reference protocol was developed during a two-day expert consultation held in Geneva, Switzerland, on 11–12 October 2018, followed by an additional review process.

The WHO reference protocol gives detailed laboratory procedures and instructions for accurate measurement of the fat content and fatty acid composition, including TFA, of food samples. The WHO reference protocol was designed to generate accurate and globally comparable fatty acid data with emphasis on industrially produced TFA. The main steps of the analytical procedure include collection of food samples, extraction of fat from the food samples, conversion of extracted fat to fatty acid methyl esters (methylation of fat) (FAMES) and analysis of the FAMES using capillary gas chromatography with flame ionization detection (GC-FID), identification of FAMES and measurement of the fatty acid composition. The analytical procedures were primarily adopted from the Association of Official Analytical Collaboration (AOAC) Official Method 996.06 and the American Oil Chemists’ Society (AOCS) Official Method Ce 1h-05.

Although the WHO reference protocol was successfully implemented in several laboratories worldwide, some laboratories, especially those operating on a tight budget, had difficulties adopting it. Some of the challenges faced were:

- not enough funds to purchase gas chromatography (GC) columns;
- not enough funds to purchase FAME reference standards, especially TFA isomers, for identifying GC peaks;
- the recommended internal standard triheneicosanoin (C21:0 TAG) is expensive and not readily available commercially; and
- some of the solvents that are recommended in the protocol, such as chloroform, hexane for solubilizing fatty acids, FAMES, triacylglycerol (TAG) standards, and reagents such as boron trifluoride in methanol (BF₃-MeOH), are toxic, expensive and prohibited to use in some countries.

To address the above challenges, WHO held a two-day expert consultation on the WHO reference protocol for measuring TFA in foods on 27 and 30 June 2022. This document is a report of the consultation.

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Consultation

Management of conflicts of interest

According to the rules in the WHO Basic documents, whenever an expert or an individual provides independent advice to WHO, including participating in WHO meetings, any financial and intellectual interests must be declared and assessed by the WHO Secretariat. Declared interests of the participants were reviewed by the WHO Secretariat in consultation with the Office of Compliance, Risk Management and Ethics before the consultation. Based on the review, the role and engagement of each participant were defined (see Annex) and followed through the consultation process.

Selection of the Chair

The expert consultation unanimously agreed to select Dr Katerina Mastovska as the Chair of the consultation.

Overview of the WHO activities to achieve the global elimination of industrially produced TFA

Dr Rain Yamamoto, Scientist at WHO, outlined the purpose of the consultation and requested the expert consultation to review the WHO reference protocol, discuss areas that need to be modified, and make suggestions for development of a simpler, fit-for-purpose protocol (hereinafter referred to as “WHO simplified protocol”) that is implementable globally, especially by countries with limited resources.

It was agreed that the focus of the WHO simplified protocol is to measure percentage TFA content of total fatty acids in foods including fats and oils (that is, weight percentage, or wt %, of total fatty acids), rather than the absolute amount (that is, weight of fatty acids). The TFA data generated can be used to check the trend of TFA content in food on the market and compliance of food products with regulations for TFA elimination. WHO recommends that countries implement either of the two best-practice policies for TFA elimination: 1) mandatory national limit of 2 g of industrially produced TFA per 100 g of total fat in all foods; and 2) mandatory national ban on the production or use of partially hydrogenated oils (PHO) as an ingredient of all foods.

Proposed revisions to the WHO global protocol

Dr Nimal Ratnayake, Consultant at WHO, presented an overview of the current WHO reference protocol. Topics included GC column type, length and operating parameters; GC-FID response factors; GC internal standard; fat extraction; methylation; solvents for preparing FAME solutions; safety of using hydrogen as a GC carrier gas; and calculation of GC data. The presentation also reviewed the revisions and potential alternatives suggested in writing by the participants on each of the above topics before the consultation. The review included pros and cons of the proposed potential alternatives, providing a basis for the consultation.

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**Item 1: GC column**

The expert consultation discussed the best columns for GC analysis of TFAs. The unanimous decision was to keep using 100 m SP-2560 or CP-Sil 88 fused capillary columns. These columns are coated with 100% biscyanopropylpolysiloxane (BCS) stationary phase. It was suggested to list other 100 m BCS commercial columns as recommended alternatives to these columns. Further, it was suggested not to include columns coated with less than 100% BCS (for example, 100 m HP-88, which is coated with 88% BCS). The resolution of C18 TFA isomers is inferior when columns with less than 100% BCS are used.

As a quick monitoring tool for checking for regulatory compliance, a few participants proposed to include alternative columns in the recommended list, such as 30 m or 60 m 100% BCS columns. Many pointed out that the resolution between C18:1 cis and trans isomers with these shorter columns is not adequate for accurate quantitation of the trans isomers. It is estimated that the total TFA is underestimated by about 20%. The consensus of the consultation was that it is important to obtain data/evidence to evaluate efficiency of the shorter columns for resolution of C18 TFAs before making a decision on this proposal. Some experts volunteered to provide evidence (publications, published GC chromatograms and other forms of supporting documents) to WHO for evaluation of the efficiency of both 30 m and 60 m columns. However, no data were provided to support the suitability of these columns for TFA analysis.

**Item 2: GC column operating parameters**

**Column temperature.** Two options were suggested: 1) operating the column isothermally at 180 °C; and 2) temperature programming as recommended in ISO 16958-2015 (60 °C for 5 min, raise at a rate of 15 °C/min up to 225 °C, hold for 20 min). After weighing the pros and cons of these two temperature operations, it was agreed to include the temperature programme in ISO 16958-2015 as an alternative to isothermal operation. Both temperature operations give the best possible separations of C18:1, C18:2 and C18:3 trans isomers, but both operations have some minor flaws. Although the isothermal operation is simpler to implement, a notable disadvantage of it is that short-chain fatty acids (especially C4:0, C6:0 and C8:0) are not fully resolved from the solvent front. Several views were expressed that isothermal operation is a better option for analysis of oils and foods containing no ruminant fats while the temperature programme is better for foods containing a mix of PHOs and ruminant fats. It was also brought to the attention of the consultation that with temperature programming, 9t,12c,15c-C18:3, which is found only in refined canola and soybean oils and not in PHOs, could elute with 11c-C20:1. One expert showed that separation of 9t,12c,15c-C18:3 and 11c-C20:1 can be improved by operating the column temperature isothermally slightly below 180 °C instead of at 180 °C. Another expert, who has done analysis using temperature programming, demonstrated using actual GC runs that this overlap is especially occurring with the 100 m SP-2560 column, but not with the 100 m CP-Sil 88 column.

It was suggested to display in the WHO simplified protocol examples of GC chromatograms, with all peaks marked, for the two GC operating temperatures. Two participants kindly volunteered to supply these GC chromatograms.1

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1 GC chromatograms were provided and are included in the WHO simplified protocol.
**GC carrier gas.** There was wide agreement that both hydrogen and helium are suitable GC carrier gases for TFA analysis. A concern, however, was expressed that hydrogen is explosive and a fire hazard so may not be safe to use. An expert indicated that, to his knowledge, there are no reports of fire incidents due to hydrogen use. Further, he alluded to the fact that modern GCs are equipped with hydrogen leak detectors and, therefore, are explosion-proof.

Helium is a good alternative for hydrogen, but is expensive and not available in some countries. A proposal was made to use nitrogen as the carrier gas, but no data are available to gauge the efficiency of the resolution of FAMEs using nitrogen. WHO requested data from those who are proposing the use of nitrogen, however, no data were provided.

**Item 3: GC-FID response factors (theoretical versus experimental)**

An expert presented data to demonstrate that the theoretical FID response factors do not match experimentally measured FID response factors, especially for fatty acids below C16 chain length. However, the expert consultation noted that the difference is small and therefore agreed to retain the FID theoretical factors. The benefit of using theoretical FID responders is that it eliminates the time-consuming steps of measuring the FID response for each FAME reference standard.

**Item 4: Internal standard**

There was an agreement that C21:0 TAG is not a suitable internal standard for the WHO simplified protocol because of its high cost. It was also pointed out that C21:0 FAME co-elutes with C18 CLA isomers. Some experts suggested using C11:0 FAME as the internal standard, which is the internal standard recommended in the ISO Official Method 16958-2015.

An expert illustrated, using a formula, that it is not necessary to have an internal standard because the aim of the simplified protocol is to measure wt% of total fatty acids, not weight of fatty acids. It was agreed not to use an internal standard and to quantify fatty acids, including TFA, as a percentage of the total peak areas of all identified fatty acids shown in the GC chromatogram.

The consultation noted that not having to use C21:0 TAG as the internal standard eliminates the use of chloroform as the solvent for making C21:0 TAG working solutions. Chloroform is a known carcinogen and its use in laboratories is prohibited in some countries.

**Item 5: Fat extraction**

There was general agreement that the AOAC hydrolytic extraction procedure (AOAC Official Method 996.06), which is applicable to all types of foods and is a commonly used procedure, is adequate for fat extraction. One expert indicated that there is a risk of lipid oxidation due to the high temperature (80 °C for 60 min) involved in the AOAC extraction procedure. However, no data were provided to support this possibility.

It was further highlighted that, for the quantification of industrially produced TFA, it is not essential to generate information on the full fatty acid profile of the food samples. The focus should be on measuring the fatty acid profile of the externally added fats,
such as PHO, shortening, and other oils and fats, that are used in food preparation. It was also agreed that, unlike lipids such as triacylglycerols, phospholipids and glycolipids that are naturally present in foods, the externally added fats are not chemically attached to the food matrix and are often present unattached on the food surface. Therefore, the extraction of these free fats, unlike the naturally bound fats present in foods, does not require elaborate extraction procedures such as acid or base hydrolysis to free up the fats. Many indicated that the added fats can be extracted either by Soxhlet or simply using solvents at room temperature.

Although Soxhlet extraction can extract non-bonded fat, several reservations were expressed about its efficiency, because Soxhlet extraction requires several hours of heating/refluxing the food samples with solvents and the duration of refluxing varies with the type of food sample. So far, refluxing has not been established for various food samples.

There was a proposal to use a mixture of solvents (50 mL petroleum ether, 50 mL diethyl ether and 10 mL heptane) at room temperature as an efficient procedure for fat extraction. The extraction is simpler, performed at room temperature, is complete in 30 min and is applicable to all types of food samples. This solvent extraction procedure was used for analysis of foods collected from central Asia, the Caucasus and Europe in the WHO’s FEEDcities Project.\(^1\) The consultation requested the experts who proposed this procedure to provide WHO with the details of the extraction method and the Fapas results from the proficiency tests. WHO would evaluate the efficiency of their extraction method, and if it is deemed satisfactory, would include the method in the WHO simplified protocol.

In summary, there was general support for including the AOAC hydrolytic extraction procedure (AOAC Official Method 996.06) in the WHO simplified protocol. The inclusion of an extraction procedure that uses organic solvents at room temperature is to be evaluated upon data submission.\(^2\) Soxhlet extraction cannot be included because the refluxing for different types of foods has not been specified.

**Item 6: Methylation**

After discussing the pros and cons of various methylating agents (such as BF\(_3\)-MeOH, sodium methoxide in methanol, hydrogen chloride in methanol, concentrated sulfuric acid in methanol), it was agreed that potassium hydroxide in methanol (KOH-MeOH) should be recommended in the WHO simplified protocol. With KOH-MeOH, methylation is fast, can be conducted at room temperature (there is no need to heat the fat sample to affect the methylation), and it can methylate triacylglycerols, diacylglycerols and monoacylglycerols. The consultation noted that KOH-MeOH is not recommended for methylating free fatty acids and polar lipids (for example, phospholipids and glycolipids), but it was indicated that this should not be considered as a drawback, as unbound fats in prepared foods are almost 100% triacylglycerols and often contain minor amounts of free fatty acids (<2% of total fat).

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1. FEEDcities is an ongoing multi-country study that describes the urban food environments of cities in central Asia, the Caucasus and south-eastern Europe (see https://www.who.int/europe/initiatives/feedcities).
2. Sufficient data have since been received on foods not including dairy products, for which the procedure is included as an alternative in the simplified protocol.
It was agreed that BF₃-MeOH is a universal reagent because it can methylate all types of lipid classes, and it is widely used in many lipid research laboratories. A drawback is that BF₃-MeOH is toxic and may be prohibited for import in some countries. However, BF₃-MeOH will be also included in the simplified protocol, given that the AOAC hydrolytic extraction procedure (AOAC Official Method 996.06) will be included as a fat extraction procedure and needs methylation by BF₃-MeOH.

**Item 7: Solvent for preparing and storing FAME solutions (reference standards and test samples for GC)**

The consultation reviewed and discussed the best and safest solvents for preparing and storing FAME reference standards and test samples. It was noted that hexane is mildly toxic, and it was agreed that, wherever possible, hexane should be replaced with solvents that are less problematic. The consensus was that good alternatives are heptane, iso-octane and petroleum ether, and that all of these should be included in the WHO simplified protocol.

**Item 8: GC FAME peak identification**

During the discussion of this subject, the consultation agreed that, if reference FAME standards are available in the laboratory, GC FAME peaks of test samples should be identified using the reference FAME standards. But the consultation realized that many of the TFA-FAME reference standards are not readily available from commercial sources and are expensive if available, so it is challenging for laboratories with limited budgets to purchase them. The consultation agreed that an inexpensive way of identifying GC FAME peaks of test samples is to compare them with the GC FAME profiles of samples such as a FAME reference standard mixture, PHO and other well characterized oils and fats that are published in the peer-reviewed literature or given in the standard official methods (for example, in AOAC and AOCS official methods) or in the WHO protocol. In addition, it was requested that WHO provide GC chromatograms of well characterized oil samples in the simplified protocol, for FAME peak identification of test food samples. It was noted that this is an inexpensive and reliable way of identifying GC FAME peaks.

Two participants kindly volunteered to supply the GC chromatograms of FAME for some common oil samples, and reference FAME standards with all peaks identified and labelled (see also item 2).

**Item 9: Calculation of TFA content as a percentage of total fatty acids**

It was agreed that, for the purpose of the WHO simplified protocol, expressing the fatty acid data as wt % of total fatty acids was sufficient by calculating the ratio (in percentages) of the individual fatty acid with respect to the total peak area of all the identified individual fatty acids in the GC chromatogram.

Analysis of dietary fats by this simplified protocol will focus only on the C₁₈ TFAs, therefore the total TFA is calculated as the summation of all the individual C₁₈ trans isomers (that is, C₁₈:1 TFA, C₁₈:2 TFA and C₁₈:3 TFA). Both in PHOs and ruminant fats,

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1 GC chromatograms were provided and are included in the simplified protocol.
TFAs are almost exclusively of C18 chain length, with no detectable levels of TFAs from other chain lengths. Small amounts of trans isomers of C14:1, C16:1 and C17:1 are present in non-hydrogenated vegetable oils and ruminant fats, but these minor TFAs have not been characterized well. In addition, authentic FAME standards of C14:1, C16:1 and C17:1 are not commercially available, which hinders positive identification of these minor TFAs.

Expressing fatty acid data as wt % of total fatty acids requires no internal standard. The simplified protocol should include a template (for example, an Excel spreadsheet) for calculating and reporting the fatty acid composition (wt % of total fatty acids).

**Item 10: EU-JRC approach for estimating the ratio of industrially produced TFA content to ruminant TFA in test food samples**

There was a brief discussion about the European Commission Joint Research Centre (EU-JRC) approach for estimating the ratio of industrially produced TFA and ruminant TFA contents. The consensus was that differentiating between industrially produced TFA and ruminant TFA was quite complicated, so such an approach should not be included in the simplified protocol. However, this will be proposed for consideration at the planned revision of the WHO reference protocol.

**Conclusion**

WHO is to draft the simplified protocol based on the outcomes of the procedures reviewed above (see Table 1, which summarizes the differences between the WHO reference protocol and the WHO simplified protocol). A draft of the WHO simplified protocol is to be prepared and circulated to all participants for their comments.

After the WHO simplified protocol is complete, WHO is planning to revise the WHO reference protocol, which will serve as the “reference method” for laboratories whose resources are not constrained and can conduct full FAME analysis. The WHO simplified protocol will not supersede the WHO reference protocol as the intended use is different (as noted above).
Table 1.
Differences between the original WHO reference protocol and the WHO simplified protocol

<table>
<thead>
<tr>
<th>Factor</th>
<th>Type of change</th>
<th>WHO reference protocol*</th>
<th>WHO simplified protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC column</td>
<td>Alternatives added</td>
<td>100 m SP-2560 or CP-Sil 88 fused capillary columns (and their equivalents)</td>
<td>100 m SP-2560 or CP-Sil 88 fused capillary columns (and their equivalents) + other 100 m BCS commercial columns as alternatives</td>
</tr>
<tr>
<td>GC column temperature</td>
<td>Operating the column isothermally at 180 °C</td>
<td>Operating the column isothermally at 180 °C + temperature programming as recommended in ISO 16958-2015</td>
<td></td>
</tr>
<tr>
<td>GC carrier gas</td>
<td>No change</td>
<td>Hydrogen or helium</td>
<td>Hydrogen or helium</td>
</tr>
<tr>
<td>GC-FID response factors</td>
<td>No change</td>
<td>Theoretical FID response factors</td>
<td>Theoretical FID response factors</td>
</tr>
<tr>
<td>Internal standard</td>
<td>Removed</td>
<td>C21:0 TAG</td>
<td>–</td>
</tr>
<tr>
<td>Solvent for preparing internal standard</td>
<td>Removed</td>
<td>Chloroform</td>
<td>–</td>
</tr>
<tr>
<td>Fat extraction</td>
<td>Alternative added</td>
<td>AOAC hydrolytic extraction procedure (AOAC Official Method 996.06)</td>
<td>AOAC hydrolytic extraction procedure (AOAC Official Method 996.06) + a procedure using organic solvents at room temperature</td>
</tr>
<tr>
<td>Methylation</td>
<td>Alternative added</td>
<td>BF₃-MeOH</td>
<td>BF₃-MeOH + KOH-MeOH</td>
</tr>
<tr>
<td>Solvent for preparing FAME standard</td>
<td>Safer alternatives added</td>
<td>Hexane (or another organic solvent of similar volatility and polarity)</td>
<td>Heptane, iso-octane and petroleum ether to replace hexane whenever possible</td>
</tr>
<tr>
<td>GC FAME peak identification</td>
<td>Alternative provided</td>
<td>Identify using FAME reference standards</td>
<td>Identify using FAME reference standards + if FAME standards are not available, identify by comparing with the GC elution pattern from representative chromatograms</td>
</tr>
<tr>
<td>Calculation of TFA content</td>
<td>Focused</td>
<td>Weight of fatty acids (absolute amount), wt % of total fatty acids (calculated with respect to C21:0 TAG internal standard)</td>
<td>wt % of total fatty acids (calculated with respect to C18:0)</td>
</tr>
</tbody>
</table>

Annex

List of participants

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