Simplified protocol

for measuring *trans*-fatty acids content as a percentage of total fatty acids in food products

WHO laboratory protocol

World Health Organization

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Contents

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Management of conflicts of interest

According to the rules in the WHO *Basic documents*,[1] 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 as either an expert or an external resource person and followed through the process.

[1] Basic documents. 49th edition. Geneva: World Health Organization; 2020. (http://apps.who.int/gb/bd/, accessed 8 March 2023).

Acronyms

AOAC	Association of Official Analytical Collaboration
AOCS	American Oil Chemists' Society
BCS	biscyanopropylpolysiloxane
FAME	fatty acid methyl ester
FID	flame ionization detector
GC	gas chromatography
IP-TFA	industrially produced *trans*-fatty acid
ISO	International Organization for Standardization
MUFA	monounsaturated fatty acid
PHO	partially hydrogenated oil
PUFA	polyunsaturated fatty acid
SFA	saturated fatty acid
TFA	*trans*-fatty acid
WHO	World Health Organization

Executive summary

In December 2020, the World Health Organization (WHO) first developed and published a laboratory protocol for measuring *trans*-fatty acids (TFA) in foods *(1)* (hereinafter referred to as "WHO reference protocol"). Although the WHO reference protocol was successfully implemented in several laboratories worldwide, some laboratories, especially those operating with a limited budget, had challenges implementing it. These laboratories requested that the procedures be simplified and suitable alternatives be included in the protocol so that it is easier to use and globally applicable. For example, WHO received feedback from laboratories in various countries that they experienced difficulties procuring certain consumables, particularly internal standards and reference standards, because of cost and availability issues. In response to the requests, a two-day, virtual WHO expert consultation meeting was held on 27 and 30 June 2022 to discuss simplified procedures and other suitable alternatives that enable laboratories operating with limited resources to conduct fatty acid methyl ester (FAME) analysis *(2)*. Based on this expert consultation, the *Simplified protocol for measuring trans-fatty acids content as a percentage of total fatty acids in food products: WHO laboratory protocol* (hereinafter referred to as "WHO simplified protocol") was developed. This fit-for-purpose protocol provides the data that are required for governments' surveillance and monitoring activities to check the trend of TFA content in food products on the market and ensure that food products comply with regulations for TFA elimination.

One of the main differences between the two protocols is that the WHO simplified protocol does not require the use of internal standards, which are often not affordable or accessible by laboratories. This reduces the costs associated with FAME analysis. Because there is no need for an internal standard, chloroform, a solvent used for preparing internal standards, is also not needed. Furthermore, there are no errors associated with the weighing of internal standards and test samples. As a trade-off, the type or format of data that can be obtained from the WHO simplified protocol are limited when compared with those from the WHO reference protocol. In the WHO simplified protocol, fatty acid composition data are calculated as weight percentage (wt %) of total fatty acids,[1] whereas the WHO reference protocol directly calculates the fatty acid composition in both grams and wt % of total fatty acids, which can be calculated by the weight of fatty acid in grams per 100 g of total fatty acids. This trade-off is considered minimal, however, as the wt % of total fatty acids is deemed sufficient for TFA surveillance and monitoring activities. The advantage of not having to use an

[1] Please note that the wt % of total fatty acids obtained in the simplified protocol is an approximation because quantitation of individual fatty acids is performed by calculating area %.

internal standard for the WHO simplified protocol surpasses its limitations, especially in resource-limited settings. In addition, the procedures included in the WHO simplified protocol have already been well tested, so the data that are produced are as good as those generated from the WHO reference protocol. The procedures have also been streamlined and made to be more fit-for-purpose than those for the WHO reference protocol, to support governments to conduct surveillance and monitoring.

Furthermore, the WHO simplified protocol allows suitable alternatives to be used. Compared to the original WHO protocol published in 2020 *(1)*, the WHO simplified protocol allows (i) a procedure for fat extraction using organic solvents at room temperature,[1] in addition to the Association of Official Analytical Collaboration (AOAC) Official Method 996.06; (ii) 2 M KOH-MeOH reagent as an alternative to 7% BF_3-MeOH for methylation of fat; and (iii) temperature programming as an alternative to isothermal operation for gas chromatography (GC) operating conditions.

Like the original WHO reference protocol, the use of theoretical flame ionization detector (FID) response factors is retained in the WHO simplified protocol, and the use of experimental response factors is not required for correcting the peak areas for variation of the FID response.

In the WHO simplified protocol, reference FAME standards are not required to determine % TFA, but if they are available, they can support the identification of GC FAME peaks in test samples. Unfortunately, many FAMEs that must be measured are not currently commercially available as reference standards, and if they are available, they are expensive. This makes it challenging for laboratories with limited budgets to purchase them. The WHO simplified protocol describes in detail how peak identification can be achieved without FAME reference standards using reliable published GC chromatograms. This approach has been used in most laboratories over the past several decades. Provided that the GC analysis is performed according to the GC parameters published in the literature, the elution of fatty acids presents the same pattern on the GC chromatogram. The identification of all fatty acids, both naturally occurring and industrially produced, based on their elution order has been well established by many analysts. The WHO simplified protocol provides many example GC chromatograms for various food types, which laboratories can refer to for peak identification.

In 2023, WHO is planning to revise the current WHO reference protocol, which will serve as the "reference method" for conducting full FAME analysis. The WHO simplified protocol will not supersede the WHO reference protocol, as the intended use is different (as noted above). Laboratories can choose to use either the "WHO reference protocol (to be revised)" or the "WHO simplified protocol", depending on which is the most suitable for the purpose of the analysis.

[1] This procedure can be applied to foods not including dairy products.

1. Goal, scope and general approach

1.1 Goal

The goal of this *WHO simplified protocol for measuring trans-fatty acids content as a percentage of total fatty acids of food products* (hereinafter referred to as "WHO simplified protocol") is to provide a simpler, fit-for-purpose protocol that can be implemented by all laboratories, including those with limited resources, to measure percentage *trans*-fatty acid (TFA) content (as weight percentage, or wt %, of total fatty acids) of foods, including fats and oils. These data provide information that is required for governments' surveillance and monitoring activities to check the trend of TFA content in food products on the market and ensure food products comply with regulations for TFA elimination.[1]

1.2 Scope

Partially hydrogenated oils (PHOs) and foods prepared using PHOs are the primary sources of industrially produced TFA (IP-TFA). Other sources of IP-TFA are fully hydrogenated oils, deodorized (but non-hydrogenated) oils, oils subjected to heat treatment (such as oils used in domestic and commercial frying of foods) and foods prepared using these oils. For surveillance and monitoring purposes, IP-TFA-containing foods, oils and fats must be analysed for IP-TFA content.

Although the primary goal of the WHO simplified protocol is to obtain data on IP-TFA, the analysis of foods containing natural TFA, such as milk, milk products and ruminant meat products, is within the scope of this protocol.

1.3 General analytical approach

The approach is simplified and inexpensive because it does not require the use of an internal standard.

Fat extraction. In addition to the hydrolytic extraction procedure (Association of Official Analytical Collaboration (AOAC) Official Method 996.06), a simpler procedure using organic solvents at room temperature is included in this protocol as an alternative for foods not including dairy products. Unlike lipids such as triacylglycerol, phospholipids and glycolipids naturally present in foods, PHOs and other fats and oils that are used in

[1] 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 PHOs as an ingredient of all foods.

the preparation of foods are externally added fats. Therefore, they are not chemically bound to the food matrix and are often present as free, unattached fats on the surface of the food. As a result, the extraction of these fats, unlike the naturally bound fats, does not require acid or base hydrolysis to "free up" the fats, but can be easily done at room temperature using a solvent mixture of petroleum ether, diethyl ether and heptane. Dairy products are extracted using a procedure from AOAC Official Method 996.06 (base or base–acid hydrolysis).

Methylation. Fat extracted by the hydrolytic extraction procedure (from AOAC Official Method 996.06) is methylated by BF_3-MeOH. Fat extracted using a solvent mixture of petroleum ether, diethyl ether and heptane is methylated using either 2 M potassium hydroxide in methanol (KOH-MeOH) or 7% boron trifluoride in methanol (BF_3-MeOH).

GC analysis. Analysis of the fatty acid methyl esters (FAMEs) for the fatty acid composition is performed using a gas chromatography–flame ionization detector (GC-FID) system equipped with a 100 m fused silica capillary column coated with 100% biscyanopropylpolysiloxane (BCS) stationary phase. FAME samples can be analysed by operating the column oven temperature either isothermally or using temperature programming.

Peak identification. Individual FAME peaks can be identified by comparing the GC elution pattern to those in representative chromatograms of a mixture of FAME reference standards, and FAME samples of fully characterized canola oil, PHO, shortening and milk fat (see Fig. 1–12). If commercial reference standards are available, the FAME peaks can also be identified by analysing the reference standards using the above-mentioned conditions and comparing their GC retention times with the GC FAME peaks of the test sample.

Calculation. The peak areas of FAMEs are corrected for the variation of the FID response of the individual fatty acids using theoretical response factors. Quantitation of individual fatty acids is performed 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. The total TFA is the summation of all the individual C18 *trans* isomers.

2. Reagents

- Acetone, analytical grade.
- Ammonium hydroxide, 58% (w/w).
- Dichloromethane, reagent grade.
- Diethyl ether, anhydrous, purity ≥99.7%, containing 1 ppm BHT inhibitor (packed in aluminium containers).
- Ethanol, 95% (v/v).
- *n*-Heptane, chromatography or GC grade.
- Hydrochloric acid, 12 M and 8.3 M. To make 8.3 M HCl, in a fume hood add 250 mL of 12 M HCl to 110 mL distilled water. Mix well and store at room temperature (20–25 °C). Care should be exercised, as the solution warms up.
- Petroleum ether (boiling range between 35–60 °C), extra-pure grade.
- Phenolphthalein pH indicator, laboratory grade.
- Potassium hydroxide pellets, reagent grade.
- Methanol, reagent grade.
- Methylation reagent, either 7% BF_3-MeOH or 2 M KOH-MeOH. The 7% BF_3-MeOH reagent can methylate all types of lipids, whereas 2 M KOH-MeOH methylates only non-polar lipids (see section 7 for more details).
  - Prepare 7% BF_3-MeOH in a fume hood from commercially available 14% BF_3-MeOH reagent.
  - Prepare 2 M KOH-MeOH reagent by weighing 11.2 g (±0.1 g) of KOH pellets in a clean, dry, 250 mL Erlenmeyer flask equipped with a glass stoper. Add 100 mL methanol and dissolve the KOH pellets by stirring the solution overnight using a magnetic stirrer. Once all the KOH pellets are completely dissolved, store the solution in a refrigerator (between 4–8 °C) until ready to use. This solution is sufficient to methylate 400 samples.
- Pyrogallic acid.
- Toluene, nanograde.
- Sodium sulfate, anhydrous, reagent grade.
- Water, HPLC grade or equivalent.

3. Laboratory equipment

3.1 Gas chromatography (GC-FID) system

- **Gas chromatograph.** Suitable for use with a capillary column; equipped with an FID, temperature-controlled split/splitless-mode injector unit,[1] oven chamber for the capillary column capable of maintaining the programmed temperature ±1 °C, computer monitor, and chromatographic software capable of manipulating the GC controls (gas flow; split ratio; oven, injector and FID temperatures; recording the GC-FID chromatogram, GC peak areas, retention times and peak integration; and data handling and storage). It is desirable to have a GC with an automated sample injector (autosampler) to allow continuous analysis of samples. The GC system should be calibrated regularly, and the system report maintained. Before starting the analysis, check the system report for any issues, especially gas leaks, the flow rate, oven temperature, and injector and seals. Replace liners and septa as required for optimal GC performance.
- **Capillary column.** Fused silica capillary column with a length of 100 m, an internal diameter of 0.25 mm, thickness of 0.20 µm, and coated with 100% BCS stationary phase. SP-2560 or CP-Sil 88 are highly recommended. Commercially prepared SP-2560, CP-Sil 88, and other 100% BCS columns are available from various chromatographic suppliers (e.g. MilliporeSigma; Sigma-Aldrich; Agilent Technologies, Inc.; Fisher Scientific; SelectScience; Karckeler Scientific, Inc.; Altmann Analytik GmbH and Co. KG). Other capillary columns that provide FAME peak resolution and elution patterns as shown in Fig. 1–12 may also be used.
- **Micro syringe for GC.** 10 µL delivery, with a hardened needle.
- **Carrier gas.** Hydrogen or helium, 99.999% pure or better, GC quality, dried, and oxygen removed by suitable filters.
- **Flame ionization detector gases.** Hydrogen and air, GC quality.
- **Injection port split liner.** Split liner with glass wool (e.g. Agilent part number 5183-4647 or equivalent).

[1] An on-column injection could be used if a split/splitless inlet is not available.

3.2 Laboratory glassware and equipment

All glassware and laboratory equipment intended for food sample preparation, sample storage, fat extraction and analysis should be cleaned thoroughly to ensure that it does not contain any residual fat or fatty acids. Thorough cleaning of glassware can be achieved by soaking glassware in a detergent and rinsing with tap water, followed by distilled water. After rinsing, dry the glassware in an oven maintained at 100 °C. Store the clean, dried glassware in a clean, dust-free glass cupboard or any other clean storage area. If necessary, just before use, rinse the stored glassware with acetone followed by petroleum ether. Wipe off excess solvent using a clean cloth or paper towel, visually inspect the cleaned glassware to ensure that they contain no traces of these extremely flammable solvents, and quickly dry in the oven.

Other laboratory equipment, especially the weighing scale, mortar and pestle, and food blender/processor should also be cleaned to remove fat and dirt. Use acetone followed by heptane, then wipe off excess solvent using a clean cloth or paper towel.

- Erlenmeyer glass flasks equipped with glass stoppers (capacity 250 mL or 500 mL).
- Glass measuring cylinders (capacity 10 mL and 50 mL).
- Disposable glass pipettes.
- Glass test tubes of various capacities (e.g. 10 mL, 15 mL and 50 mL) with Teflon-lined screw caps.
- Rotary evaporator and glass round-bottom flasks, for evaporating solvents.
- Glass funnels (both medium and large sizes).
- Weighing scale, for measuring gram quantities of food samples.
- Laboratory centrifuge equipped with adaptors for test tubes.
- Nitrogen from a nitrogen cylinder.
- Domestic food blender or a food blender with a processor.
- Laboratory oven, preferably a standard digital oven built for heating and drying, offering temperature control and safety.
- Filter papers (non-ashless, slow-flow generic filter).
- Hot-water bath with nitrogen stream supply.
- Electric heating block.
- Hot-water bath shaker (shaking water bath).
- Refrigerator capable of maintaining temperatures between 2–8 °C.
- Freezer capable of maintaining temperatures from –18 °C to –15 °C, including a refrigerator freezer (if the freezer section is large enough to accommodate the collected test food samples).
- Food containers with lids (glass or food-grade plastic).
- Vortex mixer.

4. Sampling plan, sample collection and sample storage

The sampling plan, sample collection and storage procedures presented here are to be used only as a guideline.[1] Some countries, regions or laboratories may have their own sampling plans. Sampling depends on the purpose and design of each study and survey, which depend on the landscape in each country. Whatever method is used, the collected samples should be representative of the brands or lot numbers available locally.

4.1 Sampling plan

Category 1: fats and oils available to consumers and restaurants. Foods in this category include cooking oils, salad oils, frying oils, baking fats, shortenings and vegetable ghee. These are almost 100% triacylglycerol oils, but some oils could contain diacylglycerols and monoacylglycerols.

Category 2: margarines and spreads. Foods in this category include margarines, and other types of spreads.

Category 3: processed and home-made foods. Foods in this category include many packaged and processed foods sold in retail stores or made at home. Some common examples are biscuits, breads (all varieties), buns, cakes, cookies, crackers, croissants, frozen foods (such as fried potatoes, fried fish, fried shrimps, fried noodles, fried rice, other fried foods), potato chips and other chip varieties, popcorn, pizza, pastries and rotis (all types of Indian, Middle East and Latin American flat breads).

Category 4: ready-to-eat foods from food outlets. Foods in this category include all types of ready-to-eat fried and baked goods served in restaurants, grocery stores, bakeries, fast-food outlets, street vendors, corner stores and other medium-scale and small-scale food outlets.

Category 5: dairy products. Products in this category include milk, and milk products like butter, ghee, cream, yogurt and cheese.

[1] A collection protocol of FEEDcities can also be referred to as an example TFA survey. 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).

4.2 Collection of food samples[1]

Categories 1–3 and category 5. Collect representative food samples from two or more major grocery stores in the area. If resources allow, collect PHOs from oil-manufacturing or distribution facilities in the area.

Category 4. Collect ready-to-eat foods from two or more popular food outlets in the area. In addition, if possible, collect samples of cooking/frying oils used in these food outlets.

Select 3–5 different popular brands for each of the five categories. Collect three consumer-size packages from each brand. The total weight of the three consumer-size packages should be 300 g or more. Similarly, collect three servings from each of the popular ready-to-eat food items. Here also, the total weight of the three servings should be 300 g or more.

Do not make a composite sample by mixing different brands or items. Each brand and food item should be analysed separately.

4.3 Inventory of foods purchased

An inventory of the foods purchased should be maintained.

Record the following for each sample item:

- name and address of the grocery store or ready-to-eat food outlet, or where the food item was purchased
- date the food item was purchased
- if available, date the item was manufactured as shown on the food label
- best before or use by date as shown on the food label
- food category (e.g. cookies)
- sample size or weight
- brand name
- manufacturer's name
- lot number.

Assign a code number to the food item. Use this code number for labelling the food items and recording all the analytical data pertaining to the food sample.

4.4 Storage of food samples

Immediately after purchasing food items, place perishable items (especially ready-to-eat foods) in containers (preferably glass or food-grade plastic containers) and keep the containers cool with ice packs. Without delay, transport the collected samples to the analytical laboratory.

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[1] When nutrition declaration of TFA is available on the product's label or in the country's registry of the product, it is important to ensure that the sample collection captures products of various levels of TFA content within each food category.

If the laboratory is not ready to analyse the food samples on the day they were purchased, store the samples in a refrigerator (between 2–8 °C, for oil samples) or freezer (approximately –18 °C to –15 °C, for all other food items). Make sure to analyse the food samples before the expiry date shown on the food package. If the expiry date is not given or is not known, analyse the samples within 2 months of purchase.

5.
Preparation and homogenization of food samples for analysis

Only homogenize the number of food samples that can be analysed the same day.

Experienced technicians working in a well-equipped laboratory might be able to analyse three or four samples in a day, whereas a less experienced technician might take about two days to analyse one sample. Taking into consideration the skill level and experience of the technician, it is advisable not to homogenize all the samples at once – instead, start with a few samples. If successful, adjust the schedule to analyse more samples within a certain time frame.

- When the laboratory is ready to commence the analysis, take out all the food packages of a given brand from storage. Thaw frozen samples to room temperature (20–25 °C). If needed, slightly warm the packages (in an oven or hot-water bath) to accelerate thawing, but make sure not to heat the samples above 30–32 °C.
- Take the entire contents from each of the thawed packages and combine the contents to make a composite. Do not create composites of different brands by mixing the contents together, as the goal is to identify the TFA content of each food brand.
- The composite should be homogenized thoroughly to ensure that it is a true representation of all packages.
- A composite of liquid oils, milk or thawed semi-solids (e.g. margarine) can easily be prepared by mixing the contents in a suitable large, clean glass container (e.g. bottle, beaker, Erlenmeyer flask). Mixing can be done by swirling the glass container or stirring with a clean glass rod.
- Preparing a well-homogenized composite of solid food samples is a challenge. Consider using a domestic food blender or food processor. The size of the food blender or food processor must be appropriate for the volume of the sample. The temperature must be such that the fat does not separate out or get left behind in the container in a way that is disproportionate to the total sample. Place the entire contents of solid food samples in the blender or food processor and process until the contents have been blended into the smallest sized particles possible. If the particle size is not small enough, place the entire composite in a ceramic bowl (or glass bowl) and pour in sufficient dry ice to cover the composite, and then grind it using a mortar. This should produce a good homogenized sample.

- Take a subsample from the homogenized composite for fat analysis. Place the remainder of the composite in a clean and opaque bottle (with a tight cap) or Whirl-Pak bag. Label the bottle or bag with the food item's code number and store it in a refrigerator (2–8 °C, for all fat and oil samples and packaged foods) or freezer (–18 °C to –15 °C, for ready-to-eat food samples). These samples are for future use in case the fat analysis needs to be repeated. However, analysis should be completed within one month of preparing the homogenized composite.

6. Extraction of fat

6.1 Category 1 foods: fats and oils (salad oils, cooking oils, baking fats, shortenings and vegetable ghee) (100% triacylglycerol oils)

The fat extraction step is not required for fat and oil samples. Place approximately 200 mg of the food sample in a 15 mL glass test tube equipped with a screwcap lined with a Teflon or silicone septum. Methylate the sample using either 7% BF_3-MeOH or 2 M KOH-MeOH (see section 7).

6.2 Category 2 foods: margarines and spreads

1. Place approximately 20 g of the homogenized composite margarine/spread sample in a clean and dry 250 mL Erlenmeyer flask equipped with a glass stopper.
2. Add 50 mL petroleum ether and 50 mL diethyl ether (50 : 50, v/v) to the Erlenmeyer flask containing the margarine/spread sample. Place the glass stopper on the flask and shake thoroughly to dissolve the margarine/spread sample.
3. Transfer the contents of the Erlenmeyer flask into a clean and dry 250 mL separating funnel. Add 50 mL distilled water and shake gently. Allow the layers to separate.
4. Drain out the lower aqueous layer. Decant the upper solvent layer through a filter paper (Whatman No. 42) filled with approximately 1 g of anhydrous sodium sulfate, collecting the filtrate in a 250 mL round-bottom flask.
5. Evaporate the solvent in a rotary evaporator set at 40 °C and approximately 100 mbar. Use a disposable pipette to transfer the residue into a clean 50 mL glass test tube equipped with Teflon-lined screwcap. The residue contains the extracted fat. Flush the test tube with nitrogen and cap the tube.
6. Place approximately 200 mg of the extracted fat in a 15 mL test tube equipped with a Teflon-lined screwcap. Methylate the fat immediately using either 7% BF_3-MeOH or 2 M KOH-MeOH (see section 7). If the laboratory is not ready for the methylation step, flush the tube with nitrogen (to prevent or minimize oxidation of polyunsaturated fatty acids), cap the tube and store in a refrigerator (2–8 °C). However, methylation should be done within 1–2 days of fat extraction, as prolonged storage can lead to oxidation of polyunsaturated fatty acids.

6.3 Category 3 and 4 foods: processed, home-made and ready-to-eat-foods excluding dairy products

The fat in the foods of these two categories should be extracted using either the acid hydrolysis procedure (adopted from AOAC Official Method 996.06) (see section 6.3.1) or the solvent extraction procedure (see section 6.3.2)

6.3.1 Acid hydrolysis procedure

This procedure has been adopted from AOAC Official Method 996.06 *(3)*, with some minor modifications. It is to be used for all foods, including food containing mixed fats from both ruminant and non-ruminant sources. For extraction of fat from dairy products, procedures outlined in Sections 6.4.1 (dairy products not including cheese) and 6.4.2 (cheese) should be used.

1. Make a conservative estimate of the weight of the food sample that would yield approximately 200 mg fat. Take a subsample equivalent to the estimated weight from the homogenized composite (see section 5) and place in a glass test tube (50 mL capacity with a Teflon-lined screwcap). Add approximately 100 mg pyrogallic acid and 2 mL ethanol and mix well until the entire test portion is dispersed.
2. Add 10 mL 8.3 M HCl and mix well. Place the cap on the tube and heat the test tube in an electric heating block or a water bath (preferably a shaking water bath set at moderate agitation) for 40 min at 70–80 °C. Every 10 min, mix the contents of the test tube on a vortex mixer to incorporate into solution any particles adhering to the walls of the test tube.
3. After digestion, remove the test tube from the heating block or water bath and allow to cool to room temperature (20–25 °C). Add 5 mL ethanol and mix well.
4. Add 25 mL diethyl ether. Place the cap on the test tube and vortex for 5 min.
5. Add 25 mL petroleum ether and vortex for 5 min.
6. Transfer the contents to a 25 mL separatory funnel equipped with a glass stopper. Place the glass stopper on the funnel and allow the contents to settle for at least 1 h, until the upper layer is clear.
7. Drain out the bottom layer and discard it. Slowly decant the top layer (organic layer) through a glass funnel lined with a filter paper filled with approximately 1 g anhydrous sodium sulfate and into a 250 mL round-bottom flask.
8. Slowly evaporate the collected organic layer in a rotary evaporator set at 40 °C. The residue is the extracted fat.
9. Use a disposable glass pipette to transfer the extracted fat to a 10–20 mL glass test tube equipped with a Teflon-lined screwcap. Flush the test tube with nitrogen and place the cap on the tube.
10. Methylate the extracted fat (approximately 200 mg) immediately using 7% BF_3-MeOH (see section 7.1).

Note that the fat extracted through this acid hydrolysis procedure very often contains some free fatty acids. Free fatty acids can only be methylated using BF_3-MeOH. KOH-MeOH is not recommended for methylating fats containing more than 2% free fatty acids.

6.3.2 Solvent extraction procedure

This procedure has been adopted from the method presented at the WHO/EURO joint workshop on measurement of *trans*-fatty acids, held in Turkmenistan in November 2019 *(4)*.

1. Transfer the homogenized representative sample to an Erlenmeyer flask (use approximately 20 g of sample with a high fat content, such as fries, chips, pastries and cookies; or 80 g of sample with a low fat content, such as bread, buns and soups).
2. Add 50 mL petroleum ether, 50 mL diethyl ether and 10 mL n-heptane (50 : 50 : 10, v/v/v) to the Erlenmeyer flask. Shake or stir for 30 min, then allow the solid materials to precipitate. If a clean precipitation is not achieved, slowly decant the upper layer and centrifuge it for 5 min at 500 rpm.
3. Filter the upper layer though a filter paper (Whatman No. 42) filled with anhydrous sodium sulfate into a round-bottom flask that is compatible with the rotary evaporator.
4. Evaporate to dryness in a rotary evaporator set at 40 °C and approximately 100 mbar. Use a disposable glass pipette to transfer the residue into a clean 50 mL glass test tube equipped with Teflon-lined screwcap. The residue contains the extracted fat. Flush the test tube with nitrogen and place the cap on the tube.
5. Place approximately 200 mg of the extracted fat in a 15 mL test tube equipped with a Teflon-lined screwcap. Methylate the fat immediately using either 2 M KOH-MeOH or 7% BF_3-MeOH (see sections 7.1 and 7.2).
6. If the laboratory is not ready for methylation, flush the tube with nitrogen (to prevent or minimize oxidation of polyunsaturated fatty acids), cap the tube and store in a refrigerator (2–8 °C). However, methylation should be done within 1–2 days of fat extraction, as prolonged storage can lead to oxidation of polyunsaturated fatty acids.

6.4 Category 5: dairy products

The following procedures for dairy products were adopted from AOAC Official Method 996.06 *(3)*, with some modifications.

6.4.1 Dairy products not including cheese

This base hydrolysis procedure was adopted from AOAC Official Method 996.06 *(3)*, with some minor modifications.

1. Make a conservative estimate of the weight of the food sample that would yield approximately 200 mg fat. Take a subsample equivalent to the estimated weight from the homogenized composite (see section 5) and place in a glass test tube (50 mL capacity with a Teflon-lined screwcap). Add approximately 100 mg pyrogallic acid and 2 mL ethanol and mix well until the entire test portion is dispersed.
2. Add 4 mL water and mix well. Add 2 mL 58% ammonium hydroxide and mix well.
3. Place the cap on the tube and heat the test tube in an electric heating block or a water bath (preferably a shaking water bath set at moderate agitation) for 10 min at 70–80 °C. Every 5 min, mix the contents of the test tube on a vortex mixer to incorporate into solution any particles adhering to the walls of the test tube.

4. After digestion, remove the test tube from the heating block or water bath and add a few drops of phenolphthalein. Keep the solution basic (pink) with addition of 58% ammonium hydroxide.
5. Add 5 mL ethanol to the tube and mix gently.
6. Add 25 mL diethyl ether. Place the cap on the test tube and vortex for 5 min.
7. Add 25 mL petroleum ether and vortex for 5 min.
8. Transfer the contents to a 25 mL separatory funnel equipped with a glass stopper. Place the glass stopper on the funnel and allow the contents to settle for at least 1 h, until the upper layer is clear.
9. Drain out the bottom layer and discard it. Slowly decant the top layer (organic layer) through a glass funnel lined with a filter paper filled with approximately 1 g anhydrous sodium sulfate and into a 250 mL round-bottom flask.
10. Slowly evaporate the collected organic layer in a rotary evaporator set at 40 °C. The residue is the extracted fat.
11. Use a disposable glass pipette to transfer the extracted fat to a 10–20 mL glass test tube equipped with a Teflon-lined screwcap. Flush the test tube with nitrogen and place the cap on the tube.
12. Methylate the extracted fat (approximately 200 mg) immediately using 7% BF_3-MeOH (see section 7.1).

Note that the fat extracted through this base hydrolysis procedure very often contains some free fatty acids. Free fatty acids can only be methylated using BF_3-MeOH. KOH-MeOH is not recommended for methylating fats containing more than 2% free fatty acids.

6.4.2 Cheese

This base–acid hydrolysis procedure was adopted from AOAC Official Method 996.06 *(3)*, with some minor modifications.

1. Make a conservative estimate of the weight of the food sample that would yield approximately 200 mg fat. Take a subsample equivalent to the estimated weight from the homogenized composite (see section 5) and place in a glass test tube (50 mL capacity with a Teflon-lined screwcap). Add approximately 100 mg pyrogallic acid and 2 mL ethanol and mix well until the entire test portion is dispersed.
2. Add 4 mL water and mix well. Add 2 mL 58% ammonium hydroxide and mix well.
3. Place the cap on the tube and heat the test tube in an electric heating block or a water bath (preferably a shaking water bath set at moderate agitation) for 20 min at 70–80 °C. Every 5 min, mix the contents of the test tube on a vortex mixer to incorporate into solution any particles adhering to the walls of the test tube.
4. Add 10 mL of 12 M HCl and place the test tube into a boiling steam bath or a heating block maintained at 100 °C. Heat for 20 min. Mix tube contents every 10 min on a vortex mixer.
5. After digestion, remove the test tube from the boiling steam bath or heating block and allow to cool to room temperature (20–25 °C).

6. Add 5 mL ethanol to the tube and mix gently.
7. Add 25 mL diethyl ether. Place the cap on the test tube and vortex for 5 min.
8. Add 25 mL petroleum ether and vortex for 5 min.
9. Transfer the contents to a 25 mL separatory funnel equipped with a glass stopper. Place the glass stopper on the funnel and allow the contents to settle for at least 1 h, until the upper layer is clear.
10. Drain out the bottom layer and discard it. Slowly decant the top layer (organic layer) through a glass funnel lined with a filter paper filled with approximately 1 g anhydrous sodium sulfate and into a 250 mL round-bottom flask.
11. Slowly evaporate the collected organic layer in a rotary evaporator set at 40 °C. The residue is the extracted fat.
12. Use a disposable glass pipette to transfer the extracted fat to a 10–20 mL glass test tube equipped with a Teflon-lined screwcap. Flush the test tube with nitrogen and place the cap on the tube.
13. Methylate the extracted fat (approximately 200 mg) immediately using 7% BF_3-MeOH (see section 7.1).

Note that the fat extracted through this base-acid hydrolysis procedure very often contains some free fatty acids. Free fatty acids can only be methylated using BF_3-MeOH. KOH-MeOH is not recommended for methylating fats containing more than 2% free fatty acids.

7. Methylation of fat

Methylation should be performed using either 7% BF_3-MeOH (see section 7.1) or 2 M KOH-MeOH (see section 7.2).

It should be noted that there are differences in the methylation ability between these two reagents. The 7% BF_3-MeOH reagent is universal, meaning that it can methylate all types of lipids, including triacylglycerols, diacylglycerols and monoacylglycerols; phospholipids; glycolipids; and free fatty acids. However, this reagent is mildly toxic, and some countries have banned the use of BF_3-MeOH. Although 2 M KOH-MeOH is safe, it is not a universal reagent. It can methylate triacylglycerols, diacylglycerols and monoacylglycerols, but it cannot methylate polar lipids such as phospholipids, glycolipids and free fatty acids. Therefore, 2 M KOH-MeOH is not suitable for methylating fats containing these polar lipids, nor anything greater than 2% free fatty acids.

7.1 Methylation using 7% BF_3-MeOH

This procedure was adopted from AOAC Official Method 996.06, revised 2001 *(3)*.

1. Place approximately 200 mg oil (from section 6.1) or extracted fat (as per sections 6.2, 6.3 and 6.4) into a 15 mL test tube. Add 2 mL of 7% BF_3-MeOH reagent and 1 mL toluene to the test tube. Close the tube with a screwcap lined with a Teflon or silicone septum.
2. Heat the test tube in an oven or heating block for 45 min. Gently shake the tube every 10–12 min. (Note: solvent evaporating from the tubes indicates inadequate seals. If this happens, discard the solution and repeat the entire methylation procedure using a new set of glass tubes with screwcaps lined with a Teflon or silicone septum.)
3. After 45 min, remove the tube from the oven or heating block and allow to cool to room temperature (20–25 °C). Add 5 mL distilled water, 1 mL petroleum ether and approximately 1 g anhydrous sodium sulfate. Cap the tube, shake for 1 min and allow the layers to separate for about 10 min. Use a disposable glass pipette to transfer the top layer to a clean and dry 15 mL glass tube containing approximately 1 g anhydrous sodium sulfate. When transferring, make sure not to include any of the aqueous layer. The top layer contains the prepared FAMEs.
4. Add enough petroleum ether (approximately 8 mL) to the FAME solution to make the total volume to 10 mL. The concentration of this FAME solution is approximately 15–20 mg/mL petroleum ether.
5. Flush the tube with nitrogen and cap the tube.

6. Immediately analyse 1 µL of the petroleum ether FAME solution on the GC, or transfer to an autosampler vial for GC analysis (as per section 8).

7.2 Methylation using 2 M KOH-MeOH

WARNING: Do not use 2 M KOH-MeOH to methylate fat extracted as per section 6.3.1, 6.4.1 or 6.4.2 (i.e. using the acid, base or base–acid hydrolysis procedure). Fat extracted using acid, base or base–acid hydrolysis very often contains large amounts of free fatty acids

1. Place approximately 200 mg fat/oil sample (from section 1) or extracted fat (as per sections 6.2, 6.3.3 and 6.4) into a test tube (capacity 10 or 15 mL). Add 5 mL *n*-heptane and 0.5 mL 2 M KOH-MeOH reagent to the test tube.
2. Manually shake the tube vigorously for 2–3 minutes. This converts the extracted fat into FAMEs. Allow layers to separate for 10–30 min. The top layer contains the FAMEs. The tube can also be centrifuged for 5 min at 500 × *g* to reduce waiting time.
3. Use a disposable glass pipette to transfer the upper layer to another test tube (capacity 10 or 15 mL) containing 1 g of anhydrous sodium sulfate. Allow the layers to separate, or centrifuge for 5 mins at 500 × *g*. Transfer the top solvent layer to a GC vial or glass test tube (capacity 10 or 15 mL). If the separation is still not clear, filter the solution through a syringe filter paper filled with anhydrous sodium sulfate (e.g. syringe polytetrafluoroethylene filter with sodium sulfate).
4. Add enough n-heptane (approximately 5 mL) to the top solvent layer (FAME solution) to make the total volume to 10 mL. The concentration of this FAME solution is approximately 15–20 mg/mL heptane.
5. Immediately analyse 1 µL of the n-heptane FAME solution on the GC, or transfer to an autosampler vial for GC analysis (as per section 8).

8. Gas chromatography analysis of FAMEs

8.1 GC operating conditions

FAME samples can be analysed by operating the column oven temperature either isothermally (see section 8.1.1) or using temperature programming (see section 8.1.2). The choice depends on the source of the FAME sample.

The oven temperature and the carrier gas flow rate depend on the column selected and the carrier gas used (i.e. hydrogen or helium). Therefore, laboratories need to fine tune the conditions depending on their settings. In any case, the selected conditions should produce the separation between *cis* and *trans* isomers of C18:1, C18:2 and C18:3 shown in Fig. 1–12.

For FAME samples originating from food samples containing no ruminant fat, isothermal operation at 180 °C with a carrier gas flow rate of 1 mL/min, as outlined in the American Oil Chemists' Society (AOCS) Official Method Ce 1h-05 *(5)*, is recommended. This isothermal operation gives the optimum resolution of C18:1, C18:2 and C18:3 fatty acids (see Fig. 2–5 and Fig. 11). The disadvantage with the isothermal operation is that short-chain fatty acids of chain lengths C4, C6 and C8 methyl esters are not well resolved from the solvent front, which hinders accurate measurement of these short-chain fatty acids. However, this is not a hindrance for measurement of fatty acid composition of FAME samples originating from non-ruminant sources, because short-chain fatty acids are almost absent in fat from these sources (except coconut oil and palm kernel oil) (see Fig. 2–4).

Fat from milk and milk products contains approximately 3–4% C4:0. As outlined in International Standard ISO 16958:2015 *(6)*, it is recommended that analysis of FAME samples originating from milk and milk products be done using a temperature programme operation, starting from a low temperature and increasing stepwise to higher temperature. The temperature programme operation gives a good separation of C4:0 from the solvent front, which allows accurate measurement of the fatty acid composition of milk fats (see Fig. 10 and 12).

8.1.1 Isothermal operation

The following GC operating conditions have been adopted from AOCS Official Method Ce 1h-05, revised 2017 *(5)*.

Set up the GC operating conditions as follows:

Carrier gas (hydrogen or helium)	Hydrogen: constant flow rate 1.0 mL/min; linear velocity 26 cm/s; split ratio 100 : 1. A constant flow rate of 1.0 mL/min can be achieved using a column head pressure of 169.6 kPa (24.6 psi) Helium: constant flow rate 1.0 mL/min; linear velocity 19.29 cm/s; split ratio 100 : 1. A constant flow rate can be achieved using a column head pressure of 285.85 kPa (41.42 psi)
Injector port temperature	250 °C
Detector temperature	250 °C
Oven temperature	180 °C
GC injection volume	Inject 1 µL FAME solution (concentration approximately 20 µg/µL heptane or petroleum ether) using the GC micro syringe. The FAME solution concentration of 20 µg/µL is the ideal concentration for optimum resolution of C18 TFA isomers from the *cis* isomers. If the autosampler is used, make sure to not keep the FAME samples on the tray for longer than 72 h.

Examples of GC chromatograms obtained with the above isothermal conditions are shown in Fig. 1–5. As shown in these figures, and in Fig. 11, these GC conditions should produce the best possible separation between *cis* and *trans* isomers of C18:1, C18:2 and C18:3.

8.1.2 Temperature programme operation

The following GC operating conditions have been adopted from ISO 16958:2015 *(6)*.

Set up the GC operating conditions as follows:

Carrier gas (hydrogen or helium)	Constant flow rate 1.0 mL/min; split ratio 100 : 1
Injector port temperature	250 °C
Detector temperature	275 °C
Oven temperature programme	Initial temperature 60 °C, hold 5 min; ramp at 15 °C/min to 165 °C, hold 1 min; ramp at 2 °C/min to 225 °C, hold 20 min
GC injection volume	Inject 1 µL FAME solution (concentration approximately 20 µg/µL heptane or petroleum ether) using the GC micro syringe. The FAME solution concentration of 20 µg/µL is the ideal concentration for optimum resolution of C18 TFA isomers from the *cis* isomers. If the autosampler is used, make sure to not keep the FAME samples on the tray for longer than 72 h.

See Fig. 5–10 and Fig. 12 for GC chromatograms obtained with the above temperature programme conditions. It should be noted that with temperature programming, 9t,12c,15c-C18:3 is eluting with 11c-C20:1, which affects quantitation of refined fats and oils (see Fig. 7, 9 and 12).

Note: some of the settings described above, such as head pressure, oven temperature and split ratio, could be instrument specific. Small changes to these are allowed if the resolution of FAME peaks shown in Fig. 1–12 is achieved and the elution order of the FAMEs is not affected. In addition, pay attention to the resolution between the 13t+14t-C18:1 and 9c-C18:1 FAME peaks – for PHO and dairy fat samples, these peaks should be separated with a R (resolution) factor greater than 1, as shown in Fig. 3 and Fig. 5.

8.2 Identification of GC FAME peaks

FAME reference standards are not required to determine TFA content as a percentage of total fatty acids of food products. Reference standards can support identification of GC FAME peaks (as described below), which can be also achieved by comparing the GC elution pattern and/or retention times of FAME peaks in the test sample with those of the FAME peaks in the GC reference chromatograms shown in Fig. 1–10. Comparing the elution pattern or retention times with the GC chromatograms published in the original WHO reference protocol *(1)* is also useful for confirming peak identifications. This approach is a reliable and inexpensive way of identifying GC FAME peaks. It has been used in most laboratories over the past several decades and allows them to conduct FAME analysis even when FAME reference standards cannot be procured.

If commercial reference standards are available in the laboratory, FAME peaks can be identified by analysing the reference standards using the above-mentioned GC conditions and comparing their retention times with the GC FAME peaks of the test sample. Individual and mixtures of reference standards are available from various sources (e.g. Sigma-Aldrich; MilliporeSigma; Supelco, Inc; Matreya, LLC). The following reference mixtures are especially useful to have in the laboratory:

- Supelco 37 Component FAME Mix, available from Supelco, Inc (catalogue number CRM47885); GC chromatograms of this FAME mix are provided in Fig. 1 and Fig. 6
- Linoleic acid methyl ester mix, available from MilliporeSigma (catalogue number CRM47791)
- Linolenic acid methyl ester mix, available from MilliporeSigma (catalogue number L6031).

In addition to these reference mixtures, a *cis-trans* FAME isomer mixture is available from Matreya LLC (catalogue number 1131) and Sigma-Aldrich (catalogue number 40495-U). They contain most of the *cis-trans* isomers present in partially hydrogenated oils. The isomers include 6t-C18:1, 8t-C18:1, 9t-C18:1, 10t-C18:1, 11t-C18:1, 12t-C18:1, 13t-C18:1, 14t-C18:1, 15t-C18:1, 6c-C18:1, 8c-C18:1, 9c-C18:1, 10c-C18:1, 11c-C18:1 and 12c-C18:1.

For GC analysis, prepare individual and mixed reference FAME solutions (in an organic solvent such as iso-octane or petroleum ether)[1] of approximate concentrations 1–2 mg/mL and 10 mg/mL, respectively.

[1] Hexane could be used, but it is mildly toxic. Replace hexane with the other listed solvents whenever possible.

8.2.1 Background information on TFA isomers and their FAME GC elution pattern on 100 m BCS capillary columns

The following background information is provided for laboratory analysts and their supervisors to support correct identification of the C18 TFA FAME isomer peaks.

Both in PHOs and ruminant fats, TFAs are almost exclusively of C18 chain length, with no detectable levels of TFAs from other chain lengths (see Fig. 3). 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 *trans* isomers of C14:1, C16:1 and C17:1 are not commercially available, which hinders identification of these minor TFAs. For these reasons, analysis of dietary fats should focus only on the C18 TFAs.

8.2.2 C18:1 TFA isomer GC peaks

In PHOs and dairy fats, the *trans*-oleic acid (C18:1 TFA) group represents the major fraction within *trans*-isomeric fatty acids. In PHOs, C18:1 TFA accounts for approximately 90–95% of total TFA. Some PHOs can contain up to 13 *trans*-C18:1 isomers, whose double bond position ranges from 4 to 16 (double bond counted from the carboxylic carbon). Out of these 13 isomers, 12 isomers, namely 4t-C18:1 to 14t-C18:1 (note: 14t elutes with 13t isomer) and 16t-C18:1, can be readily identified, as shown in Fig. 2–12. On the 100 m BCS capillary columns, the *trans* isomers from 4t-C18:1 to 14t-C18:1 are readily separated from the majority of *cis*-C18:1 isomers, and this cluster of *trans* isomers accounts for 95% or more of the total C18:1 TFA (see Fig. 11 and Fig. 12). Furthermore, the 16t-C18:1 isomer, often present in PHOs as a minor component, is also well separated from the *cis*-C18:1 isomers – it elutes after 13c-C18:1 and before 14c-C18:1 (Fig. 2–5 and Fig. 11). As shown in all the figures, adding up the cluster of isomers from 4t to 14t with 16t-C18:1 gives the total C18:1 TFA.

Out of the 13 possible C18:1 TFA isomers, only 15t-C18:1 is not quantifiable because it co-elutes with 10c-C18:1. Therefore, the contribution of 15t-C18:1 to the total C18:1 TFA is impossible to measure. Excluding the contribution of 15t-C18:1 to the total C18:1 TFA is not going to present a significant error because 15t-C18:1 is often a minor component (<1% of total C18:1 TFA) in PHOs. For practical purposes, 15t-C18:1 is included in the *cis*-C18:1 total.

Three *cis*-18:1 isomers (6c-C18:1, 7c-C18:1 and 8c-C18:1) elute in the *trans*-C18:1 region (Fig. 2–12). Consequently, these isomers are added indirectly to the sum of C18:1 TFA. However, these three *cis*-C18:1 isomers are also minor components, and their contribution to the total C18:1 TFA compensates the fact that 15t-C18:1 is not taken into account.

8.2.3 C18:2 TFA isomer GC peaks

PHOs often contain an assortment of *trans* isomers of linoleic acid (C18:2 TFA). The levels of these isomers could reach up to 6% (of total fatty acids) in mildly hydrogenated oils, whereas they are hardly present in heavily hydrogenated oils (<0.1% of total fatty acids). Mildly hydrogenated oils known to contain at least nine C18:2 isomers, with five of these characterized by researchers as 9c,13t-C18:2; 9c,12t-C18:2; 9t,12c-C18:2; and 9t,15c-C18:2 + 10t,15c-C18:2 (these two elute together). These isomers elute in that

order on the 100 m SP-2560 and CP-Sil columns (when operated isothermally at 180 °C), without any overlaps with *cis*-C18:2 isomers (see Fig. 2–4 and Fig. 11). These five isomers can be readily identified from their elution pattern, as shown in Fig. 2–4 and Fig. 11. For the total C18:2 TFA, include all five isomers.

Refined, deodorized (but not hydrogenated) oils, and cooking oils exposed to heat (e.g. deep fat frying), also contain small amounts (approximately 0.1–0.3% of total fatty acids) of C18:2 TFA. These are the geometrical isomers of linoleic acid (9t,12t-C18:2; 9c,12t-C18:2; 9t,12c-C18:2). Therefore, for deodorized and heated cooking oils, the total C18:2 TFA is the sum of these three geometrical isomers of linoleic acid (see Fig. 2 and Fig. 11).

8.2.4 C18:3 TFA isomer GC peaks

TFA isomers of α-linolenic acid (18:3n-3 or 9c,12c,15c-C18:3) are not found in partially hydrogenated oils. This is because during partial hydrogenation, 18:3n-3 is immediately converted to C18:2 isomers, then to C18:1 isomers, and eventually to stearic acid (C18:0). However, refined, deodorized oils containing 18:3n-3 (such as canola, soybean) can contain 0.5–2.5% (of total fatty acids) *trans*-geometrical isomers of 18:3n-3 (9t,12c,15t-C18:3; 9c,12c,15t-C18:3; 9c,12t,15c-C18:3; 9t,12c,15c-C18:3). Of these four isomers, the major ones are 9c,12c,15t-C18:3 and 9t,12c,15c-C18:3 (see Fig. 2 and Fig. 11). On the SP-2560 columns, as shown in Fig. 2 and Fig. 11, these four isomers are well separated from each other and elute after C20:0 and before 11c-C20:1 (gadoleic acid). The sum of these four isomers gives the total C18:3 TFA.

GC operating conditions influence the separation between 9t,12c,15c-C18:3 and 11c-C20:1. Good resolution of these two FAMEs is obtained when the column (especially with the 100 m SP-2560 column) is operated isothermally at 180 °C. However, the column operating temperature may need to be adjusted by a few degrees above or below 180 °C to obtain the optimum resolution of these two FAMEs. These FAMEs tend to co-elute if the column is operated at temperatures significantly different from 180 °C, so special attention should be paid to this separation when changing GC operating parameters. Ageing columns may also influence the resolution between 9t,12c,15c-C18:3 and 11c-C20:1.

8.3 Criteria for deciding the acceptability of GC chromatograms

After each GC run, the FAME chromatograms of the test food sample should be examined to ensure that the GC operating parameters are correct. The FAMEs profile should be identical to that shown in Fig. 1–5 for isothermal analysis at 180 °C, and Fig. 6–10 for temperature-programmed analysis. In addition, ensure that the separation of the following critical FAMEs is achieved, for both isothermal and temperature-programmed analyses:

- baseline separation between 9c-C18:1 and 11c-C18:1 (as shown in Fig. 2–5 and Fig. 6–10);
- separation of the 4t-C18:1 to 12t-C18:1 *trans* isomers from all the *cis*-C18:1 isomers (as shown in Fig. 2–5 and Fig. 7–10);
- partial separation between (13t+14t)-C18:1 and 9c-C18:1 (as shown in Fig. 2–5 and Fig. 7–10) (note that 13t-C18:1 and 14t-C18:1 always elute together and that the

baseline separation between (13t+14t)-C18:1 and 9c-C18:1 is impossible under any GC conditions. However, a good partial separation is sufficient for peak area measurements for (13t+14t)-C18:1 and 9c-C18:1); and

- near-baseline separation between 16t-C18:1 and 13c-C18:1, and partial separation from 14c-C18:1 (as shown in Fig. 3, 5 and 10).

Furthermore, when performing the GC analysis using 180 °C isothermal conditions, ensure that there is baseline separation between 9t,12c,15c-C18:3; 11c-C20:1 (gadoleic acid); and 9c,12c,15c-C18:3 (α-linolenic acid). Note that 11c-C20:1 elutes between 9t,12c,15c-C18:3 acid and α-linolenic acid, as shown in Fig. 2c, 3c and 4c.

Any GC runs showing poor resolution of the above critical FAMEs are not acceptable. If the resolutions are not ideal, perform the following until the desired separations are achieved:

- adjust the column oven temperature in small increments
- adjust the volume/concentration of the injected FAME sample.

If the peaks are too small and below the limit of quantitation, increase either the volume of the sample injected (from 1 to 2 µL) or the concentration of the sample (by evaporating some of the solvent using a slow stream of nitrogen). Consider also changing the split ratio if needed.

If the column oven temperature needs to be adjusted, this should be done in small increments, preferably by 1 °C. This is because, on SP-2560, CP-Sil 88 and other BCS capillary columns, the column temperature has a profound effect on the resolution between the following: (13t+14t)-C18:1 and 9c-C18:1; 16t-C18:1 and 14c-C18:1; and 9t,12c,15c-C18:3, 11c-C18:1 and 9c,12c,15c-C18:3.

With time, the performance of the GC column gradually degrades. A newly installed column with daily operation (24 hours a day, 7 days a week) is generally good for 6 months; after that, it typically loses its ability to resolve the critical FAMEs. If poor resolutions are noticed, install a new column. Test the performance of the new column with a PHO sample or, if available, FAME reference standards (see section 8.2 for examples of products).

9. Calculations of fatty acid composition and total TFAs

Calculation of the fatty acid composition should be performed using a spreadsheet (see Annex). In the shaded cells of the spreadsheet, insert the sample ID, date that the sample was analysed by GC, and GC peak area counts of all identified FAMEs in the test food sample. The spreadsheet automatically calculates the fatty acid composition. It also calculates the total C18:1 TFAs, C18:2 TFAs, C18:3 TFAs, total TFAs, total saturated fatty acids (SFAs) and total n-6 and n-3 *cis*-polyunsaturated fatty acids (PUFAs).

In this protocol, quantitation of individual fatty acids is performed 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. Area percentages are a good approximation of weight percentages, particularly for plant oils and fats. Please note that the weights obtained from this spreadsheet are *apparent* weights, not absolute weights.

The spreadsheet uses the following formulae for calculating the apparent weight of fatty acid *i* in grams (Wi) and their weight percentage of total fatty acids (Weight % W_i):

$$W_i\ (g) = (A_i \times R_i \times F_{Fai})$$

$$\text{Weight \% } W_i = [(A_i \times R_i \times F_{Fai}) \times 100]/\text{Total } W_i$$

where

A_i = GC peak area counts of fatty acid *i* in the test portion.

R_i = theoretical FID correction factor for fatty acid *i*.

F_{Fai} = conversion factor for converting the peak area of FAME $_i$ to its corresponding fatty acid form/fatty acid equivalent (see column E of the spreadsheet for the conversion factors of all individual FAMEs)

Total W_i = sum of apparent weight, in grams, of all individual fatty acids (note: when calculating the sum, include only the identified fatty acids; GC peaks of unknown identity should not be included in the summation when quantifying W_i).

Note that the area percentage from the FID response is only a close approximation of the weight percentage of fatty acids *(7,8)*. To convert to true weight percentages, appropriate response factors must be used. The FID response is dependent on the content of the carbon that hydrogen is bonded to. This means that short-chain fatty acids have lower FID responses than medium-chain and long-chain fatty acids, and they require a greater FID response correction factor. Column D of the spreadsheet includes the theoretical flame ionization correction factors. Correction factors are relative to

C18:0, which has a correction factor of 1.0000. Correction factors for the following FAMEs have been verified experimentally *(7)*: C4:0; C6:0; C8:0; C10:0; C12:0; C14:0; C16:0; C17:0; C20:0; 9c-C18:1; 9c,12c-C18:2; 9c,12c,15c-C18:3; 5c,8c,11c,14c-C20:4; and 4c,7c,10c,13c,16c,19c-22:6. One factor is given for all positional and geometrical isomers, and for branch-chain FAMEs.

The calculated apparent weight (in grams) and the weight % of all the individual fatty acids are automatically displayed in columns G and H, respectively, of the spreadsheet (Annex).

9.1 Calculation of total TFA as a percentage of total fatty acids

The spreadsheet calculates the % total TFA (as wt % of total fatty acids, i.e. apparent g per 100 g of total fatty acids, displayed in Column H of the spreadsheet) using the following formula:

% Total TFA = % C18:1 TFA + % C18:2 TFA + % C18:3 TFA

where

% C18:1 TFA, % C18:2 TFA and % C18:3 TFA are the sum of *trans* isomers of C18:1, C18:2 and C18:3, respectively (see sections 8.2.2 to 8.2.4 for a description of all C18 *trans* isomers).

9.2 Limit of quantitation

In general, GC-FID can detect fatty acids (as FAMEs) as low as 0.05 g per 100 g of total fatty acids (i.e. 0.05%). However, the chromatography operating conditions, such as column oven temperature, carrier gas flow rate, sample injection volume and concentration, and the GC operating data system may have to be adjusted to detect and record FAME peak areas corresponding to 0.05 or 0.1 g/100 g total fatty acids.

10. Test report

The test report should include at least the following:

- information needed for the complete identification of the test sample;
- the sampling method used;
- all operating details, together with details of any deviations from the WHO simplified protocol;
- the test results obtained for total TFA, C18:1 TFA, C18:2 TFA and C18:3 TFA (express the results in % total fatty acids to two decimal places); and
- the test results obtained for other individual and groups of fatty acids as appropriate to conform with requirements of each jurisdiction. In general, information on fatty acids other than TFAs is useful in assessing the overall nutritional quality of the test sample. Express the results in a format similar to that shown in Table 1.

11. References

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12. Annex

FAME calculation spreadsheet for the WHO simplified protocol

Sample ID	
Date sample analyzed by GC	

Note

- Please download the Excel sheet available at https://cdn.who.int/media/docs/default-source/nutritionlibrary/replace-transfat/fattyacid-calculation-spreadsheet-who-simplified-protocol.xlsx?sfvrsn=8b9c6f9d_3.
- Shaded cells are for input by the user in Excel. The remaining cells will be calculated automatically.
- The weights (W_{FAMEi} (g), W_i (g), g fatty acid i per 100 g total fatty acids) obtained from this Excel sheet are apparent weights, and not absolute.

FAME	**A*i***	Theoretical (Relative) FID Correction Factors (TCF) **(R_i)**	Apparent **W_{FAMEi} (g)**	Conversion factor of FAME to fatty acid equiv. **(F_{FAi})**	Apparent g fatty acid *i* **W_i (g)**	**wt % of total fatty acids** (Apparent g fatty acid *i* per 100 g total fatty acids)
C4:0		1.5396		0.8627		
C6:0		1.3084		0.8923		
C8:0		1.1927		0.9114		
C10:0		1.1233		0.9247		
C12:0		1.0771		0.9346		
Iso-C12:0		1.0771		0.9346		
C13:0		1.0593		0.9386		
Iso-C13:0		1.0593		0.9386		
Anteiso-C13:0		1.0593		0.9386		
C14:0		1.0440		0.9421		
Iso-C14:0		1.0440		0.9421		
C15:0		1.0308		0.9453		
Iso-C15:0		1.0308		0.9453		
Anteiso-C15:0		1.0308		0.9453		
C16:0		1.0193		0.9481		
Iso-C16:0		1.0193		0.9481		

FAME	**A*i***	Theoretical (Relative) FID Correction Factors (TCF) **(R_i)**	Apparent **W_{FAMEi} (g)**	Conversion factor of FAME to fatty acid equiv. **(F_{FAi})**	Apparent g fatty acid *i* **W_i (g)**	**wt % of total fatty acids** (Apparent g fatty acid *i* per 100 g total fatty acids)
C17:0		1.0091		0.9507		
Iso-C17:0		1.0091		0.9507		
Anteiso-C17:0		1.0091		0.9507		
C18:0		1.0000		0.9530		
C20:0		0.9846		0.9570		
C22:0		0.9720		0.9604		
C24:0		0.9614		0.9633		
Total SFA						
9t-C14:1		1.0354		0.9417		
9t-C16:1		1.0117		0.9477		
11t-C16:1		1.0117		0.9477		
9t-C17:1		1.0019		0.9503		
4t-C18:1		0.9932		0.9527		
5t-C18:1		0.9932		0.9527		
(6t-14t)-C18:1		0.9932		0.9527		
16t-C18:1		0.9932		0.9527		
Total 18:1 trans						
Total t-MUFA						
9t,12t-C18:2		0.9865		0.9524		
9c,12t-C18:2		0.9865		0.9524		
9t,12c-C18:2		0.9865		0.9524		
sum of other trans-C18:2 isomers[a]		0.9865		0.9524		
Total 18:2 trans						
9t,12c,15t-C18:3		0.9797		0.9520		
9c,12c,15t-C18:3		0.9797		0.9520		
9c,12t,15c-C18:3		0.9797		0.9520		
9t,12c,15c-C18:3		0.9797		0.9520		
Total 18:3 trans						
Total trans						
9c-C14:1		1.0354		0.9417		
9c-C15:1		1.0027		0.9449		
7c-C16:1		1.0117		0.9477		
9c-C16:1		1.0117		0.9477		
9c-C17:1		1.0017		0.9503		
7c-C18:1		0.9932		0.9527		
9c-C18:1		0.9932		0.9527		
10c-C18:1		0.9932		0.9527		
11c-C18:1		0.9932		0.9527		

[a] Other trans C18:2 includes several unidentfied isomers present in the trans C18:2 region. This group often includes 9c,13t-C18:2, 9t,15c-C18:2 and 10t,15c-C18:2. Lack of authentic FAME standards precludes confirmation of their identity.

FAME	**A*i***	Theoretical (Relative) FID Correction Factors (TCF) **(R_i)**	Apparent **W_{FAMEi} (g)**	Conversion factor of FAME to fatty acid equiv. **(F_{FAi})**	Apparent g fatty acid *i* **W_i (g)**	**wt % of total fatty acids** (Apparent g fatty acid *i* per 100 g total fatty acids)
12c-C18:1		0.9932		0.9527		
13c-C18:1		0.9932		0.9527		
14c-C18:1		0.9932		0.9527		
15c-C18:1		0.9932		0.9527		
16c-C18:1		0.9932		0.9527		
Total 18:1 cis						
11c-C20:1		0.9785		0.9568		
9c-C20:1		0.9785		0.9568		
13c-C22:1		0.9664		0.9602		
15c-C24:1		0.9564		0.9632		
Total MUFA						
C18:2n-6		0.9865		0.9524		
C18:3n-6		0.9797		0.9520		
C20:2n-6		0.9724		0.9565		
C20:3n-6		0.9663		0.9562		
C20:4n-6		0.9603		0.9560		
C22:2n-6		0.9609		0.9600		
C22:4n-6		0.9499		0.9595		
C22:5n-6		0.9443		0.9593		
Total n-6 LC-PUFA						
C18:3n-3		0.9797		0.9520		
C18:4n-3		0.9730		0.9517		
C20:3n-3		0.9663		0.9562		
C20:4n-3		0.9603		0.9560		
C20:5n-3		0.9452		0.9557		
C22:3n-3		0.9554		0.9538		
C22:5n-3		0.9443		0.9593		
C22:4n-3		0.9499		0.9595		
C22:6n-3		0.9388		0.9590		
Total n-3 LC-PUFA						
Total cis-PUFA						
Total W_{FAMEi}						
Total W_i						

13. Tables and figures

Table 1. Fatty acids for reporting fatty acid data for food samples[a,b]

IUPAC name (trivial name)	Shorthand notation (n minus notation)
Saturated fatty acids (SFAs)	
butanoic acid (butyric)	C4:0
hexanoic acid (caproic)	C6:0
octanoic acid (caprylic)	C8:0
decanoic acid (capric)	C10:0
dodecanoic acid (lauric)	C12:0
tridecanoic acid	C13:0
iso-tetradecanoic acid	I-C14:0
tetradecanoic acid (myristic)	C14:0
pentadecanoic acid	C15:0
iso-hexadecanoic acid (iso-palmitic)	I-C16:0
hexadecanoic acid (palmitic)	C16:0
iso-heptadecanoic acid	I-C17:0
anteiso-hetadecanoic acid	AI-C17:0
heptadecanoic acid	C17:0
octadecanoic acid (stearic)	C18:0
nonadecanoic acid	C19:0
eicosanoic acid (arachidic)	C20:0
heneicosanoic acid	C21:0
docosanoic acid (behenic)	C22:0
tricosanoic acid	C23:0
tetracosanoic acid (lignoceric)	C24:0
∑SFA (sum of all the above SFAs)	

IUPAC name (trivial name)	Shorthand notation (n minus notation)
***cis*-monounsaturated fatty acids (c-MUFAs)**	
5-*cis*-tetradecenoic acid	5c-C14:1
10-*cis*-pentadecenoic acid	10c-C15:1
7-*cis*-hexadecenoic acid	7c-C16:1
9-*cis*-hexadecenoic acid	9c-C16:1
9-*cis*-heptadecenoic acid	9c-C17:1
(9-*cis*+10-*cis*)-octadecenoic acid[c]	(9+10c)-C18:1
11-*cis*-octadecenoic acid (*cis* vaccenic)	11c-C18:1
12-*cis*-octadecenoic acid	12c-C18:1
13-*cis*-octadecenoic acid	13c-C18:1
14-*cis*-octadecenoic acid	14c-C18:1
15-*cis*-octadecenoic acid	15c-C18:1
16-*cis*-octadecenoic acid	16c-C18:1
11-*cis*-eicosenoic acid	11c-C20:1
13-*cis*-docosenoic acid (erucic)	13c-C22:1
15-*cis*-tetracosanoic acid (nervonic)	15c-C24:1
∑cis-MUFA (sum of all the above cis-MUFAs)	
n-6 polyunsaturated fatty acids (n-6 PUFAs)	
9-*cis*,12-*cis*-octadecadienoic acid (linoleic)	9c,12-C18:2 (18:2n-6)
6-*cis*,9-*cis*,12-*cis*-octadecatrienoic acid (α-linolenic)	6c,9c,12c-C18:3 (18:3n-6)
8-*cis*,11-*cis*,14-*cis*-eicosatrienoic acid	8c,11c,14c-C20:3 (20:3n-6)
5-*cis*,8-*cis*,11-*cis*,14-*cis*-eicosatetraenoic acid (arachidonic)	5c,8c,11c,14c-C20:4 (20:4n-6)
7-*cis*,10-*cis*,13-*cis*, 16-*cis*-docosatetraenoic acid	7c,10c,13c,16c-C22:4 (20:4n-6)
4-*cis*,7-*cis*,10-*cis*,13-*cis*,16-*cis*-docosapentanoic acid	4c,7c,10c,13c,16c-C22:5 (22:5n-6)
n-3 polyunsaturated fatty acids (n-3 PUFAs)	
9-*cis*,12-*cis*,15-*cis*-octadecatrienoic acid (α-linolenic)	9c,12c,15c-C18:3 (18:3n-3)
6-*cis*,9-*cis*,12-*cis*,15-*cis*-octadecatetraenoic acid	6c,9c,12c,15c-C18:4 (18:4n-3)
5-*cis*, 8-*cis*,11-*cis*,14-*cis*,17-*cis*-eicosapentaenoic acid (EPA)	5c,8c,11c,14c,17c-C20:5 (20:5n-3)
7-*cis*,10-*cis*,13-*cis*,16-*cis*,19-*cis*-docosapentaenoic acid (DPA)	7c,10c,13c,16c,19c-C22:5 (22:5n-3)
7-*cis*,10-*cis*,13-*cis*,16-*cis*,19-*cis*-docosahexaenoic acid (DHA)	4c,7c,10c,13c,16-c,9c-C22:6 (22:6n-3)
∑PUFA (sum of all the above n-6 and n-3 PUFAs)	

IUPAC name (trivial name)	Shorthand notation (n minus notation)
C18:1 TFA	
(4+5+6+7+8+9+10+11+12+13+14)-*trans*-octadecenoic acid[d]	(4t-14t)-C18:1
16-*trans*-octadecenoic acid	16t-C18:1
∑C18:1 TFA (sum of all the above C18:1 TFAs)	
C18:2 TFA	
trans,*trans*-octadecadienoic acid	tt-C18:2
9-*trans*,12-*trans*-octadecadienoic acid	9t,12t-C18:2
9-*cis*,13-*trans*-octadecadienoic acid	9c,13t-C18:2
9-*cis*,12-*trans*-octadecadienoic acid	9c,12t-C18:2
9-*trans*,12-*cis*-octadecadienoic acid	9t,12c-C18:2
(9-*trans*,15-*cis*- + 10-*trans*,15-*cis*-) octadecadienoic acid[e]	(9t,15c + 10t,15c)-C18:2
∑C18:2 TFA (sum of all the above C18:2 TFAs)	
C18:3 TFA	
9-*trans*,12-*cis*,15-*trans*-octadecatrienoic acid	9t,12c,15t-C18:3
9-*cis*,12-*cis*,15-*trans*-octadecatrienoic acid	9c,12c,15t-C18:3
9-*cis*,12-*trans*,15-*cis*-octadecatrienoic acid	9c,12t,15t-C18:3
9-*trans*,12-*cis*,15-*cis*-octadecatrienoic acid	9t,12c,15c-C18:3
∑C18:3 TFA (sum of all the above C18:3 TFAs)	
Total TFA (sum of C18:1 TFA, C18:2 TFA and C18:3 TFA isomers)	

[a] The fatty acids listed here are generally encountered in natural and prepared foods. Note that some foods may not contain all the listed fatty acids.
[b] Use the shorthand notation when reporting fatty acid data of food samples.
[c] The peak for 9c-C18:1 includes a small proportion of 10c-C18:1 and 15t-C18:1. Therefore, their peak areas are lumped (summed) together.
[d] *trans*-C18:1 isomers from 4t to 14t elute as a group, with no clear baseline separations (see Fig. 3c). Therefore, it is practical to lump (sum) their peak areas together and express as (4t-14t)-C18:1.
[e] 9t,15c-C18:2 and 10t,15c-C18:2 elute together. Therefore, their peak areas are lumped together.

Fig. 1. GC chromatogram of Supelco 37 Component FAME Mix on SP-2560 capillary column (100 m × 0.25 mm)

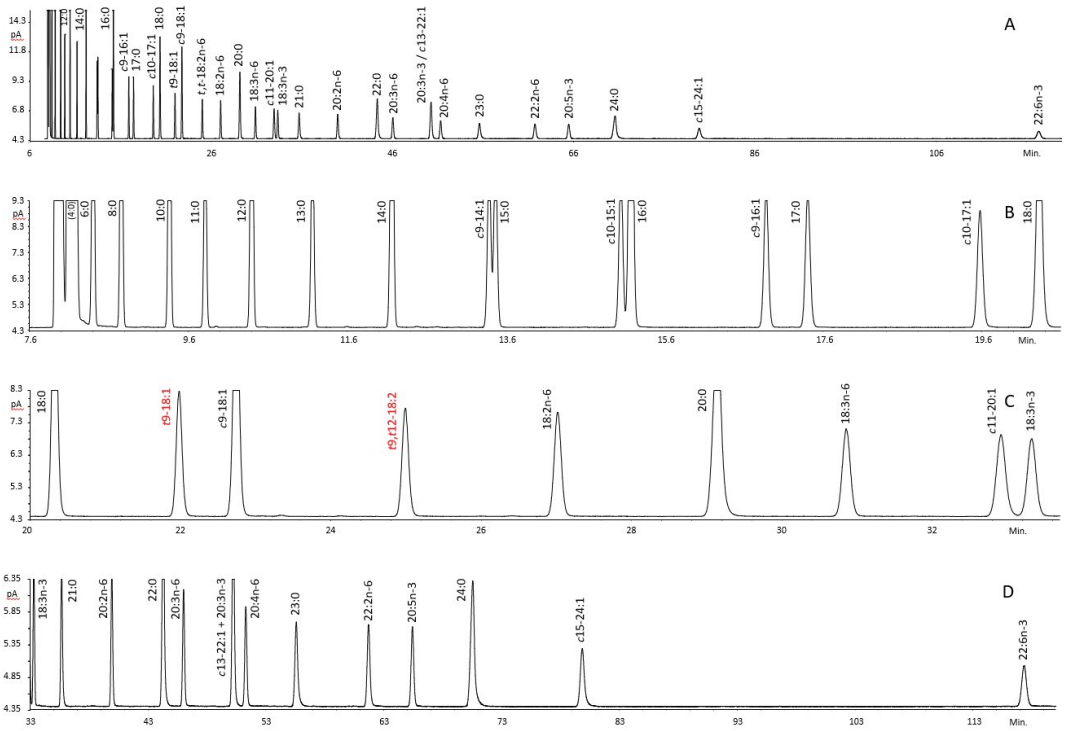

Fig. 1a: Entire GC chromatogram. Fig. 1b, 1c and 1d: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3n-3, and C18:3n-3 to 22:6n-3, respectively.

Note: 180 °C isothermal, hydrogen carrier at 1.0 mL/min constant flow

Source: Dr Pierluigi Delmonte, United States Food and Drug Administration

Fig. 2. GC chromatogram of canola oil on SP-2560 capillary column (100 m × 0.25 mm)

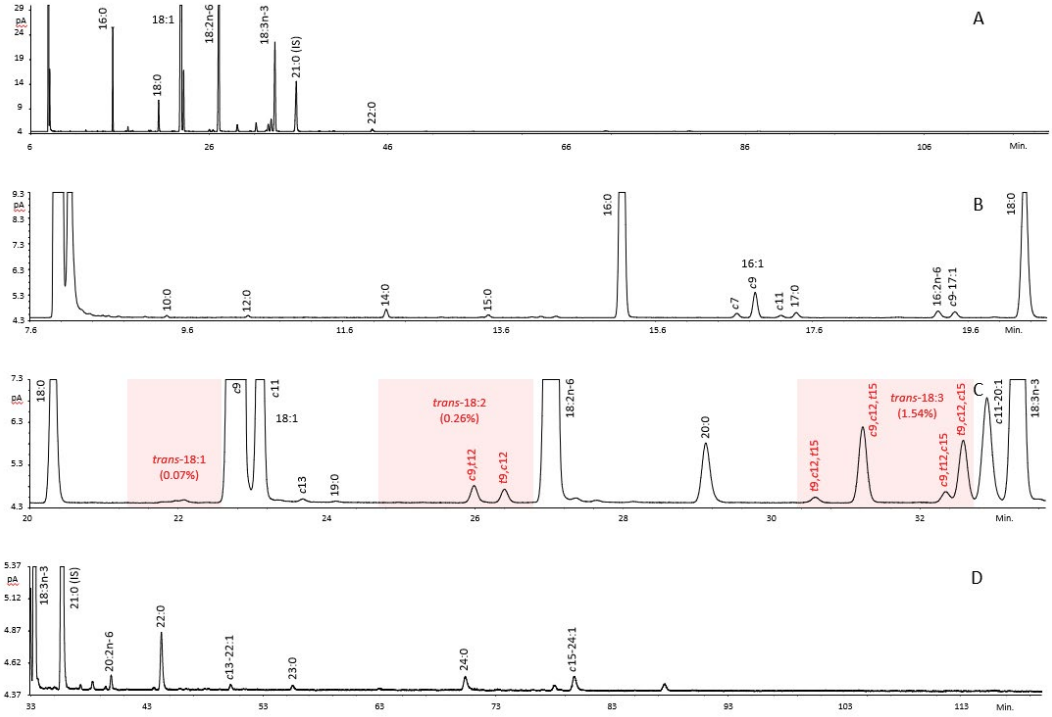

Fig. 2a: Entire GC chromatogram, Fig. 2b, 2c and 2d: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3n-3, and C18:3n-3 to 22:6n-3, respectively.

Note: 180 °C isothermal, hydrogen carrier at 1.0 mL/min constant flow

Source: Dr Pierluigi Delmonte, United States Food and Drug Administration

Fig. 3. GC chromatogram of a PHO on SP-2560 capillary column (100 m x 0.25 mm)

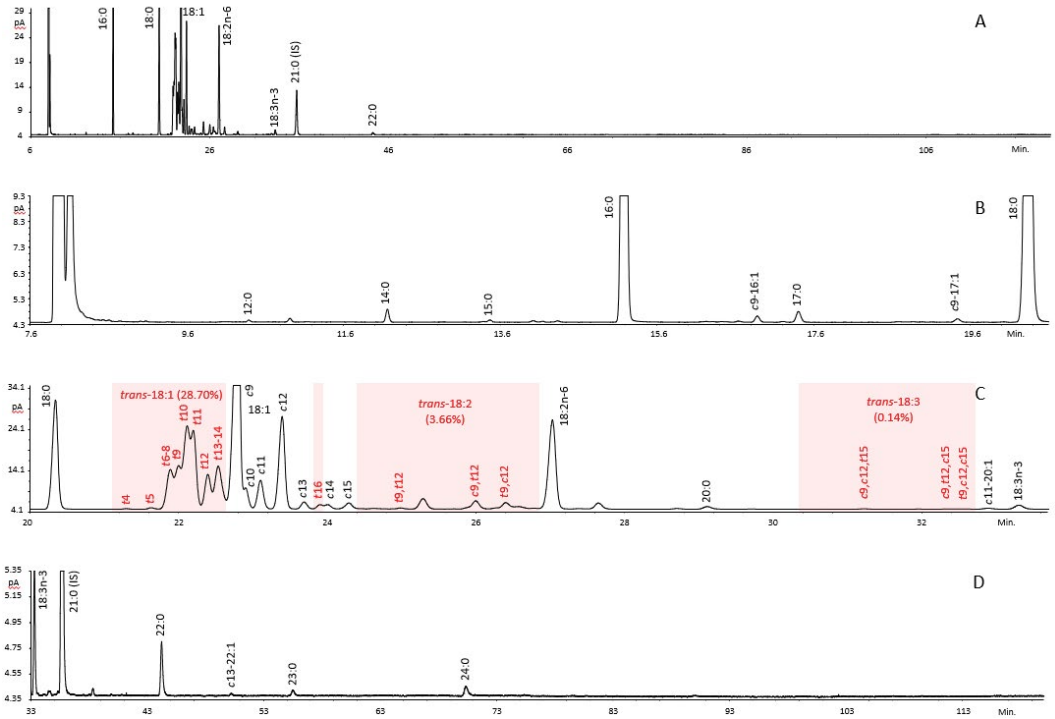

Fig. 3a: Entire GC chromatogram. Fig. 3b, 3c and 3d: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3n-3, and C18:3n-3 to 22:6n-3, respectively.

Note: 180 °C isothermal, hydrogen carrier at 1.0 mL/min constant flow

Source: Dr Pierluigi Delmonte, United States Food and Drug Administration

Fig. 4. GC chromatogram of a shortening blend on SP-2560 capillary column (100 m × 0.25 mm)

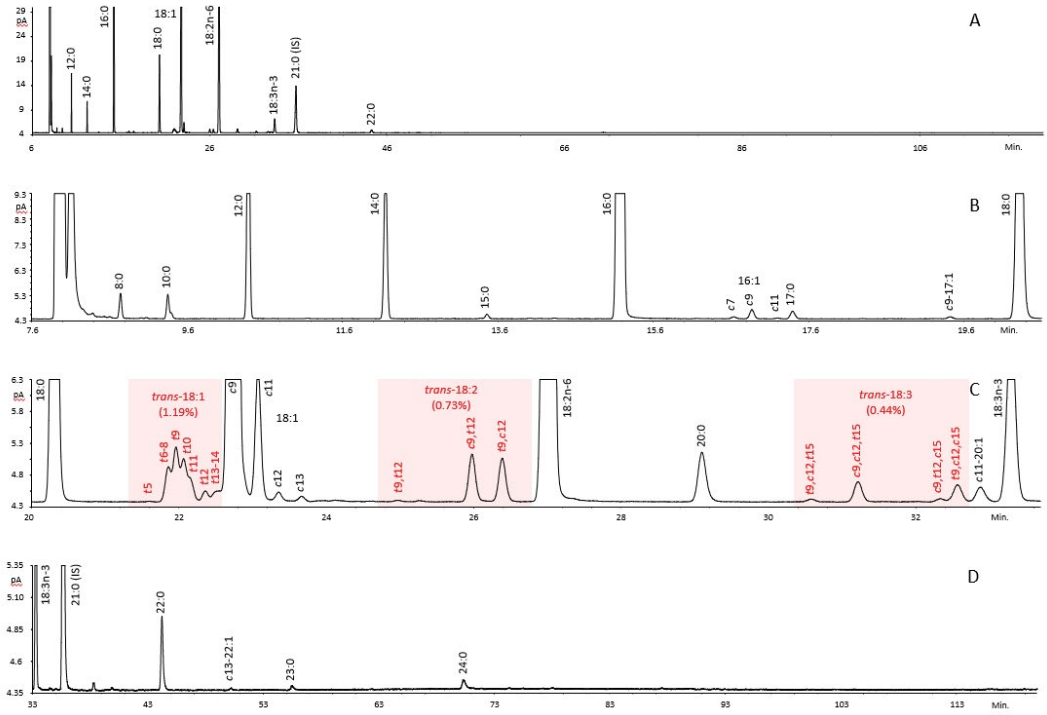

Fig. 4a: Entire GC chromatogram. Fig. 4b, 4c and 4d: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3n-3, and C18:3n-3 to 22:6n-3, respectively.

Note: 180 °C isothermal, hydrogen carrier at 1.0 mL/min constant flow

Source: Dr Pierluigi Delmonte, United States Food and Drug Administration

Fig. 5. GC chromatogram of a butter on SP-2560 capillary column (100 m × 0.25 mm)

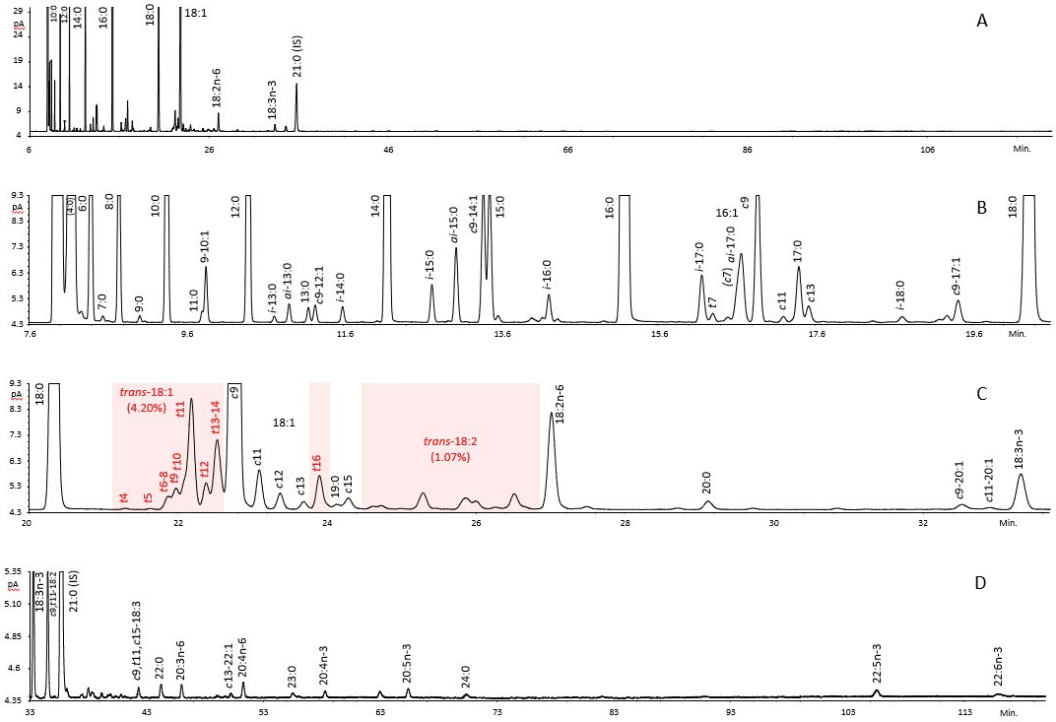

Fig. 5a: Entire GC chromatogram. Fig. 5b, 5c and 5d: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3n-3, and C18:3n-3 to 22:6n-3, respectively.

Note: 180 °C isothermal, hydrogen carrier at 1.0 mL/min constant flow

Source: Dr Pierluigi Delmonte, United States Food and Drug Administration

Fig. 6. GC chromatogram of Supelco 37 Component FAME Mix on SP-2560 capillary column (100 m × 0.25 mm)

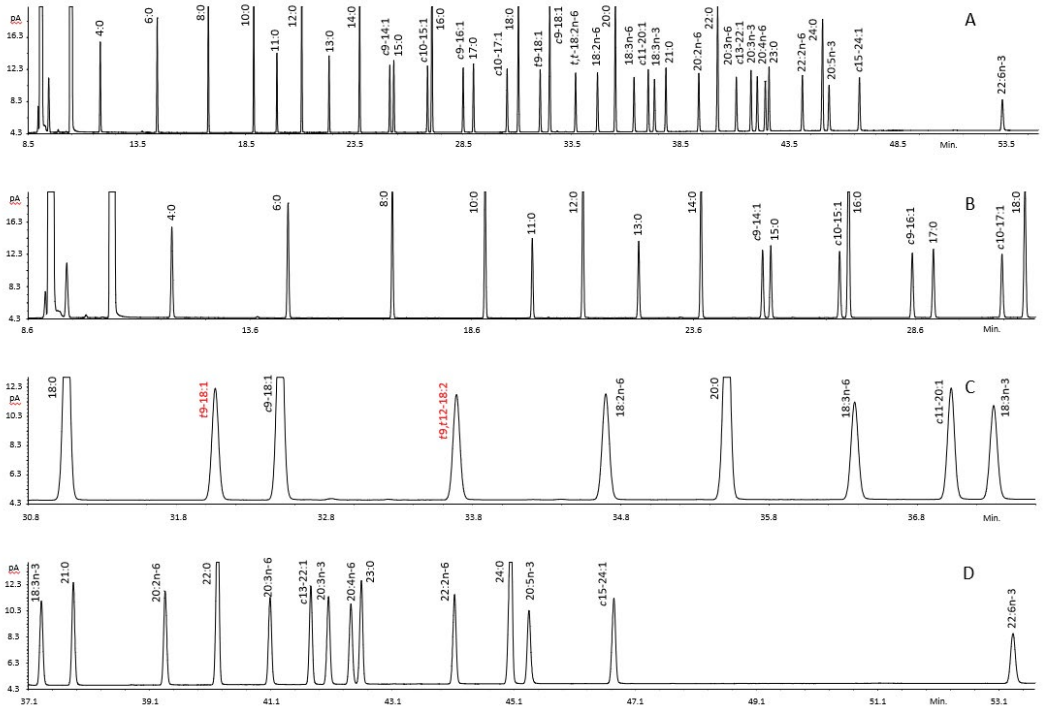

Fig. 6a: Entire GC chromatogram. Fig. 6b, 6c and 6d: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3 n-3, and C18:3n-3 to 22:6n-3, respectively.

Note: Temperature programme – initial temperature 60 °C for 5 min, ramped to 165 °C at 15 °C/min, hold 1 min, ramped to 225 °C at 2 °C/min, hold 20 min. Hydrogen carrier gas at 1.0 mL/min constant flow

Source: Dr Pierluigi Delmonte, United States Food and Drug Administration

Fig. 7. GC chromatogram of canola oil on SP-2560 capillary column (100 m × 0.25 mm)

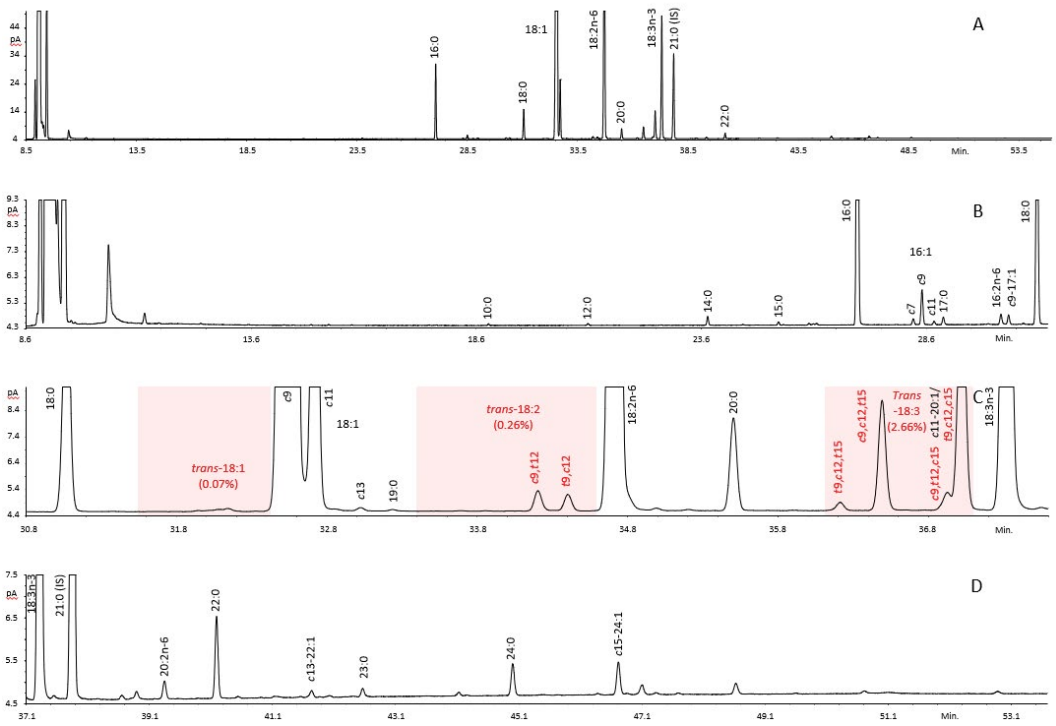

Fig. 7a: Entire GC chromatogram. Fig. 7b, 7c and 7d: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3 n-3, and C18:3n-3 to 22:6n-3, respectively.

Note: Temperature programme – initial temperature 60 °C for 5 min, ramped to 165 °C at 15 °C/min, hold 1 min, ramped to 225 °C at 2 °C/min, hold 20 min. Hydrogen carrier gas at 1.0 mL/min constant flow

Source: Dr Pierluigi Delmonte, United States Food and Drug Administration

Fig. 8. GC chromatogram of a PHO on SP-2560 capillary column (100 m × 0.25 mm)

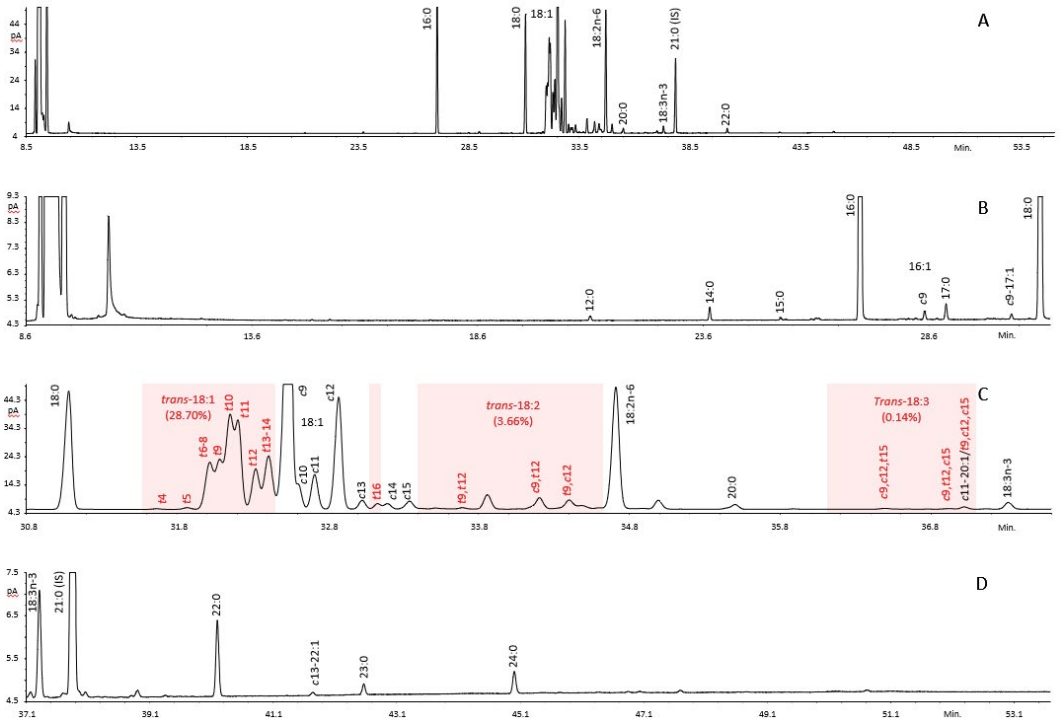

Fig. 8a: Entire GC chromatogram. Fig. 8b, 8c and 8d: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3 n-3, and C18:3n-3 to 22:6n-3, respectively.

Note: Temperature programme – initial temperature 60 °C for 5 min, ramped to 165 °C at 15 °C/min, hold 1 min, ramped to 225 °C at 2 °C/min, hold 20 min. Hydrogen carrier gas at 1.0 mL/min constant flow

Source: Dr Pierluigi Delmonte, United States Food and Drug Administration

Fig. 9. GC chromatogram of a shortening blend on SP-2560 capillary column (100 m × 0.25 mm)

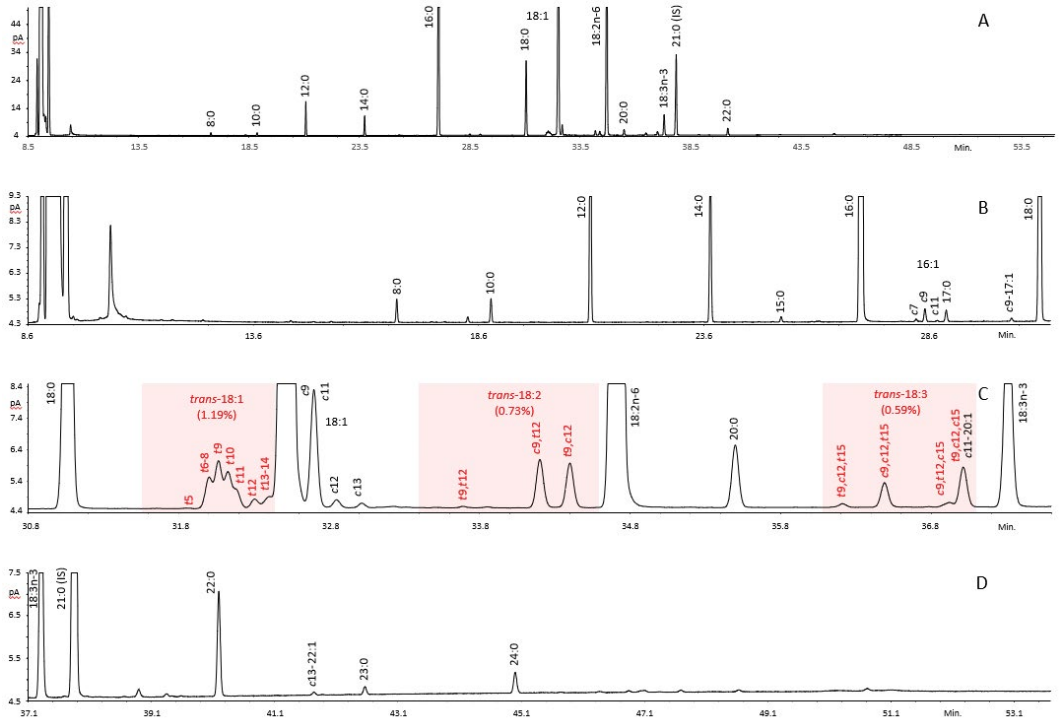

Fig. 9a: Entire GC chromatogram. Fig. 9b, 9c and 9d: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3 n-3, and C18:3n-3 to 22:6n-3, respectively.

Note: Temperature programme – initial temperature 60 °C for 5 min, ramped to 165 °C at 15 °C/min, hold 1 min, ramped to 225 °C at 2 °C/min, hold 20 min. Hydrogen carrier gas at 1.0 mL/min constant flow

Source: Dr Pierluigi Delmonte, United States Food and Drug Administration

Fig. 10. GC chromatogram of a butter on SP-2560 capillary column (100 m × 0.25 mm)

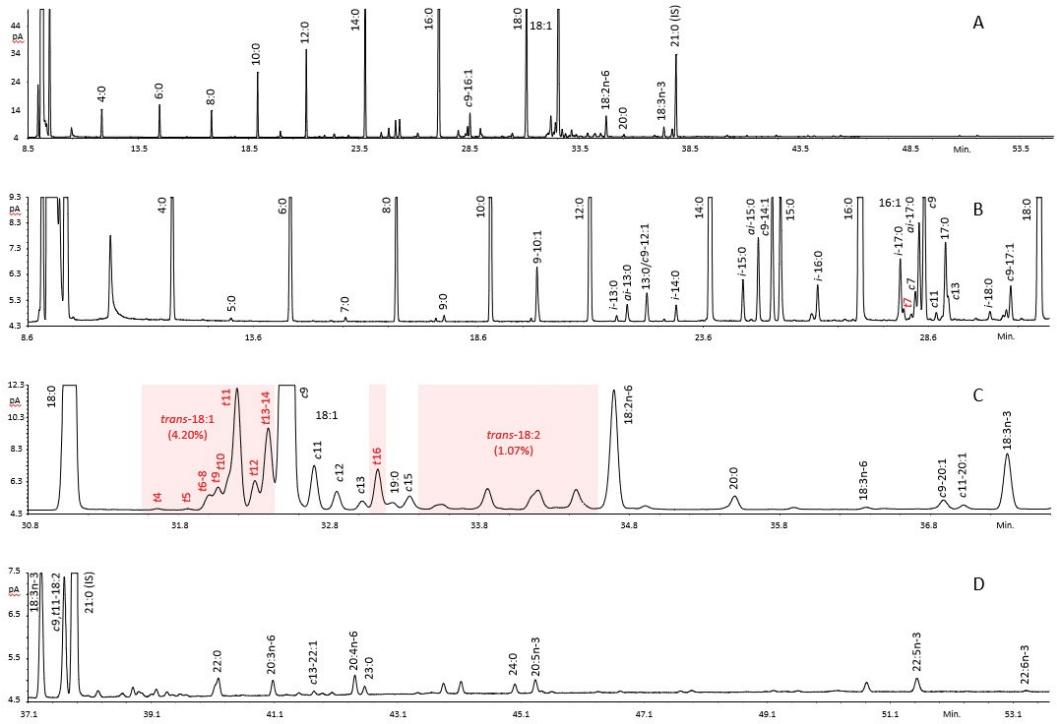

Fig. 10a: Entire GC chromatogram. Fig. 10b, 10c and 10d: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3 n-3, and C18:3n-3 to 22:6n-3, respectively.

Note: Temperature programme – initial temperature 60 °C for 5 min, ramped to 165 °C at 15 °C/min, hold 1 min, ramped to 225 °C at 2 °C/min, hold 20 min. Hydrogen carrier gas at 1.0 mL/min constant flow

Source: Dr Pierluigi Delmonte, United States Food and Drug Administration

Fig. 11. GC chromatogram of various FAME samples on SP-2560 capillary column (100 m × 0.25 mm)

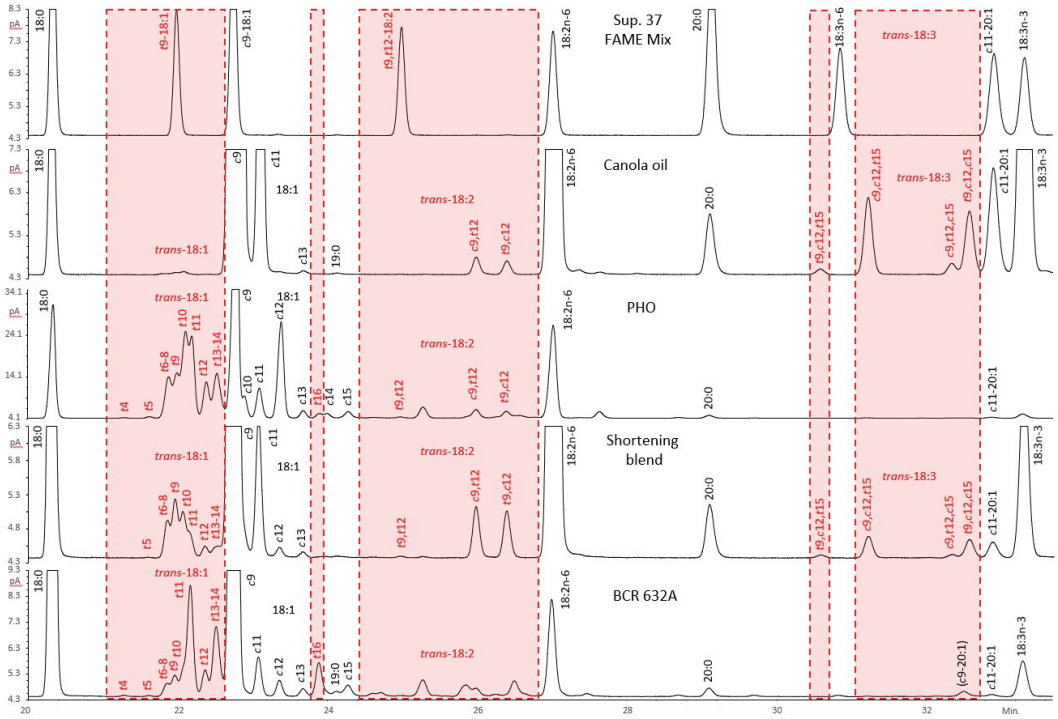

Fig. 11a: Supelco 37 Component FAME Mix. Fig. 11b: Canola oil. Fig. 11c: PHO. Fig. 11d: shortening blend. Fig. 11e: butter.

Note: 180 °C isothermal, hydrogen carrier at 1.0 mL/min constant flow

Source: Dr Pierluigi Delmonte, United States Food and Drug Administration

Fig. 12. GC chromatogram of various FAME samples on SP-2560 capillary column (100 m × 0.25 mm)

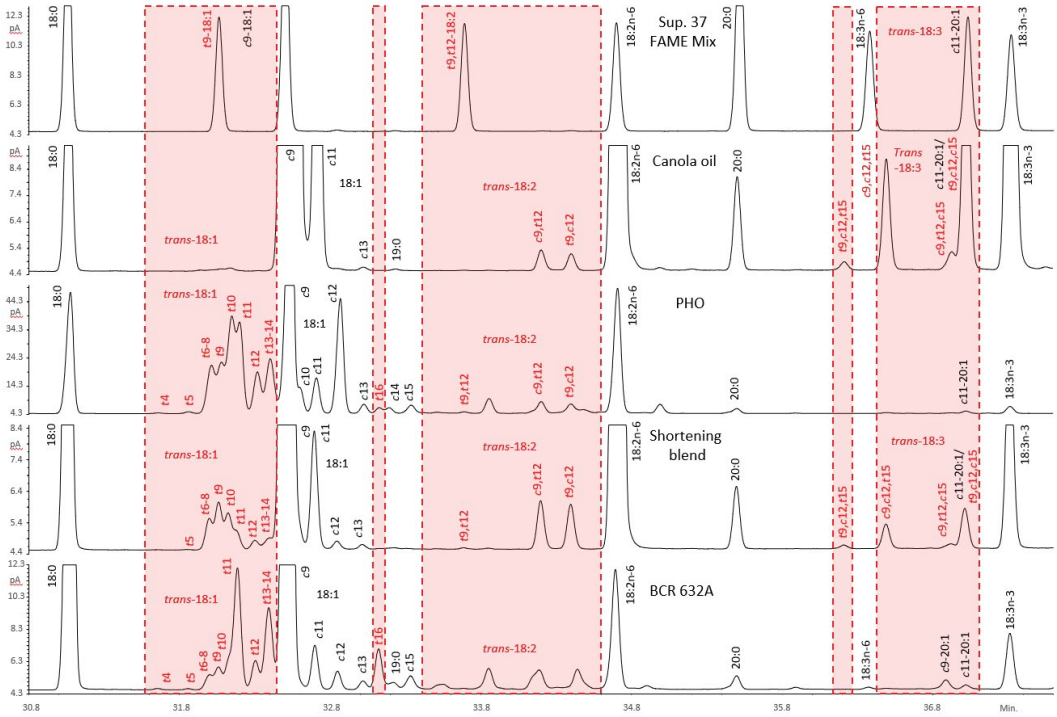

Fig. 12a: Supelco 37 Component FAME Mix. Fig. 12b: Canola oil. Fig. 12c: PHO. Fig. 12d: shortening blend. Fig. 12e: butter.

Note: Temperature programme – initial temperature 60 °C for 5 min, ramped to 165 °C at 15 °C/min, hold 1 min, ramped to 225 °C at 2 °C/min, hold 20 min. Hydrogen carrier gas at 1.0 mL/min constant flow

Source: Dr Pierluigi Delmonte, United States Food and Drug Administration

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