WHO reference protocol

for measuring fatty acids in foods, with emphasis on monitoring *trans*-fatty acids originating from partial hydrogenation of edible oils

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Contents

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Observers: Dr Scott Bloomer (American Oil Chemist Society, United States of America), Dr Chris Dayton (Bunge Limited, United States of America), Dr Francesca Giuffrida (Nestlé Research, Switzerland), Mr Pierre-Alain Golay (Nestlé Research, Switzerland), Dr Florence Lacoste (L'Institut des Corps Gras, France), Dr Katerina Mastovska (Association of Official Analytical Collaboration (AOAC) INTERNATIONAL, United States of America) and Ms Deborah McKenzie (AOAC INTERNATIONAL, United States of America) and Ms Tiffanie West (American Oil Chemist Society (AOCS), United States of America).

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Management of conflicts of interest

According to the rules in the World Health Organization (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.

Every external participant involved in the development of the reference protocol completed and submitted a Declaration of Interests. Declared interests of the participants were reviewed by the WHO Secretariat in consultation with the WHO Department of Compliance and Risk Management and Ethics, when necessary. The review found that the interests declared by 13 participants were not directly related to the topics under discussion, allowing them to contribute as experts. For eight participants, disclosed interests were somewhat linked to the topics; therefore, they participated as observers. The role and engagement defined as above were followed throughout the process. All contributing participants were acting in their individual capacity and not as institutional representatives.

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

Abbreviations

AOAC	Association of Official Analytical Collaboration
AOCS	American Oil Chemists' Society
BCS	biscyanopropylpolysiloxane
FA	fatty acid
FAME	fatty acid methyl ester
FFA	free fatty acid
FID	flame ionization detector
GC	gas chromatography
IDF	International Dairy Federation
IP-TFA	industrially produced *trans*-fatty acid
IS	internal standard
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
MTBE	methyl *tert*-butyl ether
MUFA	monounsaturated fatty acid
m/v	mass concentration of solution; mass per volume
PHO	partially hydrogenated oil
PTFE	polytetrafluoroethylene (brand name: Teflon)
PUFA	polyunsaturated fatty acid
RP-TFA	ruminant product *trans*-fatty acid
SFA	saturated fatty acid
TAG	triacylglycerol
TCF	theoretical correction factor
TFA	*trans*-fatty acid
TLC	thin-layer chromatography
WHO	World Health Organization
w/w	weight concentration of solution; weight per weight

Executive summary

In December 2020, the World Health Organization (WHO) first developed and published a laboratory protocol for measuring *trans*-fatty acids (TFAs) in foods[2] (hereinafter referred to as the "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 alternative methods and chemicals be included in the protocol so that it is easier to use and globally applicable. In response to the requests, WHO organized an expert consultation meeting in June 2022, based on which the Simplified Protocol for Measuring TFA content (hereinafter referred to as the "WHO simplified protocol") was developed.[3] This fit-for-purpose protocol can be implemented by all laboratories including those with limited budgets, and provides most of the data that are required for governments' surveillance and monitoring activities to check the trend of TFA content of fats and oils added during food preparation and processing. This will ensure that the fats and oils used in preparation comply with regulations for TFA elimination. Regarding the key differences between the original WHO reference protocol and the WHO simplified protocol, please refer to Table 1 in the report of the WHO expert consultation held in June 2022.[4]

When developing the WHO simplified protocol, new data and information were obtained on the procedures for measuring fatty acids (FA). It was realized that the WHO reference protocol needs to be revised to provide up-to-date, comprehensive and validated options for conducting full fatty acid methyl ester (FAME) analysis. A 2-day, virtual WHO Expert Consultation was held on 19 and 22 September 2023 to update the WHO reference protocol.[5] In doing so, all the critical steps of the WHO reference protocol and the WHO simplified protocol were reviewed. During this expert consultation, validated alternative procedures were recommended for increasing the versatility of the protocol and expanding its ability to analyse all types of food products that could contain TFAs.

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[2] Global protocol for measuring fatty acids in foods, with emphasis on monitoring *trans*-fatty acids originating from partially hydrogenated oils. Geneva: World Health Organization; 2020 (https://iris.who.int/handle/10665/338049).

[3] Simplified protocol for measuring *trans*-fatty acids content as a percentage of total fatty acids in food products. Geneva: World Health Organization; 2023 (https://iris.who.int/handle/10665/366690).

[4] 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. Geneva: World Health Organization; 2023 (https://apps.who.int/iris/handle/10665/366677).

[5] WHO expert consultation on the WHO protocol for measuring *trans*-fatty acids in foods: Virtual meetings report, September 2023. Geneva: World Health Organization; 2025 (https://iris.who.int/handle/10665/381142).

This revised document, entitled "WHO laboratory protocol – Reference protocol for measuring fatty acids in foods, with emphasis on monitoring *trans*-fatty acids originating from partial hydrogenation of edible oils" comprehensively includes validated procedures for fat extraction, gas chromatography (GC) peak identification, and determination of FAME flame ionization detector response. It also includes validated alternatives for internal standards, methylating agents, solvents for preparing FAMEs, GC parameters for determination of FA composition, and FAME reference standards. Furthermore, this protocol includes two sample preparation procedures in which the FA are methylated directly in the food sample before extraction, significantly reducing the time and cost of analysis. Throughout this document "methylation" is defined as the generic process of converting FA to FAME regardless of their free or bound status. The described procedures allow the determination of the TFA content as grams (g) per 100 g of food, grams per 100 g total FAs and grams per 100 g total fat. For measuring TFA in grams of FA per 100 g of food, an internal standard should be added to the test food sample portion before fat extraction or combined methylation/extraction. For the differences among the original WHO reference protocol, the WHO simplified protocol and the updated WHO reference protocol, please refer to Table 1 of this document. The updated WHO reference protocol retains the following sections outlined in the 2020 WHO reference protocol and 2022 WHO simplified protocol: clinical relevance, dietary *trans* fats, sampling plan, sample collection and storage, sample preparation for analysis, safety precautions and laboratory equipment.

This updated WHO reference protocol replaces the original WHO global reference protocol. This updated edition of the WHO reference protocol is a compendium of validated procedures for conducting FAME analysis, allowing laboratories to choose the most suitable procedure for their specific analytical needs. While this protocol is also inclusive of all the procedures outlined in the WHO simplified protocol (except for the fat extraction procedure using solvents in section 6.3.2), the simplified protocol will remain as a standalone protocol as it is a useful summary of the fit-for-purpose procedures, and is recommended for laboratories operating on a limited budget and which are aiming to obtain the minimal data required for government surveillance and monitoring activities of fats and oils externally added during food preparation and processing.

Overview of procedures

Step 1

Selection of internal standard (go to section 8)

- Tritridecanoin (C13:0 triacylglycerol; TAG)
- Triheneicosanoin (C21:0 TAG)
- Methyl undecanoate (C11:0 FAME) with C13:0 TAG 1:1 (w/w)
- No internal standard

Step 2

Extraction of fat from test food sample

- Category 1 Foods: pure oils, fats, shortenings, and vanaspati (go to section 11.2)
- Category 2 Foods: margarine, vegetable fat spreads and butter (go to section 11.3)
- Category 3 Foods: other foods (go to section 11.4)

Step 3

Methylation of extracted fat

- Option 1: using 7% BF_3-CH_3OH reagent (go to section 12.1)
- Option 2: using 2 M KOH-CH_3OH (go to section 12.2)
- Option 3: using 5% (m/v) CH_3ONa in CH_3OH (go to section 12.3)

OR

Direct methylation combined with fat extraction from the test food sample

- Option 1: using alkali hydrolysis (go to section 13.1)
- Option 2: using 5% (m/v) CH_3ONa in CH_3OH (go to section 13.2)

Step 4

GC analysis

- Option 1: isothermal operation (go to section 14.1)
- Option 2: temperature programme operation (go to section 14.2)

Step 5

Interpretation of GC separations (go to sections 14.3–14.5)

Step 6

Calculation of fat content and FA composition (go to section 15)

1. Clinical relevance

Trans-fatty acids (TFAs) are unsaturated fatty acids (FAs) that contain at least one double bond in the *trans* configuration. The three-dimensional structure of TFAs resembles saturated FAs more closely than it does natural unsaturated FAs: the double bonds in natural unsaturated FAs are in the *cis* configuration. The presence of double bonds in *trans* configuration substantially alters the physical properties of oils containing TFAs *(1)*.

Regular consumption of TFAs increases the level of low-density lipoprotein cholesterol in the blood and the risk of coronary heart disease *(2, 3)*. The World Health Organization (WHO) recommends that consumption of TFAs (including artificial and from ruminant products) should be less than 1% of total energy intake *(4)*. Elimination of industrially produced TFAs from the global food supply is a WHO priority *(5, 6)*.

2. Dietary *trans* fats

2.1 Sources

Dietary TFAs originate primarily from two sources, which are:

- industrial partial hydrogenation of dietary fats and oils; and
- biohydrogenation by microorganisms in the rumen of ruminant animals.

Several common vegetable oils (e.g. canola, soybean, corn, sunflower) are generally not the preferred choice for deep-frying and for the preparation of certain processed foods (e.g. margarine, shortenings, cookies, cakes, breads) because of their low melting points and susceptibility to oxidative deterioration due to their high unsaturation. Industrial partial hydrogenation raises the melting point and increases the oxidative stability of the oil by reducing the degree of unsaturation. In addition, partial hydrogenation causes geometrical and positional isomerization of some of the unreduced double bonds, which creates an assortment of artificial unsaturated *cis* and *trans* FAs *(1)*.

Rumen biohydrogenation is responsible for the 2–8% TFAs content (as a proportion of total fat) occurring in the milk and meat of ruminant animals, whereas industrial partial hydrogenation can produce higher concentrations of TFAs – up to 40–60% of total fat *(1)*. Rumen biohydrogenation also causes geometrical and positional isomerization of the naturally occurring unsaturated FAs. A description of chemical structures of TFAs present in industrially produced and ruminant fats is given in Annex 1.

Throughout this document, industrially produced TFAs are abbreviated as IP-TFAs. TFAs present in ruminant products are referred to as RP-TFAs.

2.2 Fatty acid nomenclature and shorthand notations

The International Union of Pure and Applied Chemistry (IUPAC) *(7)* defines FAs as alkyl or alkenyl carboxylic acids. The first portion of the name indicates the length of the linear chain, followed by the number of double bonds on the linear chain and the ending "oic acid". The presence of double bonds is reported by the suffix "en" after the chain length descriptor, preceded by their number (di-en, tri-en) if greater than one. The suffix "an" is used if no double bonds are present. The location and geometry (Z for *cis*, E for *trans*) of each double bond are reported ahead of the linear chain descriptor separated by a comma. The carboxylic group is numbered carbon 1, and the other carbons of the linear chain are numbered sequentially. Thus, for example, linoleic acid is characterized by a linear chain of 18 carbons (octadeca) with two double bonds along the linear chain (di-en), and the two double bonds are in position/configuration 9 *cis* (9Z) and 12 *cis* (12Z). Therefore, the systematic name of linoleic acid is 9Z,12Z-octadecadienoic acid. Following the same convention vaccenic acid is termed 11E-octadecenoic acid (note the

removal of final "a" from "octadeca"), *trans*-palmitoleic acid as 9E-hexadecenoic acid, and saturated arachidic acid as eicosanoic acid. In the case of branched-chain FAs, the methyl groups not part of the linear chain are considered as substituent groups and reported ahead of the linear chain definer. As an example, *iso*-palmitic acid is defined as 14-methyl pentadecanoic acid.

For simplicity, in this document, the shorthand notation is preferred over the IUPAC systematic nomenclature since all relevant FAs occurring in foods are characterized by similar structures. FAs are named based on the total number of carbons, reported in numeric format and preceded by a "C", sequentially followed by ":" and the number of double bonds, also in numeric format. As an example, stearic acid (octadecanoic acid) is abbreviated to C18:0. When double bonds are present, their location on the alkenyl chain and geometric configuration are reported ahead of the FA name as for the IUPAC nomenclature, but using the descriptors "*c*" for *cis* and "*t*" for *trans*. For example, the shorthand notation for linoleic acid (9Z,12Z-octadecadienoic acid) is 9*c*, 12*c*-C18:2. In certain instances, as for figures, the "C" may be omitted to further simplify the nomenclate (i.e. 9*c*,12*c*-18:2 instead of 9*c*,12*c*-C18:2). For branched-chain FAs, the name indicates the total number of carbons and the prefix "*iso*" or "I-" indicates that one of the carbons is attached one carbon before the end of the chain (position n – 1), while the prefix "*anteiso*" or "AI" indicates that a carbon is attached two carbons away from the end of the chain n – 1. As examples, iso-palmitic acid (14-methyl pentadecanoic acid) and *anteiso*-palmitic acid (13-methyl pentadecanoic acid) are defined as I-16:0 and AI-C16:0, respectively.

Table 2 reports a comprehensive list of FAs commonly found in natural and processed foods. In the table, the FAs are named using both the IUPAC system and the simplified delta shorthand notation. Trivial names or historical names for some common FAs are also included.

3. Goal, scope and general approach

At the highest level, the development of a reference protocol requires establishment of terms of reference. These include aims, scope and general principles.

3.1 Goal

The goal of the updated WHO reference protocol is to provide a compendium of comprehensive, validated methods for measuring the TFA content of foods, especially those containing both IP-TFA and RP-TFA. This will support generation of accurate and globally comparable TFA and FA data about foods.

3.2 Scope

The updated WHO reference protocol specifies several options for measurement of TFAs contained in foods. These options also simultaneously measure the content of all other FA contained in the sample, including the various saturated FAs (SFAs), *cis*-monounsaturated FAs (MUFAs), *cis*-polyunsaturated FAs (PUFAs, including omega-3 and omega-6 PUFAs) in:

- refined and unrefined oils, partially hydrogenated oils (PHOs), fully hydrogenated oils, milk, cheese and other milk products; and
- all types of foods prepared using unrefined oils, refined oils, PHOs, milk, cheese, other milk products, and mixtures of all these products.

The updated WHO reference protocol is not intended for the analysis of partially hydrogenated fish oils (PHFOs) or processed foods containing PHFOs. These products have been almost completely discontinued from food distribution and require dedicated methods of analysis. The myriads of mono- and polyunsaturated TFAs ranging from C16 to C24 contained in PHFO cannot be resolved by applying gas chromatographic conditions suitable for routine analysis.

3.3 Methods

A two-day virtual WHO Expert Consultation was held on 19 and 22 September 2023 to update the WHO reference protocol. Discussions were guided by participant input and responses to a pre-circulated questionnaire. Based on these discussions, the WHO Secretariat drafted a revised reference protocol, which was then shared with participants for feedback. The draft was further refined to incorporate their input and subsequently reviewed by an external peer reviewer, whose comments were integrated into the final version.

3.4 General approach

The key steps of the analytical procedure of the updated WHO reference protocol are:

1. Collection of representative test food samples from retail stores/restaurants/food outlets, and preparation of the collected samples for FA analysis.
2. Blending and homogenization of the samples.
3. Extraction of fat from representative test sample portions.

   Note: If the aim of the analysis is to express the FA data in grams per 100 g of sample, an internal standard (IS) should be added to the weighed test portion before fat extraction. For pure fats and oils samples, which do not require extraction, the IS may be added before the methylation step. If the scope of the analysis is limited to obtaining the FA composition as grams per 100 g of total FAs, the IS may be omitted.
4. Conversion of the extracted fat to FAMEs (methylation)

   The fat extraction (step 3) and methylation (step 4) may be combined by adding the methylation reagent(s) to the sample portion before fat extraction. This approach results in a significant saving of time and reagents. The American Oil Chemists' Society (AOCS) Official Method Ce 2b-11 (Direct methylation of lipids in foods by alkali hydrolysis) is based on this principle and is applicable to most types of food samples *(8)* (See section 13.1). The Association of Official Analytical Collaboration (AOAC) INTERNATIONAL 2012.13 / the International Organization for Standardization (ISO) 16958 | the International Dairy Federation (IDF) 231:2015 method *(9, 10)* (milk, milk products, infant formula and adult nutritionals – determination of fatty acids composition – capillary gas chromatographic method) is also based on the combination of these two steps, and is applicable to various types of milk and milk products.
5. Analysis of the prepared FAME by gas chromatography (GC) with flame ionization detection (GC-FID).

   Processing of the GC chromatograms and calculation of the FA content as grams/100 g of sample, and/or grams/100 g of FAs (if no IS is added).

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. Some countries, regions or laboratories may have their own sampling plans. Sampling depends on the purpose and design of each study and survey, and these depend on the landscape of each country. Whatever method is used, the collected samples should be representative of the brands or lot numbers available locally.

4.1 Sampling plan

Foods most likely to contain TFAs can be generally grouped into three categories. These categories consider factors such as the origin of TFA (IP-TFA, RP-TFA or refined deodorized oils), food collection and fat extraction. For each category, identify foods and brands that are commonly consumed in the region, make a list, and collect representative samples, as described in section 4.2.

Category 1: Pure fats and oils, available to consumers and commercial producers. These are pure fats and oils that contain no other ingredients, except minute amounts of antioxidants and other additives. No water should be present. Samples in this category include cooking oils, salad oils, frying oils,vanaspati (hydrogenated vegetable oil), most baking fats and shortenings (those with no emulsified water).

Category 2: Margarines and butters. Samples in this category include all types of butters, margarines and vegetable fat spreads containing varying amounts of emulsified water.

Category 3: All products not in categories 1 or 2. This includes:

- all dairy products (milk, various cheeses and other milk products);
- packaged foods from grocery stores (including biscuits, breads, buns, cakes, cheese, cookies, crackers, croissants, potato chips and other chip varieties, peanut butter, popcorn, pizza, pastries and various snacks); and
- ready-to-eat foods from food outlets or retail stores (including all types of cooked foods, frozen-fried/cooked foods, fresh-fried and baked foods served by restaurants, grocery stores, bakeries, street vendors, corner stores and other food outlets).

4.2 Collection of food samples

Cooking and salad fats and oils, milk, cheese, other milk products, processed food samples and ready-to-eat food items that are likely to contain TFAs originating from ruminant and edible oil sources are collected from major grocery stores, popular

restaurants, fast food chains and street vendors in a region. Collect representative food samples from two or more major grocery stores. Ideally, the major grocery stores should be sampled in various neighbourhoods of different socioeconomic statuses (at least one grocery store from high- or middle-income neighbourhoods, and at least one from low-income neighbourhoods). Collect ready-to-eat foods from two or more popular food outlets, again in neighbourhoods with different socioeconomic statuses.

In the case of traditional foods or recipes originating from different geographical regions, samples should be taken from these different regions.

If resources allow, collect PHOs from oil manufacturing facilities and distribution facilities. In addition, collect cooking and frying oils and baking fats used in popular restaurants and bakeries.

4.3 Number of brands and weight of representative samples

If available, select three to five different popular brands for each of the food categories. Collect three consumer-size packages of each brand. The total weight of the three consumer-size packages should be 300 g or more. If less than 300 g, collect more packages. Similarly, collect three regular servings from each of the popular ready-to-eat food items. Here also, the total weight of the three regular servings should be 300 g or more. If less than 300 g, collect more regular-size servings.

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

4.4 Inventory of purchased test food samples

Maintain an inventory of the foods purchased.

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.

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 item was purchased;
- date the item was manufactured;
- food category (e.g. margarines, cookies);
- sample size;
- brand name;
- manufacturer's name and address;
- lot number; and
- expiration or "best by" date, if provided.

In addition, record the presence in the food ingredient list of vegetable oils, PHOs, fully hydrogenated oils and ruminant fat sources.

For identification purposes, assign a code number to each food item. Use this code number for labelling the food items and recording all the analytical data pertaining to the food item.

4.5 Storage of food samples

Samples that are still in the original sealed packaging should be stored according to the manufacturer recommendations. The manufacturer may also state how to store samples once the packaging is opened, and for how many days. Category 3 samples should be stored in a refrigerator (2–8 °C) if analysed within a few days, and in a freezer (approximately –15 °C to –18 °C) if longer storage is necessary. Homogenized samples should also be stored following the same approach. All samples must be stored in the dark. Proper storage slows down deterioration of the samples. In any case, all samples must be analysed before the expiry date. If the expiry date is not shown on the food label, analyse the samples within 2 months of purchase.

4.6 Preparation of samples for analysis

4.6.1 When the laboratory is ready to begin the analysis, take out all the food packages of a given brand/type 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 never heat the samples above 30–32 °C. Do not use a microwave oven to thaw foods. Heating will be uneven and may go above 32 °C in some parts of the food.

4.6.2 Take the entire contents of each of the thawed packages of a given brand or type 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 TFAs content of each food brand.

4.6.3 The composite should be homogenized thoroughly to ensure that it is a true representation of all packages.

4.6.4 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. Category 2 products (margarines, spreads and butter) must not be melted because this would result in breaking the water emulsion and separating the sample into two distinct phases. If adequate blending equipment is not available, category 2 products may be considered as already homogenous.

4.6.5 Preparing a well-homogenized composite of solid food samples may require specialized large equipment. Domestic food blenders or food processors may be used if dedicated laboratory equipment is not available. The blending temperature should be monitored, to avoid fat separating out, which causes loss of homogeneity. Place the entire contents of solid food samples in the blender or food processor and process until the contents have been ground into the smallest particles possible. If the particle size is not small enough (ideally it should be a powder), place the entire composite in a mortar (or glass bowl) and pour in sufficient dry ice or liquid nitrogen to cover the composite,

and then grind it using a pestle. This should produce a good, homogenized sample.

4.6.6 Take a subsample from the homogenized composite for extraction of fat or direct methylation without previous fat extraction.

- For extraction of fat or direct methylation, the weight of the representative homogenized composite subsample should be selected to contain approximatively 200 mg of fat (50 mg for procedure 13.2). This determination may be accomplished by using the total fat content reported on the sample nutrition table. Alternatively, the expected fat content in common foods can be found online by searching nutrient file databases (e.g. Canadian Nutrient File is available from https://food-nutrition.canada.ca/cnf-fce/index-eng.jsp).

4.6.7 Place the remainder of the representative homogenized food 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.

Note: Only homogenize the number of food samples that can be analysed the same day. Work out the maximum number of samples that can be processed each day based on the selected methodology, available automated tools, and current level of analyst experience.

5. Laboratory equipment

The equipment listed below or equipment providing equivalent performance can be used.

5.1 Gas chromatography apparatus

- **Gas chromatograph.** Suitable for use with a capillary column; equipped with a flame ionization detector (FID), temperature-controlled split/split-less injection port, oven chamber capable of maintaining the programmed temperature ±1 °C, and a computerized data acquisition system capable of controlling the GC and processing the acquired chromatograms. It is strongly recommended to use a GC equipped with electronic pressure controllers, capable of operating in constant-flow mode. The use of an automated sample injector tower combined with a sample tray may permit more reproducible and continuous analysis of samples.
- **Capillary column.** Fused silica capillary column 100 m long, 0.25 mm internal diameter, coated with 0.20 µm non-bonded 100% BCS stationary phase. Supelco SP-2560 (Millipore Sigma, PN# 24056 or 23362-U) and J&W CP-Sil 88 for FAME (Agilent, PN# CP7489I5 or CP7489) are suitable commercial GC columns and are widely available. Equivalent capillary columns with the same dimensions and stationary phase may be available from other manufacturers.
- **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.

If possible, hydrogen should be preferred as carrier gas over helium based on its lower cost and the higher chromatographic resolution that it provides when 100 m × 0.25 mm capillary columns are used. Hydrogen provides optimal chromatographic resolution at 1.0–1.5 mL/min flow rate, while helium chromatographic resolution quickly deteriorates when the flow rate is set outside the 0.8–0.9 mL/min range.

- **Flame ionization detector gases.** Hydrogen and air. These should be free from hydrocarbon impurities (below 1 ppm) and of purity at least 99.995%.
- **Makeup gas.** Nitrogen or helium gas, GC quality.
- **Injection port split liner.** Preferably a 4 mm internal diameter, 6.3 mm outer diameter × 78.5 mm split liner with glass wool (e.g. Agilent part number 5183-4647 or equivalent).

5.2 Laboratory glassware and equipment

Usual laboratory equipment and glassware; specifically:

- Erlenmeyer glass flasks equipped with glass stoppers (capacities 250 mL and 500 mL);
- flat-bottomed glass boiling flasks (capacity 125 mL or 250 mL);
- glass measuring cylinders (capacities 10 mL, 50 mL, and 100 mL);
- glass volumetric pipettes, class A (capacities 1 mL, 2 mL, 5 mL, and 10 mL);
- calibrated dispensers (various volumes, as pipette replacements);
- disposable glass Pasteur pipettes;
- PYREX screw cap test tubes of various capacities (e.g. 15 mL, 25 mL and 50 mL) with PTFE ("Teflon")-lined caps;
- rotary evaporator and glass round-bottom flasks, for evaporating solvents;
- glass funnels (medium and large sizes);
- weighing scale, for measuring gram quantities of food samples;
- analytical balance for precise measurement of milligram quantities of material (accurate to at least 0.01 mg);
- laboratory centrifuge equipped with adaptors for test tubes;
- magnetic stir bars of various sizes;
- hotplate stirrers;
- nitrogen gas from a nitrogen cylinder of purity 99.999% or higher;
- domestic food blender or a food blender with a food 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;
- round-bottom glass flasks (50 mL) with glass stoppers and water condenser;
- electric heating block for test tubes;
- hot-water bath shaker (shaking water bath);
- refrigerator capable of maintaining temperatures between 2 °C and 8 °C;
- freezer capable of maintaining temperatures between –15 °C and –18 °C; could be 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;
- porous boiling chips;
- rack, to hold the Mojonnier fat-extraction flasks or tubes; and
- wash bottle, suitable for use with the mixed solvent of equal volumes of diethyl ether and light petroleum ether. A plastic wash bottle shall not be used.

All glassware and laboratory equipment intended for food sample preparation, sample storage, fat extraction and analysis should be cleaned thoroughly to remove all residual fats and FAs. Thorough cleaning of glassware can be achieved by soaking 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 hydrocarbon solvent such as n-hexane or n-heptane.

Other laboratory equipment, especially the weighing scale, mortar and pestle, and food blender/processor should also be cleaned to remove fat and dirt. If the materials constituting these laboratory tools are compatible (i.e. blender bowl), rinse them with acetone followed by a hydrocarbon solvent (n-hexane or n-heptane).

6. General laboratory reagents and solvents

Use only recognized analytical-grade reagents, and chromatographic- or analytical-grade solvents, including:

- acetone, analytical grade;
- ammonium hydroxide 58% (w/w);
- 2′,7′-Dichlorofluorescein in ethanol, reagent grade;
- disodium hydrogen citrate sesquihydrate [$HOC(COOH)(CH_2COONa)_2.1.5\ H_2O$];
- ethanol: 95% (v/v);
- n-hexane (> 99% purity, chromatographic grade);
- n-heptane (> 99% purity, chromatographic grade);
- hydrochloric acid (HCl): 12 M (equivalent to mass fraction of HCl of approximately 36%); to make 8.3 M HCl (equivalent to mass fraction of HCl approximately 25%), carefully and slowly add 250 mL of 12 M HCl to 110 mL distilled water, mix well, and store at room temperature (20–25 °C);
- diethyl ether: anhydrous, purity ≥99.7%, containing 1 ppm butylated hydroxy toluene (BHT) inhibitor (preferably packed in aluminium containers) – must be free of peroxides;
- light petroleum ether: with any boiling range between 30 °C and 60 °C;
- phenolphthalein indicator solution, 1% (m/v);
- potassium hydroxide (KOH) pellets, reagent grade;
- pyrogallic acid, laboratory grade;
- methanol (CH_3OH) , chromatographic grade;
- sodium chloride (NaCl), reagent grade;
- sodium hydroxide (NaOH) pellets, reagent grade;
- anhydrous sodium sulfate;
- distilled water;
- methyl *tert*-butyl ether (MTBE), chromatographic grade;
- toluene: nanograde; and
- neutralization solution (disodium hydrogen citrate sesquihydrate 10% (m/v), NaCl 15% (m/v) in water).

To prepare, weigh 50.0 g disodium hydrogen citrate sesquihydrate and 7.0 g NaCl in a 500 mL volumetric flask. Dissolve in 450 mL of distilled water using a magnetic stirrer. Remove the magnetic stirrer, then make to mark with distilled water. Store in the dark at 4 °C. This solution is stable for 1 month. Allow the solution to come to room temperature before use.

7. Reference fatty acid methyl ester standards

Reference FAMEs are required for establishing the GC elution pattern of FAMEs and for identification of FAME peaks of the food sample chromatograms. Table 3 reports a list of reference FAMEs that are generally used to setup GC methods for FAME analysis, and which are available from several commercial sources (e.g. Alltech Associates, Inc.; Nu-Chek-Prep; Supelco Inc.; Sigma Chemical Co.; MilliporeSigma; Cayman Chemical – formerly Matreya LLC Lipids and Biochemicals; Restek).

Several certified FAME reference mixtures are also available from the commercial sources listed above. The following FAME mixtures are available from MilliporeSigma: a mixture containing 37 saturated and unsaturated FAMEs of chain lengths ranging from C4 to C24 (Supelco 37 component FAME mix 47885-U), the four *cis*/*trans* geometric isomers of linoleic acid (CRM47791) and the eight *cis*/*trans* geometric isomers of linolenic acid (L6031). Table 4 shows the composition of these certified FAME reference mixtures.

In addition to the above, a highly hydrogenated vegetable oil (as FAMEs) is available from Cayman Chemical (*cis*-*trans* FAME isomer standard mixture 1131) and a lightly hydrogenated vegetable oil is available from MilliporeSigma (*cis*/*trans* FAME column performance mix 40495-U). These reference solutions prepared from PHOs are extremely valuable because they contain all the *cis*/*trans*-C18:1 FAs that are present in PHOs, many of which are not available as pure reference standard. These FAMEs include 6*t*-C18:1, 7*t*-C18:1, 8*t*-C18:1, 9*t*-C18:1, 10*t*-C18:1, 11*t*-C18:1, 12*t*-C18:1, 13*t*-C18:1, 14*t*-C18:1, 15*t*-C18:1, 6*c*-C18:1, 8*c*-C18:1, 9*c*-C18:1, 10*c*-C18:1, 11*c*-C18:1 and 12*c*-C18:1.

For GC analysis, prepare individual and mixed reference FAME solutions (in n-hexane or n-heptane) at the approximate concentration of 0.2 mg/mL for each FAME.

8. Internal standards

For the quantification of FAs in grams per 100 g of test food, the use of an internal standard (IS) is necessary. A known amount of IS should be added quantitatively to the weighted sample portion used for analysis (see section 4) before beginning the selected preparation procedure.

This WHO protocol recommends using one of the following ISs:

1. Tritridecanoin (C13:0 TAG)
2. Triheneicosanoin (C21:0 TAG)
3. Methyl undecanoate (C11:0 FAME) combined with C13:0 TAG, 1:1 (w/w) mix. C11:0 FAME is used for quantitative reference, and C13:0 TAG to verify the completion of the transesterification process.

8.1 Preparation of stock solutions of internal standard

Tritridecanoin (C13:0 TAG), triheneicosanoin (C21:0 TAG) and methyl undecanoate (C11:0 FAME) (99% or greater purity) are available from the same suppliers stated in section 7 for pure reference materials (e.g. MilliporeSigma).

IS solutions should be made with particular care in the weighing of the IS material and dilution to the final volume of the solution, because any inaccuracy in their preparation will systematically affect all analyses. Record the concentration of these prepared stock IS solutions as W_{IS}/mL solvent.

The IS solutions should be stored in a refrigerator (2–8 °C) and must be brought to room temperature before use. See Annex 3 for further details.

8.1.1 Preparation of C13:0 TAG internal standard solution at the concentration of 5 mg/mL in n-hexane or n-heptane

Into a 100 mL volumetric flask weigh, to the nearest 0.1 mg, 500 mg C13:0 TAG. Record the weight in milligrams to at least two decimal places. Add 80 mL n-hexane or n-heptane. Mix well to dissolve C13:0 TAG and add more solvent to reach the 100 mL mark. This TAG IS solution is stable for up to 1 month when stored tightly capped in a refrigerator (2–4 °C). Suitable for all sample preparations except for that in section 13.2.

8.1.2 Preparation of stock solution of C21:0 TAG internal standard of concentration 5 mg/mL in chloroform

Into a 100 mL volumetric flask weigh, to the nearest 0.1 mg, 500 mg of C21:0 TAG. Record the weight in milligrams to at least two decimal places. Add 80 mL chloroform and mix until the C21:0 TAG is dissolved. Dilute to the 100 mL mark with chloroform.

This IS solution is stable for up to 1 month when stored tightly capped in a refrigerator (2–4 °C). Suitable for all sample preparations except for that in section 13.2.

8.1.3 Preparation of C11:0 FAME and C13:0 TAG IS solution at 2 mg/mL concentration in MTBE

This is adapted from AOAC 2012.13 / ISO 16958 | IDF 231:2015 *(9, 10)*.

Into a 250 mL volumetric flask weigh, to the nearest 0.1 mg, 500 mg C11:0 FAME and 500 mg C13:0 TAG. Record the weights in milligrams to at least two decimal places. Add 200 mL MTBE and mix until dissolved. Dilute to the 250 mL mark with MTBE. This IS solution (concentration 2 mg C11:0 FAME/mL MTBE) is stable for up to 1 month when stored tightly capped in a refrigerator (2–4 °C). The C11:0 FAME acts as the quantitative reference, while C13:0 TAG serves for the verification of the transesterification process completion. Suitable only for the sample preparation described in section 13.2.

9. Reagents for converting fats and oils to FAMEs (methylation)

Several methylating agents have been prescribed in the lipid literature for converting fat or oil into FAMEs *(8–16)*. This updated edition of the WHO reference protocol recommends using one of the following reagents. In a case-to-case basis analysts should scrutinize the applicability and limitations of each reagent before proceeding to its use for a specific type of sample; the reagents are:

- 7% (m/v) BF_3 in CH_3OH
- 2 M KOH in CH_3OH
- 5% (m/v) CH_3ONa in CH_3OH.

The reagent 7% BF_3-CH_3OH is the most used and most versatile methylating reagent. It can methylate all relevant lipid classes contained in fats and oils, either pure or extracted from food sources. In general, it can convert into FAMEs all the FA moieties contained in most lipid materials including the ones bound to non-polar lipids (such as tri-, di- and mono-acylglycerol, wax esters, sterol esters) and polar lipids (such as phospholipids, glycolipids, and free fatty acid, FFA). The FAs found in fats and oils (including extracted from foods), which are primarily bound to glycerol or in free form, may be quantitatively converted to FAMEs in 45 min by reaction with BF_3 in CH_3OH at 100 °C. Some limitations affect the use of this reagent. This reagent is not capable of direct digestion/FA methylation of food. More significantly, BF_3 in CH_3OH is toxic and it must be used only under a fume hood while carefully following all mandated safety precautions.

The two alkali-based reagents (2 M KOH-CH_3OH and 5% [m/v] CH_3ONa-CH_3OH) can perform only transesterification reactions. Therefore, they can methylate only oils and fats containing non-polar lipids. Transesterification is a rapid reaction, and it can be carried out even at room temperature. The main disadvantage of alkali-based reagents is their inability to methylate FFAs, and other polar lipids. Degraded oils, processed foods and fats extracted from foods that contain more than 2% FFAs should not be methylated using these reagents.

9.1 Laboratory preparation of methylating reagents

9.1.1 Preparation of 7% BF_3-CH_3OH reagent

This reagent is prepared by 1:1 dilution with CH_3OH of commercially available 14% BF_3-CH_3OH solution (from MilliporeSigma and other major chemical suppliers).

Under a fume hood, transfer 50 mL 14% BF_3-CH_3OH into a 100 mL glass container, then add 50 mL of anhydrous CH_3OH. This reagent has a limited shelf life and should be stored in the refrigerator (2–4 °C). This working solution is sufficient to analyse approximately 50 samples.

Aged BF_3-CH_3OH reagent can form artifacts with unsaturated FAs during methylation *(14)*. The presence of a white precipitate at the bottom of the bottle indicates that the reagent is degraded, and it should be discarded.

9.1.2 Preparation of 2 M KOH-CH_3OH

Prepare by weighing 11.2 g (± 0.1 g) of KOH pellets in a dry 250 mL Erlenmeyer flask equipped with a stopper. Add 100 mL CH_3OH and dissolve the KOH pellets. stirring the solution until fully dissolved. Do not add more solvent, 100 mL is the final volume. The reaction is exothermic. Once the KOH is dissolved, store the solution capped in a refrigerator (2–4 °C) until ready to use. A container made of an alkali-stable polymer, such as PTFE, is preferrable for long-term storage to avoid slow dissolution of glass. This solution is sufficient to analyse approximately 400 samples.

9.1.3 Preparation of 5% CH_3ONa-CH_3OH

The simplest way to prepare the 5% (m/v) CH_3ONa solution (approx. 1 M) in CH_3OH is dilution of 30% (m/v) CH_3ONa in CH_3OH, which is commercially available from chemical suppliers. This reagent may also be prepared by direct dissolution of pure sodium methoxide (in powder form) in CH_3OH, and by reaction of metallic sodium with CH_3OH (this is a last resort and is not recommended).

Into a 300 mL volumetric flask, pipette 50 mL of 30% (m/v) CH_3ONa-CH_3OH reagent and add 250 mL CH_3OH. Cap the container and mix gently for approximately 5–8 min using a magnetic stir bar of suitable size. Remove the magnetic stir bar and make up to the mark with CH_3OH. Store in the dark at 4 °C in a sealed glass container. This solution is stable for 1 week. Allow the solution to come to room temperature (20–25 °C) before use. This solution is sufficient to analyse approximately 40 samples. In the case of a smaller number of food samples for analysis, the preparation of the 5% (m/v) CH_3ONa-CH_3OH reagent volume can be adjusted accordingly. Whenever the container is open, the reagent will adsorb carbon dioxide and develop a white sodium carbonate precipitate.

9.1.4 Preparation of 0.5 M NaOH-CH_3OH

The 0.5 M NaOH-CH_3OH reagent may be prepared by direct dissolution of NaOH in CH_3OH. In a 1 L volumetric flask (preferably made of a polymer resistant to alkali, such as PTFE), weigh (or transfer) 20 g of NaOH pellets, then add CH_3OH, about 100 mL at a time. After every addition, mix gently, but keep in mind that the reaction is exothermic. Once all the NaOH pellets have dissolved, bring the solution volume to the 1 L mark.

10. Reagents for preparation of FAMEs directly from food samples (transesterification reagents)

Several transesterification methods have been described in the literature for directly converting FAs in the food product to FAMEs without the prior step of fat extraction *(8, 9, 10, 13, 14)*. This updated WHO reference protocol recommends two approaches.

10.1 0.5 M NaOH in CH_3OH followed by 14% (m/v) BF_3 in CH_3OH

The sequential reaction of lipids with 0.5 M NaOH in CH_3OH and 14% BF_3 in CH_3OH (m/v) is a highly efficient transesterification procedure that exploits the complementary benefits of acidic and alkaline catalysis *(8)*. Alkaline catalysis provides fast transesterification of TAGs but is unable to esterify FFAs, while acidic catalysis transesterifies TAGs slowly but it esterifies FFAs very efficiently. The ability of alkali to perform digestion of sample matrices and to free bound lipids permits the use of the combined sequential alkaline–acidic catalysis to analyse food samples without prior fat extraction. An international collaborative study performed by the AOCS *(8)* has demonstrated that this alkali–acid combined method is applicable to several food types including natural foods, processed foods, ready-to-eat foods, fats and oils, milk, cheese, other milk products, beverages, and animal and human tissue. Another advantage of this approach is that high-moisture food samples do not require removal of water before analysis. Further, 14% BF_3 in CH_3OH (m/v) is commercially available, and 0.5 M NaOH in CH_3OH may be purchased or prepared as described in section 9.1.4.

10.2 5% (m/v) CH_3ONa in CH_3OH

This reagent has been shown to be effective in the transesterification of FAs present in milk, milk products, infant formulae, and adult nutritional formulae *(9, 10)*. This reagent should be used only to methylate samples in which FAs occur only in the form of TAGs, and FFA content is less than 2% of total FAs.

11. Extraction of fat

Safety precautions

General laboratory precautions should be always observed. Protective gloves, laboratory coats, and safety glasses must always be worn during all steps of this method. See Annex 2 for a detailed description of safety precautions.

11.1 General information on the preparation of a test sample for fat extraction

For expressing the FA data as grams per 100 g of food or 100 g total fat, an IS should be added to the weighed test portion that will be used for analysis. See section 8 for the list of suitable internal standards and preparation of their solutions.

If the objective of the analysis is to obtain only the FA composition data as a weight percentage of total FAs, then addition of IS is not required.

The solvent extraction procedure detailed in section 6.3.2 on page 13 of the WHO simplified protocol is not included in this updated WHO reference protocol. There are two primary reasons for its exclusion: (a) the solvent extraction method has not yet been published in peer-reviewed journals, and (b) it has not been validated through an interlaboratory collaborative study. Furthermore, the solvent extraction procedure is limited to extracting only added fats and oils used in food preparation – it cannot extract naturally occurring fats that are chemically bound within the food matrix.

11.2 Category 1 foods: Pure oils, fats, shortenings, and vanaspati

Since these samples are constituted almost wholly of TAGs, there is no need to perform an extraction. These pure fats and oils can be directly converted to FAMEs via methylation as described in section 12. For the methylation step, prepare the test sample as follows.

Into a 25 mL screw cap glass test tube, or 50 mL round-bottom flask with glass stopper (if applying section 13.1 method), weigh to the nearest 0.1 mg an approximately 200 mg (sections 12 and 13.1 methods) or 50 mg (section 13.2 method) subsample of the homogenized representative test sample. Record the weight of the subsample, denote it as W_{TS} and add the IS of your choice (see below).

11.2.1 C13:0 TAG or C21:0 TAG IS

C13:0 TAG or C21:0 TAG should be selected as internal standard when applying the methylation procedures described in sections 12.1, 12.2 and 13.1.

Add 2 mL of either C13:0 TAG or C21:0 TAG (of approximate weight 10 mg; see section 8) to the test tube containing the test sample portion. Denote the actual weight of the

added IS as W_{IS}. Then evaporate the IS solvent in a stream of nitrogen, flush the tube with nitrogen, place the cap and proceed to methylation step as per section 12 or combined methylation/extraction as described in section 13.1.

11.2.2 C11:0 FAME combined with C13:0 TAG IS

This IS solution should be used when applying the combined methylation/extraction described in section 13.2. The addition of the combined C11:0 FAME and C13 TAG IS solution will be described in section 12.3.

If C11:0 FAME in combination with C13:0 TAG is selected as the IS, add accurately, using a volumetric pipette, 5 mL of the C11:0 FAME + C13:0 TAG IS solution (see section 8). The weight of the C11:0 FAME in 5 mL of the combined stock solution is approximately 10 mg. Denote the actual weight of C11:0 FAME as W_{IS}. Flush the tube with nitrogen, place the cap and proceed to transesterification step as per section 13.2. Do not remove the IS solvent (MTBE).

11.3 Category 2 Foods: Margarine, vegetable oil spread and butter samples

1. Make a conservative estimate of the weight of the sample that would yield 200 mg of fat if applying methylation methods from sections 12 and 13.1, or 50 mg if applying methods from section 13.2. In a 25-mL screw cap glass test tube, weigh the chosen amount of homogenized sample composite (see section 4.6). Record its weight (W_{TS}).
2. Add the selected IS as outlined in section 8. If applying methylation methods from sections 12.3 or 13.1, or the goal of the analysis is to determine the FA composition as a weight percentage of total FAs, no IS should be added at this point.
3. Dissolve the test portion containing the added IS in 10 mL n-hexane or n-heptane. A warm-water bath (50–60 °C) may be used to help dissolve the sample. Transfer quantitatively to a 250 mL separatory funnel. To ensure complete transfer, rinse the tube three or four times with 5 mL portions of n-hexane or n-heptane and add the rinsing solvent to the separatory funnel.
4. Add 50 mL n-hexane or n-heptane, shake thoroughly for 3–4 min, add 50 mL distilled water. Shake gently for about 1 min. Allow the layers to separate. The top layer contains the extracted fat plus the added IS.
5. Slowly transfer the top layer to an Erlenmeyer flask (capacity 200 mL). When collecting the n-hexane or n-heptane layer, make sure not to pipet any of the bottom (aqueous) layer.
6. Add again 50 mL n-hexane or n-heptane to the aqueous layer in the separatory funnel, shake gently for 1–2 min and allow the layers to separate.
7. Drain out the bottom layer. Transfer the top n-hexane or n-heptane layer to the 200 mL Erlenmeyer flask containing the extract from step 5. Again, make sure not to transfer any of the bottom layer.
8. Add anhydrous sodium sulfate. The amount should be sufficient to dry the n-hexane or n-heptane extract. Gently swirl the flask and allow the sodium sulfate to settle. The presence of free-floating sodium sulfate indicates that sufficient sodium sulfate has been added. Allow it to dry for about 15 min.

9. Filter and collect the n-hexane or n-heptane extract into a round-bottom flask (capacity 200 or 250 mL). Slowly evaporate the extract in a roto-evaporator. The remaining residue in the flask consists of the extracted lipids and the added IS.
10. Dissolve the fat extract in the round-bottom flask in 4–5 mL n-hexane or n-heptane. Transfer it quantitatively to a 25 mL glass test tube (fitted with PTFE-lined screw cap). To assure complete transfer, rinse the round-bottom flask three times with 3 mL of n-hexane or n-heptane.
11. Evaporate the solvent using a hot-water bath (or heating block) and a slow stream of nitrogen. If the extraction procedure was followed accurately, the weight of the extracted material should be approximately 210 mg.
12. Methylate the above residue (extracted fat + IS) according to the procedure described in section 12.

11.4 Category 3 foods (all products not in categories 1 or 2)

For all the other foods, fats are extracted by applying the hydrolytic extraction methods described in the AOAC Official Method 996.06 (acidic hydrolysis for most products, alkaline hydrolysis for dairy products, and a combination of alkaline-acidic hydrolysis for cheese) *(11)*. The fat released by the hydrolysis is then extracted using a mixture of n-hexane (or n-heptane) and diethyl ether. The fat extract is recovered by evaporating the solvent.

For milk, milk products, infant formula and adult nutritionals, the method described in section 13.2 (adapted from the International Standard AOAC 2012.13 / ISO 16958 | IDF 231:2015 *(9, 10)*) may be used as an alternative to the hydrolytic extraction procedures of AOAC 996.06 *(11)*.

11.4.1 Fat Extraction from all foods (excluding ruminant milk, milk products and cheese) applying the AOAC 996.06 acidic hydrolysis method

The acidic hydrolysis procedure described below is adapted from the AOAC Official Method 996.06 *(11)* with some minor modifications. This procedure is applicable to all foods prepared using unrefined oils, refined fats and oils, PHOs, fully hydrogenated oils or mixtures of PHOs and ruminant fats. This procedure, however, is not recommended for ruminant milk and cheese. For ruminant milk and cheese, use the alkaline and the combined alkaline–acidic hydrolysis procedures described in sections 11.4.2 and 11.4.3, respectively.

Make a conservative estimate of the weight of the food sample that would yield approximately 200 mg fat. Weigh to the nearest 0.1 mg in a glass test tube or bottle (100–125 mL capacity with a PTFE-lined screw cap) the estimated amount of homogenized composite (see section 4.6). Record the weight as W_{TS}. Add approximately 100 mg pyrogallic acid, 2.0 mL of either C13:0 TAG or C21:0 TAG IS solution (see section 8 for the preparation of IS stock solutions) and 2.0 mL ethanol. Mix well until the entire test portion is dispersed. If the objective of the analysis is to obtain only the FA composition data as a weight percentage of total FAs, then addition of IS is not required.

1. Add 10 mL of 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.

2. After digestion, remove the test tube from the heating block or water bath and allow it to cool to room temperature (20–25 °C). Add 5 mL ethanol and mix well.
3. Add 25 mL diethyl ether. Place the cap on the test tube and vortex for 5 min.
4. Add 25 mL n-hexane or n-heptane and vortex for 5 min.
5. Transfer the contents to a 250 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.
6. 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, into a 250 mL round-bottom flask.
7. Slowly evaporate the collected organic layer in a rotary evaporator set at 40 °C. The residue is the extracted fat.
8. Dissolve the extracted fat in 2–3 mL n-hexane or n-heptane and 2–3 mL diethyl ether. Quantitatively transfer the mixture using a disposable glass pipette to the pre-weighed test tube that will be used for methylation (a 15–25 mL screw cap glass test tube) and then evaporate to dryness in 40 °C water bath under a nitrogen stream. Weigh the test tube containing the extracted oil. Record the weight and calculate the weight of the extracted oil (W_{FAT}) as the difference in weight of the test tube with and without the oil. W_{FAT} should be approximately 200 mg.
9. Methylate the extracted fat (approximately 200 mg) immediately as per section 12. If the laboratory is not ready for methylation immediately, flush the tube with nitrogen, cap the tube and store it in a refrigerator (2–8 °C). However, methylation should be done within 1–2 days.

11.4.2 Fat Extraction from ruminant milk, and milk products (excluding cheese) using the AOAC alkaline hydrolysis method.

This alkaline hydrolysis procedure was adapted from AOAC Official Method 996.06 *(11)*, with some minor modifications.

1. Make a conservative estimate of the weight of the milk sample that would yield approximately 200 mg fat. Weigh to the nearest 0.1 mg the selected amount of homogenized composite (see section 4.6) in a glass test tube or bottle (100–125 mL capacity with a PTFE-lined screw cap). Record the weight as W_{TS}. Add approximately 100 mg pyrogallic acid, 2 mL C13:0 TAG or C21:0 TAG IS solution (see section 8 for the preparation of IS solutions) and 2 mL ethanol. 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 indicator solution. Keep the solution basic (pink) with the 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 n-hexane or n-heptane and vortex for 5 min.
8. Transfer the contents to a 250 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 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. Dissolve the extracted fat in 2–3 mL n-hexane or n-heptane and 2–3 mL diethyl ether. Quantitatively transfer the mixture using a disposable glass pipette to the pre-weighed test tube that will be used for methylation (a 15–25 mL screw cap glass test tube) and then evaporate to dryness in 40 °C water bath under nitrogen stream. Weigh the test tube containing the extracted crude oil. Record the weight and calculate the weight of the extracted oil (W_{FAT}) as the difference in weight of the test tube with and without oil. W_{FAT} should be approximately 200 mg.
12. Methylate the extracted fat (approximately 200 mg) immediately as per section 12. If the laboratory is not ready for methylation immediately, flush the tube with nitrogen, cap the tube, and store it in a refrigerator (2–8 °C). However, methylation should be done within 1–2 days.

11.4.3 Fat extraction from cheese using the AOAC combined alkaline–acidic hydrolysis.

This base–acid hydrolysis procedure was adapted from AOAC Official Method 996.06 *(11)*, with some minor modifications.

1. Make a conservative estimate of the weight of the cheese sample that would yield approximately 200 mg fat. Weigh to the nearest 0.1 mg the selected amount of homogenized composite (see section 4.6) in a glass test tube or bottle (100–125 mL capacity with a PTFE-lined screw cap). Record the weight as W_{TS}. Add approximately 100 mg pyrogallic acid. If the objective is to obtain FA data as grams per 100 g food or 100 g fat, add 2 mL of either C13:0 TAG or C21:0 TAG IS solution (see section 8) and 2 mL ethanol. 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 steam bath or heating block and allow it 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 n-hexane or n-heptane and vortex for 5 min.
9. Transfer the contents to a 250 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.
0. 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. Dissolve the extracted fat in 2–3 mL n-hexane or n-heptane and 2–3 mL diethyl ether. Quantitatively transfer the mixture using a disposable glass pipette to a pre-weighed test tube that will be used for methylation (a 15–20 mL screw cap glass test tube) and then evaporate to dryness in 40 °C water bath under nitrogen stream. Weigh the test tube containing the extracted oil. Record the weight and calculate the weight of the extracted oil (W_{FAT}) as the difference in weight of the test tube with and without oil. W_{FAT} should be approximately 200 mg.
13. Methylate the extracted fat (approximately 200 mg) immediately as per section 12. If the laboratory is not ready for methylation immediately, flush the tube with nitrogen, cap the tube and store it in a refrigerator (2–8 °C). However, methylation should be done within 1–2 days.

12. Methylation

Methylation is defined as the process to convert free and bound FAs to FAMEs to determine the FA composition by GC. This protocol recommends using one of the following methylating reagents: 7% (m/v) BF_3-CH_3OH, 2 M KOH-CH_3OH or 5% (m/v) CH_3ONa-CH_3OH. See section 8 for details of preparation of these reagents and for their application limitations.

12.1 Methylation using 7% BF_3 in CH_3OH

Methylation using this reagent is applicable to all types of fats and oils, including fat extracted from food samples.

12.1.1 Addition of the methylating agent

Category 1 sample refined (salad or cooking) oils and fats, shortenings (from section 11.2)

Bring to room temperature the test tubes prepared in section 11.2, containing the IS (if added) and the 200 mg fat sample test portion.

Add 2.0 mL 7% BF_3-MeOH reagent (section 9.1.1) and 1.0 mL toluene. Purge the test tube with nitrogen and seal it with the PTFE-lined screw cap. Then follow steps 1–5 in section 12.1.2 below.

Fats extracted from category 2 and 3 foods (from sections 11.3 and 11.4)

Bring to room temperature the test tubes prepared in section 11.3, and 11.4 containing the IS (if added) and the 200 mg of fat extracted from the sample test portion.

Add 2.0 mL 7% BF_3-CH_3OH reagent (see section10.4) and 1.0 mL toluene to the extracted fat sample (approximate weight 200 mg). Seal the test tube with the PTFE-lined screw cap. Then follow steps 1–5 in section 12.1.2.

12.1.2 FAME formation and extraction

1. Heat the test tube in an oven or heating block for 45 min at 100 °C. Gently shake the tube every 10–12 min. Note: solvent evaporation from the tubes indicates inadequate sealing. If this happens, discard the solution, and repeat the entire FAME preparation procedure. Investigate the cause of the leak and discard the test tube if dents are discovered in the opening.
2. 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 n-hexane or n-heptane, cap the tube, shake for 1 min, and allow the layers to separate for about 10 min. Carefully transfer the top layer containing the FAMEs using a disposable glass pipette

to another 15–20 mL glass tube containing approximately 1 g anhydrous sodium sulfate. When transferring, make sure not to include any of the aqueous layer.

3. Add sufficient n-hexane or n-heptane to the FAME solution to reach the 10 mL volume. The expected concentration of this FAME solution is approximately 20 mg/mL. If the amount of the methylated fat, oil or sample extract is outside the 150–200 mg range, adjust the volume of added n-hexane or n-heptane accordingly to achieve the desired FAME concentration.
4. Fill an autosampler vial with the FAME solution and proceed to GC analysis as per section 14, or store it in a freezer (–15 °C to –18 °C), ensuring the container is tightly capped.

12.2 Methylation using 2 M KOH in CH_3OH

WARNING: Do not use this reagent for fats and oils containing more than 2% FFAs. Fat extracted using the AOAC hydrolytic methods (as per sections 11.4.1, 11.4.2 and 11.4.3) often contains large amounts of FFAs. Therefore, this reagent should not be used for methylating fats extracted using these sample digestions.

1. Bring to room temperature the test tubes prepared following instructions in sections 11.2 and 11.3, which contain the IS (if added) and the 200 mg of fat from the sample test portion.
2. Add 5 mL of n-hexane or n-heptane and 0.5 mL 2 M KOH in CH_3OH (see section 8) to the test tube.
3. Place the cap and manually shake the tube vigorously for 2–3 min. Even though the reagent or n-heptane or n-hexane (the latter contains the extracted fat) remain as two distinct phases, this procedure quantitatively converts the triacylglycerol components of the lipid extract into FAMEs. Allow layers to separate for 10 to 15 min. The top layer contains the FAMEs dissolved in n-heptane (or n-hexane). The tube can be centrifuged for 5 min at 500 × g to reduce the waiting time.
4. Using a disposable glass pipette, transfer the upper layer to another 10–20 mL test tube containing 1 g of anhydrous sodium sulfate. Add 5 mL more n-heptane (or n-hexane), mix for 30 sec and wait for the sodium sulfate to settle. Centrifuge at 500 × g if necessary. The expected concentration of the FAME solution is approximately 20 mg/mL. If the amount of methylated fat, oil or sample extract is outside the 150–200 mg range, adjust the volume of added n-hexane or n-heptane to achieve the desired FAME concentration.
5. Fill an autosampler vial with the FAME solution and proceed to GC analysis as per section 14, or store it in a freezer at –15 °C to –18 °C, ensuring the container is tightly capped.

12.3 Methylation using 5% (m/v) CH_3ONa in CH_3OH

WARNING: Do not use this reagent for fats and oils containing more than 2% FFAs. Fat extracted using hydrolytic methods *(11)* (as per sections 11.4.1, 11.4.2 and 11.4.3) methods often contains large amounts of FFAs. Therefore, this reagent should not be used for methylating fats extracted via such hydrolysis procedures.

The procedure described below was taken from AOAC 2012.13 / ISO 16958 | IDF 231:2015 *(9, 10)* with minor modifications.

1. Bring to room temperature the test tubes prepared following instructions in sections 11.2, and 11.3, which contain the two IS (if added, including their solvent) and about 50 mg of fat from the sample test portion.
2. Add 5 mL of 11:0 FAME + 13:0 TAG in MTBE IS solution. Do not remove the solvent.
3. Add 5 mL of 5% (m/v) CH_3ONa in CH_3OH solution (see section 9.1.3). The methylation time starts with the addition of the first drop of the 5% (m/v) CH_3ONa in CH_3OH. Close the tube tightly and shake well for 10 sec using a vortex mixer.
4. At 180 sec after the start time, open the tube and add 2 mL n-hexane or n-heptane. At 210 sec after the start time, add 10 mL disodium hydrogen citrate and NaCl in water (see section 6 for the preparation of this solution). Shake gently using a vortex mixer for 30 sec. The total reaction time must not exceed 240 sec from the addition of the first drop of CH_3ONa in CH_3OH. Centrifuge the tube to obtain a clear separation between the bottom layer and the upper layer. Transfer 1 mL of upper layer to a GC autosampler vial and add 0.250 mL of n-hexane or n-heptane to obtain 20 mg/mL FAME concentration.
5. Proceed to GC analysis (as per section 14) or store the FAME solution in a freezer at –15 °C to –18 °C, ensuring the container is tightly capped.

13. Preparation of FAMEs directly from foods by combination extraction and methylation

The two steps of FAME sample preparation – fat extraction followed by methylation – can be replaced by a combined fat extraction and methylation. The FA moieties are methylated while still in the food matrices, and successively extracted as FAME derivatives ready for GC analysis.

This WHO protocol recommends the two procedures given below for direct preparation of FAMEs from food matrices.

13.1 Direct transesterification of lipids in foods using alkali hydrolysis

The procedure described below is taken from the AOCS Official Method Ce 2b-11 *(8)*. The collaborative study supporting the AOCS Official Method Ce 2b-11 shows that this method is applicable to analysis of various types of food samples including solid and liquid foods, beverages, animal tissues, and oils and fats. Also, the collaborative study demonstrated that material with high moisture content does not require drying before analysis.

Some selected foods – for example, extruded cat and dog foods – were reported to provide an underestimation of the total fat content. However, this is not a drawback, as this AOCS method gives accurate data for all human foods and the analyses of animal foods are out of the scope of this WHO protocol.

Procedure:

1. Bring the homogenized representative composite test sample (prepared as per section 4.6) to room temperature (20–25 °C).
2. Add (using a 0.5–5 mL class A volumetric pipette) enough IS (either C13:0 TAG or C21 TAG, as outlined in sections 8.1.1 and 8.1.2, respectively) into a tared 50 mL round flat-bottom flask, so that after the test portion is added, the concentration of the final solution is between 0.05 and 0.1 mg per 1 mg of oil/fat contained in the subsample of the homogenized composite test food sample. See Table 6 to determine the appropriate volume of IS to add based on upon the expected fat/oil concentration of the test portion. Evaporate the IS solvent (n-hexane, n-heptane or chloroform). Skip this step if analysing fats extracted according to sections 11.2 and 11.3 (IS already added), or the goal of the analysis is to determine the FA composition as a weight percentage of total FAs.
3. Add the appropriate mass of the subsample from the homogenized representative composite (point 1, above) into the tared 50 mL round flat-bottom flask containing the IS (see Table 6 for the ideal amount). This will yield a final concentration of

15–20 mg fat/oil per mL of final solution. When samples contain less than 20% fat or oil, the range will be <20 mg fat/oil. For samples containing less than 10% fat, use 1000 mg of sample (maximum). Record the weight to a precision of at least 0.1 mg, as this amount will be used for the total fat content and FA composition determinations. Record this weight as W_{TS}.

4. Add 2 or 3 porous boiling chips.
5. Add 1 mg pyrogallol per milligram of oil analysed.
6. Add 5 mL of 0.5 M NaOH-CH_3OH reagent to the round flat-bottom flask (for preparation see section 10.1)
7. Attach the water condenser to the round flat-bottom flask.
8. Reflux for 15 min after boiling begins.
9. Add 5 mL 14% BF_3-CH_3OH reagent to the boiling flask. Reflux for an additional 2 min.
10. Add 5 mL of n-hexane or n-heptane.
11. Remove heat source, cool the flask to room temperature, then add saturated NaCl solution until the organic layer is in the neck of the 50-mL round flat-bottom flask. Stopper the flask and shake. Allow the layers to separate and transfer a clear portion of the top layer (n-hexane or n-heptane) with a transfer pipette into a 5 mL test tube containing 50 mg sodium sulfate or into a 2 mL autosampler vial containing 50 mg sodium sulfate. Analyse 1 µL of the clear solution (FAME in n-hexane or n-heptane) by GC for FA composition determination.

13.2 Direct transesterification of milk, milk products, infant formulas and adult nutritionals using 5% (m/v) CH_3ONa in CH_3OH

This procedure was adopted from AOAC 2012.13 / ISO 16958 | IDF 231:2015 (Fat extraction from milk, milk products, infant formula and adult nutritionals) *(9, 10)*). This combined transesterification/extraction method can be used only for direct preparation of FAMEs from milk, milk products, infant formulas and adult nutritionals. It can be used as an alternative to the hydrolytic extraction procedures of AOAC 996.06 *(11)* for milk and milk products, infant formula and adult nutritionals. Cheese samples cannot be transmethylated directly using 5% (m/v) CH_3ONa-CH_3OH reagent because analysis of cheese products requires prior fat extraction, Alternatively, cheese samples can be directly transmethylated using the alkali hydrolysis method of AOCS Official Method Ce 2b-11 *(8)*.

Furthermore, it is not known whether 5% (m/v) CH_3ONa-CH_3OH reagent is capable of transmethylating other types of foods, a matrix extension study may be necessary.

WARNING: Do not use this reagent for fats and oils containing more than 2 g FFAs per 100 g of total fat. This procedure cannot be applied without the addition of the 11:0 FAME/13:0 TAG IS solution.

Sample preparation:

1. Liquid and powder milk and infant formula with a fat content ≥ 1.5% m/m

Bring the sample to room temperature and shake vigorously before use. Ensure that the sample is homogenous.

2. Liquid and powder milk and infant formula with a fat content <1.5% m/m

Analysis of these products requires prior fat extraction in accordance with ISO 14156 | IDF 172: 2001 *(15)*, taking care to evaporate the extraction solvents by heating them to a temperature not higher than 40 °C to avoid the degradation of long-chain polyunsaturated FAs (LC-PUFA).

Procedure:

1) Into a 25 mL centrifuge tube with a screw cap, weigh to the nearest 0.1 mg a quantity of sample that will give 50 mg fat in the tube. For example, for a product containing 26 g fat per 100 g, the corresponding sample weight is approximately 190 mg.
2) For a powder sample, add 2.0 mL water using a micropipette. For liquid samples, water addition is not required. Close the tube and dissolve the sample gently using a vortex mixer. Wait for 15 min at room temperature.
3) Add 5 mL C11:0 FAME/C13:0 TAG in MTBE IS solution (section 8.1.3).
4) Add 5 mL of 5% (m/v) CH_3ONa in CH_3OH solution (section 9.1.3). The methylation time starts with the addition of the first drop of 5% (m/v) CH_3ONa in CH_3OH solution. Close the tube tightly and shake well using a vortex mixer.
5) At 180 sec after the start time, open the tube and add 2 mL n-hexane or n-heptane. At 210 sec after the start time, add 10 mL disodium hydrogen citrate and NaCl in water (see section 6 for the preparation of this solution). Shake gently using a vortex mixer for 30 sec. The total reaction time must not exceed 240 sec from the addition of the first drop of 5% (m/v) CH_3ONa in CH_3OH solution. Centrifuge the tube to obtain a clear separation between the bottom layer and the upper layer. Transfer 1 mL of the upper layer to a GC autosampler vial and add 0.250 mL of n-hexane or n-heptane to obtain 20 mg/mL FAME concentration.
6) Proceed to GC analysis (as per section 14) or store the FAME solution in a freezer at –15 °C to –18 °C, ensuring the container is tightly capped.

14. Gas chromatography analysis of FAMEs

The prepared FAMEs are analysed by GC employing 100 m × 0.25 mm capillary columns coated with 100% BCS. The GC may be operated either in isothermal (see section 14.1) or temperature programme mode (section 14.2). Although both approaches may be used to analyse all types of FAME samples considered in this protocol, pure vegetable fats and oils are best analysed using the isothermal method, and the temperature programme is preferred for the analysis of samples containing fats from ruminant sources.

The carrier gas flow rate depends on the carrier gas used (hydrogen or helium). Laboratories may need to fine-tune the column oven temperature to compensate for column-to-column variability and column ageing. The selected conditions must provide the separation between *cis* and *trans* isomers of C18:1, C18:2 and C18:3 as shown in Fig. 1–12.

As outlined in the AOCS Official Method Ce 1h-05 using hydrogen as the carrier gas for FAMEs originating from food samples containing no ruminant fats, isothermal operation at 180 °C with 1 mL/min carrier gas flow rate is recommended *(16)*. Isothermal operation provides the optimum resolution of C18:1, C18:2 and C18:3 TFAs from other FAs (see Fig. 2–5 and Fig. 11). However, the main disadvantages of isothermal operation are the incomplete resolution of C4–C8 short-chain FA from the solvent front and short-chain contaminants, and the long elution time of long-chain FA, especially the highly unsaturated ones (see Fig. 2–4). These limitations do not affect the analysis of samples not containing fats or oils of ruminant or marine origin.

The GC temperature programmes described in AOAC 2012.13 / ISO 16958 | IDF 231:2015 *(9, 10)* and its modified version *(17)* are recommended for analysis of FAME samples containing milk fats, dairy products, and ruminant fats in general.

14.1 Isothermal operation

The following GC operating conditions have been adopted from AOCS Official Method Ce 1h-05, revised 2017 *(16)*. 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; and
- GC injection volume
  - inject 1 µL FAME solution (concentration approximately 20 µg/µL in n-hexane/n-heptane); higher FAME concentration may compromise the resolution 13*t*/14*t*-C18:1 from 9*c*-C18:1, but lower concentration may result in unsuitable limit of quantification
  - if the autosampler is used, do not keep the FAME samples on the tray for longer than 72 h.

Examples of GC chromatograms obtained applying the above isothermal conditions are shown in Fig. 1–5 and Fig. 16. These GC conditions are optimized for the separation of C18:1, C18:2 and C18:3 TFAs from FA, including the resolution of C18:3 TFA isomers from 11*c*-C20:1.

14.2 Temperature programme operation

14.2.1 AOAC 2012.13 / ISO 16958 | IDF 231:2015 temperature programme operation

The following GC operating conditions have been adopted from AOAC 2012.13 / ISO 16958 | IDF 231:2015 Official Method *(9, 10)*.

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; and
- GC injection volume
  - inject 1 µL FAME solution (concentration approximately 20 µg/µL in n-hexane/n-heptane) using the GC micro syringe (a FAME solution concentration of 20 µg/µL is ideal 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. 6–10 and Fig. 17 for GC chromatograms obtained with the above temperature programme conditions. Note that with this temperature programming 9*t*,12*c*,15*c*-C18:3 is eluting with 11*c*-C20:1, which affects quantitation of refined fats and oils (see Fig. 7, 9 and 17)

14.2.2 Modified temperature programme operation

The following GC operating conditions have been adapted from P Delmonte *(17)* who made an improvement to the temperature programme of AOAC 2012.13 / ISO 16958 | IDF 231:2015 *(9, 10)*. The addition of a temperature plateau at 184 °C optimized the elution of C18:3 FAs versus C20:1, obtaining the elution of *c*11-C20:1 after the mono-

trans-C18:3n-3 but before C18:3n-3. Regarding the elution of the other FAMEs contained in refined/hydrogenated vegetable oils and ruminant fats, the modified method provides a nearly undistinguishable elution profile compared to the one obtained applying the chromatographic conditions described in AOAC 2012.13 / ISO 16958| IDF 231:2015 *(9, 10)*. This minor modification permits a more accurate quantification of mono-*trans*-C18:3n-3 FA, still preserving the resolution of C18:1, C18:2 TFA, thus improving the total TFA measurement accuracy for samples containing refined oils. See Fig. 11–15 and Fig. 18 for GC chromatograms obtained by applying these temperature programme conditions with hydrogen as the carrier gas.

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 184 °C, hold 25 min, ramp at 4 °C/min to 225 °C, hold 20 min; and
- GC injection volume
  - inject 1 µL FAME solution (concentration approximately 20 µg/µL in n-hexane/n-heptane) 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.

Some of the settings described above, such as head pressure, oven temperature and split ratio, could be instrument specific. Minor modifications to these parameters may be necessary to achieve resolution of C18 TFAs as shown in Fig. 1–18. The minor modifications, however, must not affect the elution order and the resolution of other FAs. In addition, the resolution factor between the 13*t*+14*t*-C18:1 and 9*c*-C18:1 must be greater than 1, as shown in Fig. 3 and Fig. 5.

14.3 Identification of FAMEs in GC separations

Whenever available, reference FAME standards should be used for identification of FAMEs in GC separations. In the absence of other identity information, such as mass spectra, FAMEs are identified by comparing their retention times with those of reference FAME standards. Individual and mixed reference FAME standards (see section 7 for a description of reference FAMEs) are used to define the resolution pattern and to verify that the chromatographic separation is suitable for the quantification of TFAs, as outlined in section 14.4.

Many FAMEs found in dietary fats and oils, including TFAs, are not readily available as reference materials. In other instances, samples contain FAs with the same retention times as those of reference FAME standards. Thus, the identification achieved using reference FAMEs may be misleading and, therefore, the identification of FAMEs in the separations of samples should be complemented and verified by other means – for example, by comparing the GC patterns shown in Fig. 1–18. Comparison of the elution

pattern or retention times with the GC chromatograms published in the literature is also helpful for establishing the identification of FAMEs. These approaches are reliable and inexpensive ways of identifying GC FAME peaks. Comparison with chromatograms published in literature has been used in most laboratories over the past several decades and allows them to conduct FAME analysis, especially when FAME reference standards cannot be procured.

14.4 Background information on TFAs and their GC separation using 100 m × 0. 25 mm BCS capillary columns.

The following background information is provided to help laboratory personnel establish accurate identification of the C18 TFA peaks (as FAME).

Both in PHOs and ruminant fats, TFAs occur almost exclusively with the C18 chain length *(9, 10, 17–24)* (see Fig. 3). C14:1, C16:1 and C17:1 TFAs may be present in non-hydrogenated vegetable oils and ruminant fats in amounts close to their limits of quantification but it can be assumed that these FA provide a negligible contribution to the total TFA content. In addition, very few C14:1, C16:1 and C17:1 TFA reference materials are commercially available. For these reasons, quantification of TFA in dietary fats should focus on the measurement of C18 TFAs.

14.4.1 C18:1 TFAs

In PHOs and dairy fats, the FAs with 18 carbons and one *trans* double bond (C18:1 TFA) represent the major group of TFA isomers *(9, 10, 17–24)*. In highly hydrogenated PHOs, the C18:1 TFA isomer group accounts for approximately 90–95% of total TFAs. In PHOs and dairy fats, C18:1 TFAs occur with double bond positions from 4 to 16 *(18–19, 24)*. Not all these 13 TFAs are available as reference materials, but they may be identified based on the chromatographic separation and elution patterns shown in Fig. 3, 8 and 13. Applying either isothermal or temperature programme methods, the C18 TFAs with double bond from 4*t* to 14*t* are eluted ahead of oleic acid (9*c*-C18:1) and only partially separated from each other; therefore, for convenience they may be integrated as an unseparated cluster (see Fig. 16–18). If the GC separation is tuned correctly, the 16*t*-C18:1 should be eluted between 13*c*-C18:1 and 14*c*-C18:1 (see Fig. 2–5 and Fig. 16). The total C18:1 TFA content is determined by summing the amount of the 4*t*-14*t* cluster and 16*t*-C18:1.

The only C18:1 TFA isomer that cannot be measured using 100 m × 0.25 mm BCS column is 15*t*-C18:1, because it co-elutes with 9*c*-C18:1 (oleic acid) or 10*c*-C18:1 *(18, 20, 21, 23, 24)*. Exclusion of 15t-C18:1 from the total C18:1 TFA quantification is a commonly accepted underestimation. Whenever present, 6*c*/7*c*/8*c*-C18:1 FA are eluted concurrently with the 4*t*-14*t*-C18:1 cluster (Fig. 3, 8 and 13), providing an overestimation of C18:1 TFA. From a practical point of view, the underestimation from the missing 15*t*-C18:1 and overestimation from the 6*c*/7*c*/8*c*-C18:1 contribution may compensate each other.

14.4.2 C18:2 TFA (including CLA)

Fatty acids characterized by an 18-carbon chain and two double bonds, at least one of them in the *trans* configuration, are defined as C18:2 TFAs. Those C18:2 TFAs in which the two double bonds are conjugated are commonly called conjugated linoleic acid (CLA) and they are discussed independently because they have significantly longer GC retention times than unconjugated C18:2 TFAs.

PHOs often contain an assortment of C18:2 TFAs. The amount of these TFAs could reach up to 6% (of total FAs) in mildly hydrogenated oils and 0.1% in heavily hydrogenated oils *(18, 21, 23, 24)*. Various nonconjugated C18:2 TFAs were identified in mildly hydrogenated oils including 9*c*,13*t*-C18:2; 9*c*,12*t*-C18:2; 9*t*,12*c*-C18:2; and 9*t*,15*c*-C18:2 + 10*t*,15*c*-C18:2 (the latter two elute together) (*18, 21, 23, 24)*. Ruminant fats also contain non-negligible amounts of various nonconjugated C18:2 TFAs, many of which are unresolvable from each other. The refining of fats and oils, and the thermal stress due to food preparation (e.g. deep-frying), also result in formation of nonconjugated C18:2 TFAs (generally 0.1–0.3% of total FA) *(21, 24)*, consisting of the three nonconjugated geometrical isomers of linoleic acid (9*t*,12*t*-C18:2; 9*c*,12*t*-C18:2; 9*t*,12*c*-C18:2).

All nonconjugated C18:2 TFAs are eluted between 15*c*-C18:1 and linoleic acid (9*c*,12*c*-C18:2) and they may be integrated as a single cluster (see Fig. 2–4 and Fig. 11). Alternatively, as when the baseline is not stable, the contribution from all FAMEs between 15*c*-C18:1 and 9*c*,12*c*-C18:2 may be summed. The amount of 16*c*-C18:1 should be excluded from this summation. The same approach may also be applied to the analysis of deodorized and thermally stressed oils, but in this simpler case, the three geometrical isomers of linoleic acid may be summed (see Fig. 2 and Fig. 11).

Conjugated C18:2 TFAs (CLA) are present primarily in ruminant fats, and in minor amounts in refined and thermally stressed oils. Ruminant fats contain primarily 9*c*,11t- and 7*t*,9*c*-C18:2, which are eluted as a single peak after C18:3n-3 (α-linolenic acid). Other CLA isomers (including 10*t*,12*c*-, 9*t*,11*c*- and 10*c*,12*t*-C18:2) may also occur in minor amounts, and are eluted after 9*c*,11*t*-C18:2. The C21:0 FA is also eluted in the same retention region, and its elution relative to CLA isomers is highly variable. Before including CLA in the total C18:2 TFA quantification, especially the ones occurring in lower amounts, it should be verified that C21:0 is not included.

14.4.3 C18:3 TFA

With very few exceptions, the C18:3 TFAs found in fats and oils consist of the geometric isomers of α-linolenic acid (C18:3n-3 or 9*c*,12*c*,15*c*-C18:3). These TFAs are not formed in appreciable amounts during partial hydrogenation because 9*c*,12*c*,15*c*-C18:3 is quickly depleted as hydrogenation progresses. Ruminant fats are also not significant sources of C18:3 TFAs.

The C18:3 TFAs primarily occur in refined and thermally stressed oils containing high amounts of 9*c*,12*c*,15*c*-C18:3, such as canola and soybean oils, and may account for up to 3.0% of total FAs. These C18:3 TFAs consists of mono- and di-*trans* isomers (9*t*,12*c*,15*t*-C18:3; 9*c*,12*c*,15*t*-C18:3; 9*c*,12*t*,15*c*-C18:3; and 9*t*,12*c*,15*c*-C18:3) *(23)*. Of these four isomers, the most abundant ones in refined oils are 9*c*,12*c*,15*t*-C18:3 and 9*t*,12*c*,15*c*-C18:3 (see Fig. 2 and Fig. 11).

Applying the isothermal elution method (Fig. 2 and Fig. 16) and the revised temperature programme (Fig. 12 and Fig. 18), the C18:3 TFAs generated from the 9*c*,12*c*,15*c*-C18:3 isomerization, are eluted between C20:0 and 11*c*-C20:1 (gadoleic acid) and may be integrated as a single cluster. Alternatively, all FAs occurring in this elution interval may be quantified independently and summed up to determine the total C18:3 TFA content.

The isothermal GC elution temperature may need to be adjusted to optimize the separation between 11*c*-C20:1 and 9*t*,12*c*,15*c*-C18:3 *(17, 18, 19, 23, 24)*. For

optimal separation, 11*c*-C20:1 should elute precisely in the centre point between 9*t*,12*c*,15*c*-C18:3 and 9*c*,12*c*,15*c*-C18:3. Increasing the elution temperature (by 0.5–1.0 °C increments) moves the elution time of 11*c*-C20:1 towards that of 9*t*,12*c*,15*c*-C18:3, while lowering elution temperature moves the elution time of 11*c*-C20:1 towards that of 9*c*,12*c*,15*c*-C18:3. Since ageing of the column stationary phase alters the relative retention of FAMEs with different numbers of double bonds, it is necessary to periodically re-optimize the elution temperature.

The quantification of 9*c*,12*c*,15*c*-C18:3 TFAs by applying the temperature programme method described in section 14.2.1 can be performed using the same approach of integrating as a cluster of all FAs eluting between C20:0 and 11*c*-C20:1. The co-elution of 11*c*-20:1 and 9*t*,12*c*,15*c*-C18:3 may result in an underestimation or overestimation of the sample TFA content depending on the inclusion or exclusion of this peak in the TFA quantification. For all methods, the presence of 9*c*-C20:1 results in a minor overestimation of the total C18:3 TFAs, especially for ruminant fats.

14.5 Criteria for assessing the acceptability of GC chromatograms.

After each GC analysis, the FAME chromatograms of the test food sample should be examined to ensure that the acquired GC separation is suitable for the determination of the TFA content. The FAME profiles should be comparable to those shown in Fig. 1–5 for isothermal analyses at 180 °C, and Figs 6–10 or 11–15 for temperature-programmed analyses. In addition, it must be verified that the separation of the following critical FAMEs is achieved, for both isothermal and temperature-programmed analyses:

- Baseline separation between 9*c*-C18:1 and 11*c*-C18:1, as shown in Fig. 2–5, Fig. 7–10 and Fig. 12–15.
- Partial separation between (13*t*+14*t*)-C18:1 and 9*c*-C18:1, as shown in Fig. 2–5, Fig. 7–10 and Fig. 12–15; note that 13*t*-C18:1 and 14*t*-C18:1 always elute together.
- Near-baseline separation between 16*t*-C18:1 and 13*c*-C18:1, and partial separation from 14*c*-C18:1, as shown in Fig. 3, 5, 10 and 15.
- Resolution factors (R-value) for the above critical pairs should be equal to or greater than 1. See Table 5 for a list of R values reported for 100 m SP-2560 and CP-Sil columns operated isothermally at 180 °C with hydrogen and helium carrier gases at a flow rate of 1.0 mL/min *(19)*.

Applying the isothermal elution method, the elution temperature must be fine-tuned to optimize the separation of 9*t*,12*c*,15*c*-C18:3, 11*c*-C20:1 (gadoleic acid) and 9*c*,12*c*,15*c*-C18:3 (α-linolenic acid). As described above, the elution temperature should be adjusted to elute 11*c*-C20:1 precisely at the centre point between 9*t*,12*c*,15*c*-C18:3 and α-linolenic acid, as shown in Fig. 2c, 3c and 4c. The adjustment of the GC column, however, should be done in small increments – for example, by 0.5 to 1 °C.

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 time, it typically loses its ability to resolve the critical FAMEs. If the adjustment of the elution temperature does not fix the lack of chromatographic resolution, install a new column. Test the performance of the new column with a PHO sample or, if available, FAME reference standards.

15. Calculations: fat content and fatty acid composition

For calculations, four spreadsheets have been developed (see Annex 4).[6]

Depending on the internal standard used (C11:0 FAME, C13:0 TAG, or C21:0 TAG), perform the calculations using spreadsheet A, B, or C, respectively. If an internal standard was not used, perform the calculations using spreadsheet D.

Spreadsheets A, B and C calculate the fat content as TAG equivalents and the fatty acid composition in two different formats:

- weight of individual FAs as free or unbound FAs (Wi); and
- weight of FAMEs (W_{FAMi}) and TAG equivalents (TAG).

Additionally, the A, B and C spreadsheets calculate the weight of individual FAs in four different formats:

- weight of individual FAs in grams per test portion used for analysis;
- weight of individual FAs in grams per 100 g of the test food sample;
- weight of individual FAs in grams per 100 g of total fat of the test food sample; and
- FA composition as a weight percentage (wt.%) of total FAs.

Use spreadsheet D, if the analysis was performed without an internal standard. Since an internal standard was not used, it calculates only the FA composition as a weight percentage of total FAs.

15.1 Instructions for inserting data to the spreadsheets:

From Annex 4, select the spreadsheet relevant to your choice of internal standard. Insert the following experimentally derived data into the shaded cells of the spreadsheet. The spreadsheet then automatically performs the calculations (total fat content and the FA composition data if an internal standard was used or the FA composition if an internal standard was not used):

- sample ID;
- date that the test sample FAME was analysed by GC;
- weight (W_{TS}) in grams of the homogenized representative test food portion used for fat extraction or direct transesterification; express the weight to a precision of 0.1 mg or better (hereafter, this representative test sample used for analysis is referred to as "test portion");

[6] The spreadsheets are available at https://www.who.int/publications/m/item/WHO-HEP-NFS-SSA-2025-4.1.

- weight (W_{IS}) in grams of the internal standard C11:0 FAME (in combination with C13:0 TAG), C13:0 TAG or C21:0 TAG (see section 11) added to the test portion – express the weight to a precision of 0.1 mg or better; express the weight up to 0.0001 g
- GC FAME peak area counts (PA_{IS}) of the internal standard (C11:0 FAME, C13:0 FAME or C21:0 FAME); and
- GC peak area counts (PA_i, where i denotes a specific FAME) of all the individual FAMEs in the GC chromatogram; insert the values in column B of the relevant spreadsheet.

Description of the formulae used in spreadsheets A, B and C for step-by-step calculation is given below in sections 15.2 to 15.9

15.2 Calculation of weight of individual FAs in the test sample as FAME (W_{FAMEi})

Calculate using the following formulae (adopted from AOAC Official Method 996.06 *(11)*):

$$W_{FAMEi} = \frac{PA_i \times W_{IS} \times 1.0000 \times TCF_i}{PA_{IS}}$$ Formula 1: apply if 11:0 FAME was the IS;

$$W_{FAMEi} = \frac{PA_i \times W_{IS} \times 1.0059 \times TCF_i}{PA_{IS}}$$ Formula 2: apply if 13:0 TAG was the IS; and

$$W_{FAMEi} = \frac{PA_i \times W_{IS} \times 1.0059 \times TCF_i}{PA_{IS}}$$ Formula 3: apply if 21:0 TAG was the IS.

Where:

W_{FAMEi} = weight of individual FAs as FAMEs in grams in test portion. Computed values are in column D in each spreadsheet.

PA_i = GC FAME peak area of FA i in the test portion (enter individual peak areas in column B of each spreadsheet).

W_{IS} = weight in grams (to nearest 0.0001 g) of the IS added to the test portion (see section 8 for IS solution preparations, section 10 for fat extraction and section 12 for transmethylation). If the instruction in this protocol is followed, W_{IS} should be approximately 0.0100 g for all three internal standards.

The factors 1.0000, 1.0059 and 1.0039 are for converting the internal standards C11:0 FAME, C13:0 TAG and C21:0 TAG to their corresponding FAME forms, respectively. Since C11:0 IS was added to the test portion in the FAME form, its conversion factor is 1.0000.

TCF_i = theoretical FID correction factor for FA i (as FAME) relative to C11:0, C13:0 or C21:0 FAME[7] internal standards. TCFi values for individual FAs relative to C11:0, C13:0

[7] Theoretical FID correction factors relative to C11:0 FAME, C13;0 and C21:0 for each FA are given in column C of spreadsheets A, B and C, respectively, in Annex 4. The GC FAME peak area percentage from the FID response is only a close approximation of the weight percentage of FAs *(27,28)*. To convert to true weight percentage, appropriate FID response factors must be used. The FID response is dependent on the bonding of the carbon that hydrogen is bonded to. This means that the FID responds only to active carbon atoms (CH, CH_2 and CH_3), and not to carbonyl carbon, so that the short-chain FAs have lower responses to FID than do longer-chain FAs. Therefore, this discrepancy must be corrected for by applying the TCF to the GC FAME peak area data. This will optimize the accuracy of FAME data and minimize variation between laboratories because the TCFs are independent of the GC instrument, type of column and GC operating parameters. TCFs are calculated in the spreadsheet with respect to the internal standards (as FAMEs) added to the test portions (C11:0 FAME, C13:0 FAME and C21:0 FAME).

and C21:0 FAMEs internal standards are shown in column C of spreadsheets A, B and C, respectively.

PA_{IS} = GC FAME peak area of the internal standard C11:0, C13:0 or C21:0 FAMEs.

15.3 Calculation of weights of individual FAs in the test portion as triacylglycerol (TAG) equivalents (W_{TAGi})

$$W_{TAGi} = W_{FAMEi} \times F_{TAGi} \qquad \text{Formula 4}$$

Where:

W_{TAGi} = weight of FA i in grams of the test portion as its TAG equivalent. Computed results for the individual FAs are in column F of the relevant spreadsheet.[8]

F_{TAGi} = conversion factor for individual FAME to the corresponding TAG equivalent. The conversion factors for all the FAs are shown in column E of spreadsheets A, B and C.

15.4 Calculation of total fat content (true fat content) in test portion and in 100 g test food sample

The total true fat content in the test portion is the sum of all the individual W_{TAGi} (calculated using Formula 1).[7] The results (i.e. $\sum W_{TAGi}$) are shown in row 102 of column F of the spreadsheet,

The total true fat content (W_{FAT}) in grams per 100 g test food sample is calculated using Formula 5:

$$W_{FAT} = \frac{\sum W_{TAG}}{W_{TS}} \times 100 \qquad \text{Formula 5}$$

Where W_{TS} = weight of test portion in grams used for fat extraction or transesterification as per sections 10 and 12.

Results for W_{FAT} are shown in Row 103 of column G of spreadsheets A, B and C. Column G also displays the weights in g of the individual FAs as TAG equivalents per 100 g test food sample.

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[8] The fat residue extracted from food primarily consists of tri-, di-, mono-acylglycerols, glycerophospholipids, glycosylglycerols and FFAs. These lipid classes contain FA moieties that entirely govern the nutritional properties of dietary fats. Hence, the total content of the above-mentioned lipid classes gives a more accurate measure of the true fat content of food. True fat is generally referred to as fat.

The fat residue extracted from foods using solvents often contains small amounts of several other lipid groups such as plant pigments, hydrocarbons, waxes, free sterols, fatty alcohols and aldehydes. These minor lipid groups are devoid of FA moieties and therefore, they do not contribute to the nutritional properties of dietary fats. For this reason, the extracted fat residue is called "crude fat".

Recently, from a nutritional point of view, there has been more interest in reporting the true fat content rather than the crude fat content. In this protocol, true fat content is calculated as the sum of FAs from all lipid sources present in the extracted fat and is expressed as TAGs equivalents. Expressing the GC measured FAs as TAG equivalents requires the mathematical equivalent of condensing each FA with glycerol *(11)*. For every three FA molecules, one glycerol molecule is required.

15.5 Calculation of weight (Wi in grams) of each FA as free/ unbound-FA in the test portion

This is calculated using Formula 6:

$$W_i = W_{FAMEi} \times F_{FAi} \qquad \text{Formula 6}$$

Where F_{FAi} = conversion factor for conversion of FAME i to its corresponding unbound-FA (FFA). Conversion factors for each FAME are listed in column H of spreadsheets A, B and C. The results for W_i are shown in column I

15.6 Calculation of weight in g ($W_{i/100g\ test\ food\ sample}$) of each FA as free (or unbound) FA per 100 g food sample

Calculate using:

$$W_{i/100g\ test\ food\ sample} = \frac{W_i}{W_{TS}} \times 100$$

The results are in column J of spreadsheets A, B and C.

15.7 Calculation of FA composition (the percentage of each individual FA per 100 g total FAs)

Calculate using Formula 7:

$$F_{FAi} \text{ as a percentage of total FAs} = \frac{W_i}{\sum W_i} \times 100 \qquad \text{Formula 7}$$

Where:

$\sum W_i$ = sum of all individual FAs in grams. The results are in column K of the spreadsheet.

15.8 Calculation of weight in grams of individual FAs per 100 g of total fat (W_i g/100 g fat)

This is calculated using Formula 8:

$$W_i \text{ (g/100 g fat)} = \frac{W_i \text{ in 100 g test food sample}}{\text{Total fat content in 100 g test food sample}} \times 100 \qquad \text{Formula 8}$$

The weight of each FA (W_i) (in grams) in the 100 g test food sample test is in column I and the total fat content in 100 g test food sample is given in row 103 of column G of the spreadsheet.

The calculated results for the individual FAs per100 g fat (W_i per 100 g fat) are displayed in column L of the spreadsheet.

15.9 Calculation of TFAs content and other FA classes of fatty acids

The FA composition, TFA and other FA classes data can be directly extracted from the spreadsheets and expressed in grams as TAG equivalents per 100 g test food sample (column G), grams of FAs per 100 g of test food sample (column J), grams of FAs per 100 g fat of the test food sample (column L) or weight percentage of total FAs (column K) of spreadsheets A, B and C (Annex 4).

15.9.1 Total TFA

Calculate the total TFAs using Formula 9:

Total TFAs (g/100 g test food sample) = ($\sum$ all TFAs W_i/W_{TS}) × 100 Formula 9

Individual TFAs found in partially hydrogenated oils, refined vegetable oils and ruminant fats are listed in rows 9*t*-C14:1 to 16*t*-C18:1, rows tt-C18:2 to 9*t*,15*c*-C18:2 and rows 9*t*,12*c*,15*t*-C18:3 to 9*t*,12*c*,15*c*-C18:3 of column A in each spreadsheet. Often, there is no clear baseline separation of *t*-C18:1 isomers from Δ6*t* to Δ14*t* (see GC chromatograms 1–12). Therefore, count their peak areas as one group and express as (6*t*-14*t*)-C18:1 (see column B row (6*t*-14*t*)-C18:1).

The calculated results for the TFAs are in row 61 of column J of each spreadsheet. If required, total TFA can be expressed as grams per 100 g fat or as weight percentage of total FAs using TFA data in columns K and L.

15.9.2 Total SFA

Calculate the total saturated FAs (g/100 g test food sample) using Formula 10:

($\sum$ all SFAs W_i/W_{TS}) × 100 Formula 10

Common dietary SFAs from C4:0 to C24:0 is listed in column A from row 11 to 35 and the total SFA content (g/100 g test food sample) is shown in row 36 of column J. If required, total TFA can be expressed as grams per 100 g fat or as weight percentage of total FAs using SFA data in columns K and L.

15.9.3 *cis*-MUFA

Calculate the total *cis*-MUFAs in grams per 100 g food sample using Formula 11:

Total *cis*-MUFAs (g/100 g food sample) = ($\sum$ all *cis*-MUFAs W_i/W_{TS}) × 100 Formula 11

Common dietary *cis*-MUFAs are listed in rows 9*c*-C14:1 to 16*c*-C18:1 and rows 11*c*-C20:1 to 15*c*-C24:1 of column A of the spreadsheet. The calculated results for the total *cis*-MUFA are given in row 81 of column J of the spreadsheets. If required, total *cis*-MUFA can be expressed as grams per 100 g fat or as weight percentage of total FAs using PUFA data in columns K and L, respectively.

15.9.4 Total *cis*-PUFAs

Calculation of total *cis*-PUFAs in grams (sum of all *cis*-PUFAs) in 100 g food sample using Formula 12:

Total *cis*-PUFAs (g/100 g food sample) = ($\sum$ all *cis*-PUFAs W_i/W_{TS}) × 100 Formula 12

Common dietary *cis*-PUFAs are listed in column B, in rows n-6's and n-3's of the spreadsheets. The calculated results for the total *cis*-PUFA are shown in row 101 of column J of the spreadsheet. If required, total *cis*-PUFA can be expressed as grams per 100 g fat or as weight percentage of total FAs using PUFA data in columns K and L, respectively.

15.10 Test portion analysed without an internal standard

Calculation of FA composition as weight percentage of total FAs using spreadsheet D located in Annex 4.

If the analysis of the test portion was performed without an added internal standard, the FA data then can be used only for calculation of individual FAs as weight of percentage total FAs. Use spreadsheet D for calculation using Formulae 13, 14 and 15:

$$W_{FAMEi} = PA_i \times TCF_i \qquad \text{Formula 13}$$

$$W_{FAi} = W_{FAMEi} \times F_{Ai} \qquad \text{Formula 14}$$

$$\text{Weight percentage of total FAs} = \frac{\sum W_{TAG}}{W_{TS}} \times 100 \qquad \text{Formula 15}$$

Where:

W_{FAMEi} = weight of individual FAs in grams as FAMEs in test portion. Calculated values are in column D of spreadsheet D.

PA_i = GC FAME peak area of individual FA in the test portion (enter individual peak areas in column B of spreadsheet D).

TCF_i = theoretical FID correction factor for individual FA (as FAME) relative to C18:0 FAME. Values are shown in column C of spreadsheet D.

W_{FAi} = weight of individual FA in grams as unbound FA in test portion. Calculated values are in column F of spreadsheet D.

FA_i = factor for converting individual FAMEs to the corresponding unbound FAs. Values are in column E of spreadsheet D.

$\sum W_{FAi}$ = Sum of all the individual fatty acids in grams as unbound FAs in test portion. Calculated values are in row 101 of column F of spreadsheet D.

16. Reporting data

The report of data should include a detailed description of the food sample (name, date collected, place collected, brand name, sample ID, date sample analysed). Report in tabulated form the following analytical data:

- total fat content (g/100 g food sample);
- all the identified individual FAs of concentration ≥0.1% of total FAs. Express their amounts as both percentages of total FAs (% w/w; i.e. the FA composition), grams per 100 g fat, and grams per 100 g food. In the tabulated data table include the totals for SFAs, TFAs, *cis*-MUFAs and *cis*-PUFAs.

The data should be reviewed by a person who is a chemist trained in TFA analysis, including reviewing results and chromatograms. Many issues that might arise would not be identified by analysts without the necessary knowledge.

17. Quality assurance and control

17.1 Training of analysts

For obtaining reliable data, the analysis should be conducted by experienced and well-trained analysts. As a minimum, the analyst must have basic knowledge of chromatographic separations and evaluating peaks in chromatograms; they must also have basic problem-solving skills.

Analysts performing this analytical procedure must successfully complete training in:

- general laboratory safety
- hazardous chemical waste management
- records management.

Analysts must also have received training on the specific GC instrumentation and software (such as Agilent ChemStation) used for the measurement procedure from a qualified laboratory or from the instrument manufacturer. Analysts should be trained to correctly calculate and interpret results and transfer data to an appropriate database.

Analysts should also gain experience in the analytical procedure described here by participating in the AOCS Laboratory Proficiency Programme. The AOCS also offers a series of Quality Reference Materials (QRMs) for analysts to gain practice with calibrating equipment and testing new methods. The QRMs include non-hydrogenated soybean oil and hydrogenated soybean oil for TFA determination; they come with GC chromatograms.

17.2 Criteria for selecting a laboratory to conduct TFA analysis

In selecting a suitable laboratory to perform TFA analysis, the following criteria should be taken into consideration.

- The laboratory is reputable and follows the Workplace Hazardous Materials Information System (WHIMS) or Globally Harmonized System of Classification and Labelling of Chemicals (GHS) safety standards (or equivalent safety standards).
- The laboratory has the equipment and supplies outlined in section 5, and adequate storage capacity (freezer and refrigerator).
- The analysts performing the analysis can demonstrate previous experience and training related to the analysis of dietary FA composition, including
  - general laboratory safety training
  - hazardous chemical waste management training

  - records management training
  - training on the specific GC instrumentation and software (such as Agilent ChemStation) used for the measurement procedure from a qualified laboratory or from the instrument manufacturer.
  - training to correctly calculate and interpret results and transfer data to an appropriate database.
- The laboratory team includes experienced technicians who are familiar with preparation of homogeneous composites of food types to be tested and capillary GC procedures for analysing FA composition of dietary fats.
- The analysts have the knowledge and skill to understand and successfully adapt standard analytical procedures and related quality assurance procedures, such as the TFA analysis procedure described here.
- The laboratory has a quality assurance system in place to ensure production of valid and reliable measurement results for the FAs outlined in this protocol.

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Annex 1

Structures of industrially produced and ruminant TFAs

In both ruminant fats and partially hydrogenated vegetable oils (PHVOs), octadecenoic acid (C18:1) represents the major fatty acid (FA) group with *trans* and unnatural *cis* isomers. The double bond positions of both the *cis* and trans C18:1 usually range from Δ4 to Δ16. The positional distribution of C18:1 *trans*-fatty acids (TFAs) in PHVO depends on the starting oil and the extent of hydrogenation – often the distribution centres around 9*t*-C18:1 and 10*t*-C18:1 *(1–3)*. The C18:1 TFA positional distribution of ruminant fats is distinctly different from that of PHVOs. Vaccenic (11*t*-C18:1) is the major C18:1 TFA in ruminant fats. It accounts for 30–60% of total C18:1 TFAs, whereas 9*t*-C18:1 and 10*t*-C18:1 occur in relatively lower amounts *(4)*.

In addition to the C18:1 TFAs, PHVOs contain several *cis*-C18:1 FAs, whose double bond position generally ranges from Δ6 to Δ16 *(4–10)*. The naturally occurring oleic acid (9*c*-C18:1) is always the major isomer, followed by 10*c*-C18:1 and 11*c*-C18:1. Ruminant fat also contains several *cis*-C18:1 FAs; however, the double bond positional distribution is less complex than for PHVO. Oleic acid is the major isomer, accounting for approximately 95% of the total *cis*-C18:1 FAs *(4)*.

Dietary fats also contain several positional and geometric isomers of linoleic (C18:2 TFAs) and α-linolenic acids (C18:3 TFAs), which are often present in low concentrations in both PHVOs and non-hydrogenated oils *(1, 2, 4–10)*. PHVOs contain 10 or more C18:2 TFAs; the major ones generally are 9*c*,13*t*-C18:2, 9*c*,12*t*-C18:2 and 9*t*,12*c*-C18:2. These are often present in mildly hydrogenated vegetable oils, whereas they are hardly detectable in heavily hydrogenated oils *(1, 2, 5–7, 9)*.

The C18:1 TFAs, C18:2 TFAs and C18:3 TFAs present in non-hydrogenated oils and in many foods are the result of exposure of their all-*cis* precursor to high temperatures, as during the deodorization or stripping during refining of edible oils, or simple heating during deep-frying. In these processes, the double bonds do not shift in position, but are isomerized from *cis* to *trans*, resulting in formation of small amounts of *trans* isomers. Generally, three isomers – 9*t*,12*c*-C18:2; 9*c*,12*t*-C18:2; and 9*t*,12*t*-C18:2 – are produced from linoleic acid. α-Linolenic acid also produces four isomers – 9*t*,12*c*,15*t*-C18:3; 9*t*,12*c*,15*c*-C18:3; 9*c*,12*c*,15*t*-C18:3; and 9*c*,12*t*,15*c*-C18:3 *(1, 2, 5–7, 9)*. Some refined oils may contain minute amounts of 9*t*-C18:1

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[1] All references were accessed on 01 September 2025.

Annex 2
Laboratory safety precautions

Introduction

This annex lists elements involved in laboratory safety; however, it is not a complete listing. These precautionary notes are reminders of possible hazards involved in operations or the use of substances in the fat analysis method given in this protocol. Users of this protocol should refer to standard texts on laboratory safety for more complete information. They should also follow the safety requirements of government agencies and experts in the field of laboratory safety.

Protective gloves, laboratory coats and safety glasses must always be worn during the steps of this method.

Equipment

Blenders, food processors, electrical equipment. Accidents involving blenders, food processors and electrical heating blocks may result in injury (e.g. fingers caught in chopping mill knives, electric shock). Ground all electrical equipment. Installation, maintenance, and repair operations should be performed by qualified electricians.

Compressed gas cylinders. Identify contents (by means of tags) of compressed gas cylinders using the name of the gas in the cylinder, rather than colour codes. Secure cylinders in an upright position using straps, chains, or a non-tip base. Use only correct pressure gages, pressure regulators and flow regulators, as specified by the supplier.

Distillations, extractions, evaporations. For flammable solvents (including acetone, diethyl ether, hexane, acetone), perform operations in a fume hood or behind a safety barrier, with hot water or electric heating. Do not use open flames to evaporate solvents.

Solvents

Acetone. Acetone is highly flammable and forms explosive peroxides with oxidizing agents. Do not mix with chloroform.

Diethyl ether. Diethyl ether is extremely volatile and flammable. Handle it with extreme care. It is irritating to the eyes and the respiratory tract and can de-fat the skin. Diethyl ether can form explosive peroxides under the influence of light and air. Keep it away from heat and light. Handle only inside a fume hood. Store in a tightly sealed container in a cool room (preferably a refrigerator) protected from light, moisture, and air.

Ethanol (ethyl alcohol). Ethanol is highly flammable. Use a fume hood when heating or evaporating.

Hexane. Hexane is irritating to the eyes, the respiratory system and skin. It is flammable and harmful. Avoid contact with skin and eyes. Store container in a designated flammable cabinet.

Methanol (methyl alcohol). Methanol is flammable and highly toxic. Avoid contact with eyes. Avoid breathing vapour. Use an effective fume-removal device.

Toluene. Toluene is irritating to the eyes, the respiratory system and skin. Avoid contact with skin and eyes. Toluene is also flammable. Keep the container in a cool, well-ventilated area.

Additional materials

Boron trifluoride in methanol. This reagent presents an extreme hazard, combining the systemic toxicity of methanol with the corrosive and irritant properties of boron trifluoride. It is fatal if inhaled and toxic if ingested or upon contact with the skin.

Hydrochloric acid. Hydrochloric acid is a strong acid that will cause severe burns. Protective clothing must be worn when working with this acid. It is toxic by ingestion and inhalation, and a strong irritant to eyes and skin. When diluting the acid, always add the acid slowly to water, never the reverse. Use a properly operating fume hood.

Ammonium hydroxide. Handle ammonium hydroxide with extreme care. Avoid contact with eyes and skin. Eye contact may result in eye burns and temporary loss of sight. If inhaled, mild exposure can cause nose irritation. Handle only inside a fume hood.

Annex 3

Thin-layer chromatography–gas chromatography (TLC-GC) procedure for checking the purity of IS

For TLC analysis, dissolve about 10 mg of the internal standard (IS) – C11:0 fatty acid methyl ester (FAME) and C13-TAG (triacylglycerol) in n-hexane (2 mL) and C21:0 TAG in chloroform (1 mL). Place a drop of each of these solutions on an analytical TLC plate (analytical TLC plates are available from many chromatographic suppliers). In addition, prepare a hexane solution of another FAME standard (e.g. methyl oleate) and triacylglycerol standard (e.g. tristearin, which is commercially available) and place a drop of each of these solutions on the TLC plate adjacent to the C11:0 FAME, C13:0 TAG and C21:0 spots. Develop the TLC plate in a solvent mixture of n-hexane, diethyl ether and acetic acid (80:20:1 v/v/v). After developing, dry the plate briefly on a laboratory bench at room temperature (20–25 °C). Visualize the plate by spraying with a 0.1% solution of 2,7-dichorofluorescein in ethanol and viewing under UV light (254 nm). If the purity of C11:0 FAME is 99% or greater, it should only be one spot on the TLC plate – that corresponding to the spot for methyl oleate. Similarly, if the purities of C13:0 TAG and C21:0 are 99% or greater, they should also show only one spot – that corresponding to methyl tristearin. The presence of more than one spot indicates that the IS is of questionable purity and should not be used.

If the TLC confirms the purity, the next step is to ensure that each of three IS contains only one FA corresponding to C11:0, C13:0 and C21:0. For this, take about 5–10 mg of the IS in a glass test tube (with a PTFE-lined screw cap), dissolve in 1 mL toluene, and convert them to FAMEs using 2 mL 7% BF_3-MeOH reagent (as described in section 12.1). Analyse the FAMEs by capillary GC (as described in section 5.8). If the GC run shows only one FAME peak for each of the IS, with no other FAME peaks, then it can be deduced that the IS contains only C11:0, with no other FAs. The presence of additional peaks on the GC run indicates that the IS are contaminated with other FAs. If the sum of the FAME additional peaks is greater than 1% of the total GC FAME area, discard the IS. Try to obtain IS from another supplier.

Annex 4
FAME calculation spreadsheets

Note:

- Please download the spreadsheets available at https://www.who.int/publications/m/item/WHO-HEP-NFS-SSA-2025-4.1.
- Shaded cells are for input by the user. The remaining cells will be calculated automatically.

Spreadsheet A

Spreadsheet for calculation of fat content and fatty acid composition of samples analyzed using C11:0 FAME as the Internal Standard

Sample ID:	
Date Sample Run:	
Weight of test portion used for fat extraction (g) ($\mathbf{W_{TS}}$):	
Weight of C11:0 FAME IS added to test portion (g) ($\mathbf{W_{IS}}$):	
Peak area of C11:0 FAME IS ($\mathbf{PA_{IS}}$)	

FAME	GC FAME peak area (PA_i)	Theoretical FID correction factor relative to C11:0 FAME (TCF_i)	Weight of FAME (W_{FAMEi}) in g in test portion	Conversion factor of FAME to TAG equivalent (F_{TAGi})	Weight of fatty acid as TAG equivalents (W_{TAGi}) in g in test portion	Weight of fatty acids in g as TAG equivalents (W_{TAGi}) per 100 g test food sample	Conversion factor of FAME to free fatty acid equiv (F_{FAI})	Weight of fatty acid as free fatty acids (W_i) in test portion	Weight of fatty acids as free fatty acids in 100g test food sample	Fatty acid as % total fatty acids	Weight of fatty acids as free fatty acids per 100 g fat of the test food sample
C4:0		1.4020		0.9868			0.8627				
C6:0		1.1914		0.9897			0.8923				
C8:0		1.0861		0.9915			0.9114				
C10:0		1.0229		0.9928			0.9247				
C11:0		1.0000		0.9933			0.9300				
C12:0		0.9808		0.9933			0.9346				
Iso-C12:0		0.9808		0.9937			0.9346				
C13:0		0.9646		0.9941			0.9386				
Iso-C13:0		0.9646		0.9941			0.9386				
Anteiso-C13:0		0.9646		0.9941			0.9386				
C14:0		0.9507		0.9945			0.9421				
Iso-C14:0		0.9507		0.9945			0.9421				
C15:0		0.9387		0.9948			0.9453				
Iso-C15:0		0.9387		0.9948			0.9453				

FAME	GC FAME peak area (PA_i)	Theoretical FID correction factor relative to C11:0 FAME (TCF_i)	Weight of FAME (W_{FAMEi}) in g in test portion	Conversion factor of FAME to TAG equivalent (F_{TAGi})	Weight of fatty acid as TAG equivalents (W_{TAGi}) in g in test portion	Weight of fatty acids in g as TAG equivalents (W_{TAGi}) per 100 g test food sample	Conversion factor of FAME to free fatty acid equiv (F_{FAI})	Weight of fatty acid as free fatty acids (W_i) in test portion	Weight of fatty acids as free fatty acids in 100g test food sample	Fatty acid as % total fatty acids	Weight of fatty acids as free fatty acids per 100 g fat of the test food sample
Anteiso-C15:0		0.9387		09948			0.9453				
C16:0		0.9282		0.9950			0.9481				
Iso-C16:0		0.9282		0.9950			0.9481				
C17:0		0.9189		0.9953			0.9507				
Iso-C17:0		0.9189		0.9953			0.9507				
Anteiso-C17:0		0.9189		0.9953			0.9507				
C18:0		0.9106		0.9955			0.9530				
C20:0		0.8966		0.9959			0.9570				
C21:0		0.8906		0.9961			0.9588				
C22:0		0.8852		0.9962			0.9604				
C24:0		0.8755		0.9965			0.9963				
Total SFA											
9t-C14:1		0.9429		0.9944			0.9417				
9t-C16:1		0.9213		0.9950			0.9477				
11t-C16:1		0.9213		0.9950			0.9477				
9t-C17:1		0.9123		0.9952			0.9503				
4t-C18:1		0.9044		0.9955			0.9527				
5t-C18:1		0.9044		0.9955			0.9527				
(6t-14t)-C18:1		0.9044		0.9955			0.9527				
16t-C18:1		0.9044		0.9955			0.9527				
Total 18:1 trans											
Total t-MUFA											

FAME	GC FAME peak area (PA_i)	Theoretical FID correction factor relative to C11:0 FAME (TCF_i)	Weight of FAME (W_{FAMEi}) in g in test portion	Conversion factor of FAME to TAG equivalent (F_{TAGi})	Weight of fatty acid as TAG equivalents (W_{TAGi}) in g in test portion	Weight of fatty acids in g as TAG equivalents (W_{TAGi}) per 100 g test food sample	Conversion factor of FAME to free fatty acid equiv (F_{FAI})	Weight of fatty acid as free fatty acids (W_i) in test portion	Weight of fatty acids as free fatty acids in 100g test food sample	Fatty acid as % total fatty acids	Weight of fatty acids as free fatty acids per 100 g fat of the test food sample
tt-C18:2		0.8983		0.9954			0.9524				
9t,12t-C18:2		0.8983		0.9954			0.9524				
9c,12t-C18:2		0.8983		0.9954			0.9524				
9t,12c-C18:2		0.8983		0.9954			0.9524				
9c,13t-C18:2		0.8983		0.9954			0.9524				
9t,15c-C18:2		0.8983		0.9954			0.9524				
CLA Isomers		0.8983		0.9954			0.9524				
Total 18:2 trans		0.0000									
9t,12c,15t-C18:3		0.8921		0.9954			0.9520				
9c,12t,15t-C18:3		0.8921		0.9954			0.9520				
9c,12c,15t-C18:3		0.8921		0.9954			0.9520				
9c,12t,15c-C18:3		0.8921		0.9954			0.9520				
9t,12c,15c-C18:3		0.8921		0.9954			0.9520				
Total 18:3 trans											
Total trans											
9c-C14:1		0.9429		0.9944			0.9417				
9c-C15:1		0.9313		0.9947			0.9449				
7c-C16:1		0.9213		0.9950			0.9477				
9c-C16:1		0.9213		0.9952			0.9477				
9c-C17:1		0.9123		0.9952			0.9503				
7c-C18:1		0.9044		0.9955			0.9527				
9c-C18:1		0.9044		0.9955			0.9527				

FAME	GC FAME peak area (PA_i)	Theoretical FID correction factor relative to C11:0 FAME (TCF_i)	Weight of FAME (W_{FAMEi}) in g in test portion	Conversion factor of FAME to TAG equivalent (F_{TAGi})	Weight of fatty acid as TAG equivalents (W_{TAGi}) in g in test portion	Weight of fatty acids in g as TAG equivalents (W_{TAGi}) per 100 g test food sample	Conversion factor of FAME to free fatty acid equiv (F_{FAI})	Weight of fatty acid as free fatty acids (W_i) in test portion	Weight of fatty acids as free fatty acids in 100g test food sample	Fatty acid as % total fatty acids	Weight of fatty acids as free fatty acids per 100 g fat of the test food sample
10c-C18:1		0.9044		0.9955			0.9527				
11c-C18:1		0.9044		0.9955			0.9527				
12c-C18:1		0.9044		0.9954			0.9527				
13c-C18:1		0.9044		0.9954			0.9527				
14c-C18:1		0.9044		0.9954			0.9527				
15c-C18:1		0.9044		0.9954			0.9527				
16c-C18:1		0.9044		0.9954			0.9527				
Total 18:1 cis											
11c-C20:1		0.8910		0.9959			0.9568				
9c-C20:1		0.8910		0.9959			0.9568				
13c-C22:1		0.8800		0.9962			0.9602				
15c-C24:1		0.8709		0.9965			0.9632				
Total MUFA											
C18:2n-6		0.8983		0.9954			0.9524				
C18:3n-6		0.8921		0.9954			0.9520				
C20:2n-6		0.8855		0.9958			0.9565				
C20:3n-6		0.8799		0.9958			0.9562				
C20:4n-6		0.8745		0.9958			0.9560				
C22:2n-6		0.8750		0.9962			0.9600				
C22:4n-6		0.8650		0.9961			0.9595				
C22:5n-6		0.8599		0.9961			0.9593				
Total n-6 LC-PUFA											

FAME	GC FAME peak area (PA_i)	Theoretical FID correction factor relative to C11:0 FAME (TCF_i)	Weight of FAME (W_{FAMEi}) in g in test portion	Conversion factor of FAME to TAG equivalent (F_{TAGi})	Weight of fatty acid as TAG equivalents (W_{TAGi}) in g in test portion	Weight of fatty acids in g as TAG equivalents (W_{TAGi}) per 100 g test food sample	Conversion factor of FAME to free fatty acid equiv (F_{FAI})	Weight of fatty acid as free fatty acids (W_i) in test portion	Weight of fatty acids as free fatty acids in 100g test food sample	Fatty acid as % total fatty acids	Weight of fatty acids as free fatty acids per 100 g fat of the test food sample
C18:3n-3		0.8921		0.9954			0.9520				
C18:4n-3		0.8860		0.9954			0.9517				
C20:3n-3		0.8799		0.9958			0.9562				
C20:4n-3		0.8745		0.9958			0.9560				
C20:5n-3		0.8608		0.9958			0.9557				
C22:3n-3		0.8700		0.9958			0.9538				
C22:5n-3		0.8599		0.9961			0.9593				
C22:4n-3		0.8650		0.9961			0.9595				
C22:6n-3		0.8549		0.9961			0.9590				
Total n-3 LC-PUFA											
Total cis-PUFA											
Total Fatty Acid Methyl esters W_{FAMEi}											
Total Triglyceride equivalents W_{TAGi}											
Total Fat content as TAG equivalents per 100g test food sample											
Total Fatty Acids in test portion											
Total trans											

Spreadsheet B

Spreadsheet for calculation of the fat content and fatty acid composition of samples analysed using C13:0 TAG as the Internal Standard

Sample ID:	
Date Sample Run:	
Weight of test portion used for fat extraction (g) (**W_{TS}**):	
Weight of C13:0 TAG IS added to test portion (g) (**W_{IS}**):	
Peak area of C13:0 FAME IS (**PA_{IS}**)	

FAME	GC FAME peak area (PA_i)	Theoretical FID correction factor relative (to C13:0 FAME (TCF_i)	Weight of FAME (W_{FAMEi}) in g in test portion	Conversion factor of FAME to TAG equivalent (F_{TAGi})	Weight of fatty acid as TAG equivalents (W_{TAGi}) in g in test portion	Weight of fatty acids in g as TAG equivalents (W_{TAGi}) per 100 g test food sample	Conversion factor of FAME to free fatty acid equiv (F_{FAi})	Weight of fatty acid as free fatty acids (W_i) in test portion	Weight of fatty acids as free fatty acids in 100g test food sample	Fatty acid as % total fatty acids	Weight of fatty acids as free fatty acids per 100 g fat of the test food sample
C4:0		1.4534		0.9868			0.8627				
C6:0		1.2352		0.9897			0.8923				
C8:0		1.1259		0.9915			0.9114				
C10:0		1.0605		0.9928			0.9247				
C11:0		1.0367		0.9933			0.9300				
C12:0		1.0168		0.9933			0.9346				
Iso-C12:0		1.0168		0.9937			0.9346				
C13:0		1.0000		0.9941			0.9386				
Iso-C13:0		1.0000		0.9941			0.9386				
Anteiso-C13:0		1.0000		0.9941			0.9386				
C14:0		0.9856		0.9945			0.9421				
Iso-C14:0		0.9856		0.9945			0.9421				
C15:0		0.9731		0.9948			0.9453				
Iso-C15:0		0.9731		0.9948			0.9453				
Anteiso-C15:0		0.9731		09948			0.9453				

FAME	GC FAME peak area (PA_i)	Theoretical FID correction factor relative (to C13:0 FAME (TCF_i)	Weight of FAME (W_{FAMEi}) in g in test portion	Conversion factor of FAME to TAG equivalent (F_{TAGi})	Weight of fatty acid as TAG equivalents (W_{TAGi}) in g in test portion	Weight of fatty acids in g as TAG equivalents (W_{TAGi}) per 100 g test food sample	Conversion factor of FAME to free fatty acid equiv (F_{FAi})	Weight of fatty acid as free fatty acids (W_i) in test portion	Weight of fatty acids as free fatty acids in 100g test food sample	Fatty acid as % total fatty acids	Weight of fatty acids as free fatty acids per 100 g fat of the test food sample
C16:0		0.9622		0.9950			0.9481				
Iso-C16:0		0.9622		0.9950			0.9481				
C17:0		0.9526		0.9953			0.9507				
Iso-C17:0		0.9526		0.9953			0.9507				
Anteiso-C17:0		0.9526		0.9953			0.9507				
C18:0		0.9440		0.9955			0.9530				
C20:0		0.9295		0.9959			0.9570				
C21:0		0.9233		0.9961			0.9588				
C22:0		0.9176		0.9962			0.9604				
C24:0		0.9076		0.9965			0.9963				
Total SFA											
9t-C14:1		0.9775		0.9944			0.9417				
9t-C16:1		0.9551		0.9950			0.9477				
11t-C16:1		0.9551		0.9950			0.9477				
9t-C17:1		0.9458		0.9952			0.9503				
4t-C18:1		0.9376		0.9955			0.9527				
5t-C18:1		0.9376		0.9955			0.9527				
(6t-14t)-C18:1		0.9376		0.9955			0.9527				
16t-C18:1		0.9376		0.9955			0.9527				
Total 18:1 trans											
Total t-MUFA											
tt-C18:2		0.9313		0.9954			0.9524				
9t,12t-C18:2		0.9313		0.9954			0.9524				
tt-C18:2		0.9313		0.9954			0.9524				

FAME	GC FAME peak area (PA_i)	Theoretical FID correction factor relative (to C13:0 FAME (TCF_i)	Weight of FAME (W_{FAMEi}) in g in test portion	Conversion factor of FAME to TAG equivalent (F_{TAGi})	Weight of fatty acid as TAG equivalents (W_{TAGi}) in g in test portion	Weight of fatty acids in g as TAG equivalents (W_{TAGi}) per 100 g test food sample	Conversion factor of FAME to free fatty acid equiv (F_{FAi})	Weight of fatty acid as free fatty acids (W_i) in test portion	Weight of fatty acids as free fatty acids in 100g test food sample	Fatty acid as % total fatty acids	Weight of fatty acids as free fatty acids per 100 g fat of the test food sample
9t,12t-C18:2		0.9313		0.9954			0.9524				
9c,12t-C18:2		0.9313		0.9954			0.9524				
9t,12c-C18:2		0.9313		0.9954			0.9524				
9c,13t-C18:2		0.9313		0.9954			0.9524				
9t,15c-C18:2		0.9313		0.9954			0.9524				
CLA Isomers		0.9313		0.9954			0.9524				
Total 18:2 trans		0.0000									
9t,12c,15t-C18:3		0.9248		0.9954			0.9520				
9c,12t,15t-C18:3		0.9248		0.9954			0.9520				
9c,12c,15t-C18:3		0.9248		0.9954			0.9520				
9c,12t,15c-C18:3		0.9248		0.9954			0.9520				
9t,12c,15c-C18:3		0.9248		0.9954			0.9520				
Total 18:3 trans											
Total trans											
9c-C14:1		0.9775		0.9944			0.9417				
9c-C15:1		0.9655		0.9947			0.9449				
7c-C16:1		0.9551		0.9950			0.9477				
9c-C16:1		0.9551		0.9952			0.9477				
9c-C17:1		0.9458		0.9952			0.9503				
7c-C18:1		0.9376		0.9955			0.9527				
9c-C18:1		0.9376		0.9955			0.9527				
10c-C18:1		0.9376		0.9955			0.9527				
11c-C18:1		0.9376		0.9955			0.9527				
12c-C18:1		0.9376		0.9954			0.9527				

FAME	GC FAME peak area (PA_i)	Theoretical FID correction factor relative (to C13:0 FAME (TCF_i)	Weight of FAME (W_{FAMEi}) in g in test portion	Conversion factor of FAME to TAG equivalent (F_{TAGi})	Weight of fatty acid as TAG equivalents (W_{TAGi}) in g in test portion	Weight of fatty acids in g as TAG equivalents (W_{TAGi}) per 100 g test food sample	Conversion factor of FAME to free fatty acid equiv (F_{FAi})	Weight of fatty acid as free fatty acids (W_i) in test portion	Weight of fatty acids as free fatty acids in 100g test food sample	Fatty acid as % total fatty acids	Weight of fatty acids as free fatty acids per 100 g fat of the test food sample
13c-C18:1		0.9376		0.9954			0.9527				
14c-C18:1		0.9376		0.9954			0.9527				
15c-C18:1		0.9376		0.9954			0.9527				
16c-C18:1		0.9376		0.9954			0.9527				
Total 18:1 cis											
11c-C20:1		0.9237		0.9959			0.9568				
9c-C20:1		0.9237		0.9959			0.9568				
13c-C22:1		0.9123		0.9962			0.9602				
15c-C24:1		0.9029		0.9965			0.9632				
Total MUFA											
C18:2n-6		0.9313		0.9954			0.9524				
C18:3n-6		0.9248		0.9954			0.9520				
C20:2n-6		0.9180		0.9958			0.9565				
C20:3n-6		0.9122		0.9958			0.9562				
C20:4n-6		0.9066		0.9958			0.9560				
C22:2n-6		0.9071		0.9962			0.9600				
C22:4n-6		0.8968		0.9961			0.9595				
C22:5n-6		0.8914		0.9961			0.9593				
Total n-6 LC-PUFA											
C18:3n-3		0.9248		0.9954			0.9520				
C18:4n-3		0.9186		0.9954			0.9517				
C20:3n-3		0.9122		0.9958			0.9562				

FAME	GC FAME peak area (PA_i)	Theoretical FID correction factor relative (to C13:0 FAME (TCF_i)	Weight of FAME (W_{FAMEi}) in g in test portion	Conversion factor of FAME to TAG equivalent (F_{TAGi})	Weight of fatty acid as TAG equivalents (W_{TAGi}) in g in test portion	Weight of fatty acids in g as TAG equivalents (W_{TAGi}) per 100 g test food sample	Conversion factor of FAME to free fatty acid equiv (F_{FAi})	Weight of fatty acid as free fatty acids (W_i) in test portion	Weight of fatty acids as free fatty acids in 100g test food sample	Fatty acid as % total fatty acids	Weight of fatty acids as free fatty acids per 100 g fat of the test food sample
C20:4n-3		0.9066		0.9958			0.9560				
C20:5n-3		0.8923		0.9958			0.9557				
C22:3n-3		0.9019		0.9958			0.9538				
C22:5n-3		0.8914		0.9961			0.9593				
C22:4n-3		0.8968		0.9961			0.9595				
C22:6n-3		0.8863		0.9961			0.9590				
Total n-3 LC-PUFA											
Total cis-PUFA											
Total Fatty Acid Methyl esters W_{FAMEi}											
Total Triglyceride equivalents W_{TAGi}											
Total Fat content as TAG equivalents per 100g test food sample											
Total Fatty Acids in test portion											
Total trans											

Spreadsheet C

Spreadsheet for calculation of the fat content and fatty acid composition of samples analyzed using to C21:0 as the TAG Internal Standard

Sample ID:	
Date Sample Run:	
Weight of test portion used for fat extraction (g) (**W_{TS}**):	
Weight of C21:0 TAG IS added to test portion (g) (**W_{IS}**):	
Peak area of C21:0 FAME IS (**PA_{IS}**)	

FAME	GC FAME peak area (PA_i)	Theoretical FID correction factor relative to C21:0 FAME (TCF_i)	Weight of FAME (W_{FAMEi}) in g in test portion	Conversion factor of FAME to TAG equivalent (F_{TAGi})	Weight of fatty acid as TAG equivalents (W_{TAGi}) in g in test portion	Weight of fatty acids in g as TAG equivalents (W_{TAGi}) per 100 g test food sample	Conversion factor of FAME to free fatty acid equiv (F_{FAi})	Weight of fatty acid as free fatty acids (W_i) in test portion	Weight of fatty acids as free fatty acids in 100g test food sample	Fatty acid as % total fatty acids	Weight of fatty acids as free fatty acids per 100 g fat of the test food sample
C4:0		1.5742		0.9868			0.8627				
C6:0		1.3378		0.9897			0.8923				
C8:0		1.2195		0.9915			0.9114				
C10:0		1.1486		0.9928			0.9247				
C11:0		1.1228		0.9933			0.9300				
C12:0		1.1013		0.9933			0.9346				
Iso-C12:0		1.1013		0.9937			0.9346				
C13:0		1.0831		0.9941			0.9386				
Iso-C13:0		1.0831		0.9941			0.9386				
Anteiso-C13:0		1.0831		0.9941			0.9386				
C14:0		1.0675		0.9945			0.9421				
Iso-C14:0		1.0675		0.9945			0.9421				
C15:0		1.0540		0.9948			0.9453				
Iso-C15:0		1.0540		0.9948			0.9453				

FAME	GC FAME peak area (PA_i)	Theoretical FID correction factor relative to C21:0 FAME (TCF_i)	Weight of FAME (W_{FAMEi}) in g in test portion	Conversion factor of FAME to TAG equivalent (F_{TAGi})	Weight of fatty acid as TAG equivalents (W_{TAGi}) in g in test portion	Weight of fatty acids in g as TAG equivalents (W_{TAGi}) per 100 g test food sample	Conversion factor of FAME to free fatty acid equiv (F_{FAi})	Weight of fatty acid as free fatty acids (W_i) in test portion	Weight of fatty acids as free fatty acids in 100g test food sample	Fatty acid as % total fatty acids	Weight of fatty acids as free fatty acids per 100 g fat of the test food sample
Anteiso-C15:0		1.0540		09948			0.9453				
C16:0		1.0422		0.9950			0.9481				
Iso-C16:0		1.0422		0.9950			0.9481				
C17:0		1.0318		0.9953			0.9507				
Iso-C17:0		1.0318		0.9953			0.9507				
Anteiso-C17:0		1.0318		0.9953			0.9507				
C18:0		1.0225		0.9955			0.9530				
C20:0		1.0067		0.9959			0.9570				
C21:0		1.0000		0.9961			0.9588				
C22:0		0.9939		0.9962			0.9604				
C24:0		0.9830		0.9965			0.9963				
Total SFA											
9t-C14:1		1.0587		0.9944			0.9417				
9t-C16:1		1.0345		0.9950			0.9477				
11t-C16:1		1.0345		0.9950			0.9477				
9t-C17:1		1.0244		0.9952			0.9503				
4t-C18:1		1.0155		0.9955			0.9527				
5t-C18:1		1.0155		0.9955			0.9527				
(6t-14t)-C18:1		1.0155		0.9955			0.9527				
16t-C18:1		1.0155		0.9955			0.9527				
Total 18:1 trans											
Total t-MUFA											
tt-C18:2		1.0087		0.9954			0.9524				

FAME	GC FAME peak area (PA_i)	Theoretical FID correction factor relative to C21:0 FAME (TCF_i)	Weight of FAME (W_{FAMEi}) in g in test portion	Conversion factor of FAME to TAG equivalent (F_{TAGi})	Weight of fatty acid as TAG equivalents (W_{TAGi}) in g in test portion	Weight of fatty acids in g as TAG equivalents (W_{TAGi}) per 100 g test food sample	Conversion factor of FAME to free fatty acid equiv (F_{FAI})	Weight of fatty acid as free fatty acids (W_i) in test portion	Weight of fatty acids as free fatty acids in 100g test food sample	Fatty acid as % total fatty acids	Weight of fatty acids as free fatty acids per 100 g fat of the test food sample
9t,12t-C18:2		1.0087		0.9954			0.9524				
9c,12t-C18:2		1.0087		0.9954			0.9524				
9t,12c-C18:2		1.0087		0.9954			0.9524				
9c,13t-C18:2		1.0087		0.9954			0.9524				
9t,15c-C18:2		1.0087		0.9954			0.9524				
CLA Isomers		1.0087		0.9954			0.9524				
Total 18:2 trans											
9t,12c,15t-C18:3		1.0017		0.9954			0.9520				
9c,12t,15t-C18:3		1.0017		0.9954			0.9520				
9c,12c,15t-C18:3		1.0017		0.9954			0.9520				
9c,12t,15c-C18:3		1.0017		0.9954			0.9520				
9t,12c,15c-C18:3		1.0017		0.9954			0.9520				
Total 18:3 trans											
Total trans											
9c-C14:1		1.0587		0.9944			0.9417				
9c-C15:1		1.0457		0.9947			0.9449				
7c-C16:1		1.0345		0.9950			0.9477				
9c-C16:1		1.0345		0.9952			0.9477				
9c-C17:1		1.0244		0.9952			0.9503				
7c-C18:1		1.0155		0.9955			0.9527				
9c-C18:1		1.0155		0.9955			0.9527				
10c-C18:1		1.0155		0.9955			0.9527				
11c-C18:1		1.0155		0.9955			0.9527				

FAME	GC FAME peak area (PA_i)	Theoretical FID correction factor relative to C21:0 FAME (TCF_i)	Weight of FAME (W_{FAMEi}) in g in test portion	Conversion factor of FAME to TAG equivalent (F_{TAGi})	Weight of fatty acid as TAG equivalents (W_{TAGi}) in g in test portion	Weight of fatty acids in g as TAG equivalents (W_{TAGi}) per 100 g test food sample	Conversion factor of FAME to free fatty acid equiv (F_{FAi})	Weight of fatty acid as free fatty acids (W_i) in test portion	Weight of fatty acids as free fatty acids in 100g test food sample	Fatty acid as % total fatty acids	Weight of fatty acids as free fatty acids per 100 g fat of the test food sample
12c-C18:1		1.0155		0.9954			0.9527				
13c-C18:1		1.0155		0.9954			0.9527				
14c-C18:1		1.0155		0.9954			0.9527				
15c-C18:1		1.0155		0.9954			0.9527				
16c-C18:1		1.0155		0.9954			0.9527				
Total 18:1 cis											
11c-C20:1		1.0005		0.9959			0.9568				
9c-C20:1		1.0005		0.9959			0.9568				
13c-C22:1		0.9881		0.9962			0.9602				
15c-C24:1		0.9779		0.9965			0.9632				
Total MUFA											
C18:2n-6		1.0087		0.9954			0.9524				
C18:3n-6		1.0017		0.9954			0.9520				
C20:2n-6		0.9943		0.9958			0.9565				
C20:3n-6		0.9880		0.9958			0.9562				
C20:4n-6		0.9819		0.9958			0.9560				
C22:2n-6		0.9825		0.9962			0.9600				
C22:4n-6		0.9713		0.9961			0.9595				
C22:5n-6		0.9655		0.9961			0.9593				
Total n-6 LC-PUFA											
C18:3n-3		1.0017		0.9954			0.9520				
C18:4n-3		0.9949		0.9954			0.9517				

FAME	GC FAME peak area (PA_i)	Theoretical FID correction factor relative to C21:0 FAME (TCF_i)	Weight of FAME (W_{FAMEi}) in g in test portion	Conversion factor of FAME to TAG equivalent (F_{TAGi})	Weight of fatty acid as TAG equivalents (W_{TAGi}) in g in test portion	Weight of fatty acids in g as TAG equivalents (W_{TAGi}) per 100 g test food sample	Conversion factor of FAME to free fatty acid equiv (F_{FAI})	Weight of fatty acid as free fatty acids (W_i) in test portion	Weight of fatty acids as free fatty acids in 100g test food sample	Fatty acid as % total fatty acids	Weight of fatty acids as free fatty acids per 100 g fat of the test food sample
C20:3n-3		0.9880		0.9958			0.9562				
C20:4n-3		0.9819		0.9958			0.9560				
C20:5n-3		0.9665		0.9958			0.9557				
C22:3n-3		0.9769		0.9958			0.9538				
C22:5n-3		0.9655		0.9961			0.9593				
C22:4n-3		0.9713		0.9961			0.9595				
C22:6n-3		0.9599		0.9961			0.9590				
Total n-3 LC-PUFA											
Total cis-PUFA											
Total Fatty Acid Methyl esters W_{FAMEi}											
Total Triglyceride equivalents W_{TAGi}											
Total Fat content as TAG equivalents per 100g test food sample											
Total Fatty Acids in test portion											
Total trans											

Spreadsheet D

Spreadsheet for calculation of the fatty acid composition of samples analyzed without an internal standard

Sample ID:	
Date Sample Analyzed	
Gravimetrically Determined Total Fat	

FAME	GC FAME Peak Area (PA_i)	Theoretical FID Correction Factors (TCF_i) relative to C18:0 FAME	Weight of fatty acids as FAME W_{FAMEi} (g)	Conversion factor for converting FAMEs to unbound fatty acids (F_{FAi})	Weight of fatty acids as unbound fatty acids (W_{FAi}) (g)	Weight % of total fatty acids	Weight % of total fat (ie. ,g of fatty acid i per 100 g total fat)
C4:0		1.5396		0.8627			
C6:0		1.3084		0.8923			
C8:0		1.1927		0.9114			
C10:0		1.1233		0.9247			
C11:0		1.0917		0.9300			
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			
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			
C21:0		0.9780		0.9961			
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			

FAME	GC FAME Peak Area (PA_i)	Theoretical FID Correction Factors (TCF_i) relative to C18:0 FAME	Weight of fatty acids as FAME W_{FAMEi} (g)	Conversion factor for converting FAMEs to unbound fatty acids(F_{FAi})	Weight of fatty acids as unbound fatty acids (W_{FAi}) (g)	Weight % of total fatty acids	Weight % of total fat (ie. ,g of fatty acid i per 100 g total fat)
Total 18:1 trans							
Total t-MUFA							
tt-C18:2		0.9865		0.9524			
9t,12t-C18:2		0.9865		0.9524			
9c,12t-C18:2		0.9865		0.9524			
9t,12c-C18:2		0.9865		0.9524			
9c,13t-C18:2		0.9865		0.9524			
9t,15c-C18:2		0.9865		0.9524			
CLA Isomers		0.9865		0.9524			
Total 18:2 trans							
9t,12c,15t-C18:3		0.9797		0.9520			
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			
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			

FAME	GC FAME Peak Area (PA_i)	Theoretical FID Correction Factors (TCF_i) relative to C18:0 FAME	Weight of fatty acids as FAME W_{FAMEi} (g)	Conversion factor for converting FAMEs to unbound fatty acids(F_{FAi})	Weight of fatty acids as unbound fatty acids (W_{FAi}) (g)	Weight % of total fatty acids	Weight % of total fat (ie. ,g of fatty acid i per 100 g total fat)
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							
Total trans							

Note: The weights (WFAMEi (g), Wi (g), g Fatty acid i per 100 g total fatty acids) obtained from this spreadsheet are apparent weights, and not absolute.

Tables and figures

Table 1. Differences among the original WHO reference protocol, the WHO simplified protocol and the updated WHO reference protocol

Factor	Original WHO reference protocol[a]	WHO simplified protocol[b]	Updated WHO reference protocol
GC column	• 100 m SP-2560 or CP-Sil 88 fused capillary columns (and their equivalents)	• 100 m SP-2560 or CP-Sil 88 fused capillary columns (and their equivalents); or • Other 100 m BCS commercial columns	• 100 m SP-2560 or CP-Sil 88 fused capillary columns (and their equivalents)
GC column temperature	• Operating the column isothermally at 180 °C	• Operating the column isothermally at 180 °C; or • Temperature programming as recommended in AOAC 2012.13 / ISO 16958 | IDF 231:2015 *(9, 10)*	• Operating the column isothermally at 180 °C; or • Temperature programming as recommended in AOAC 2012.13 / ISO 16958 | IDF 231:2015 *(9, 10)* or by P Delmonte *(17)*
GC carrier gas	• Hydrogen; or • Helium	• Hydrogen; or • Helium	• Hydrogen; or • Helium
GC-FID response factors	• Theoretical FID response factors	• Theoretical FID response factors	• Theoretical FID response factors
Internal standard	• C21:0 TAG	No internal standard	• C13:0 TAG; or • C21:0 TAG; or • C11:0 FAME + C13:0 TAG; or • no internal standard
Solvent for preparing internal standard	• Chloroform	No internal standard	For C13:0 TAG, C11:0 FAME + C13:0 TAG: • n-Hexane; or • n-Heptane For C21:0 TAG: • Chloroform

Factor	Original WHO reference protocol[a]	WHO simplified protocol[b]	Updated WHO reference protocol
Fat extraction	• AOAC hydrolytic extraction procedure (AOAC Official Method 996.06 *(11)*)	• AOAC hydrolytic extraction procedure (AOAC Official Method 996.06 *(11)*); or • A procedure using organic solvents at room temperature	• AOAC hydrolytic extraction procedure (AOAC Official Method 996.06 *(11)*) for all foods; or • AOAC 2012.13 / ISO 16958 | IDF 231:2015 as alternative procedure for milk, milk products, infant formula, and adult nutritionals *(9, 10)*
Methylation	• BF_3-CH_3OH	• BF_3-CH_3OH; or • KOH-CH_3OH	• BF_3-CH_3OH; or • KOH-CH_3OH; or • 5% CH_3ONa-CH_3OH
Direct trans-esterification	No direct transesterification	No direct transesterification	• Transesterify test food directly using alkali hydrolysis with NaOH-CH_3OH reagent followed by methylation using BF_3-CH_3OH
Solvent for preparing FAME standard	• Hexane (or another organic solvent of similar volatility and polarity)	• Heptane, iso-octane and petroleum ether to replace hexane whenever possible	• n-Hexane; or • n-Heptane
GC FAME peak identification	• Identify using FAME reference standards	• Identify using FAME reference standards; or • If FAME standards are not available, identify by comparing with the GC elution pattern from representative chromatograms	• Identify using FAME reference standards; or • If FAME standards are not available, identify by comparing with the GC elution pattern from representative chromatograms
Calculation of TFA content	• Weight of FAs (absolute amount) • Weight percentage of total FAs (calculated with respect to C21:0 TAG IS)	• Weight percentage of total FAs (calculated with respect to C18:0)	• Option of calculating weight of FAs (absolute amount with respect to the added internal standard), or weight percentage of FAs (calculated with respect to C18:0)

[a] WHO global protocol for measuring fatty acids in foods *(25)*

[b] WHO simplified protocol for measuring *trans*-fatty acid content *(26)*

Table 2. 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 (butyric)	C4:0
hexanoic (caproic)	C6:0
octanoic (caprylic)	C8:0
decanoic (capric)	C10:0
iso-dodecanoic	I-C12:0
dodecanoic (lauric)	C12:0
iso-tridecanoic	I-C13:0
anteiso-tridecanoic	AI-C13:0
tridecanoic	C13:0
iso-tetradecanoic	I-C14:0
tetradecanoic (myristic)	C14:0
iso-pentadecanoic	I-C15:0
anteiso-pentadecanoic	AI-C15:0
pentadecanoic	C15:0
iso-hexadecanoic	I-C16:0
hexadecanoic (palmitic)	C16:0
iso-heptadecanoic	I-C17:0
anteiso-heptadecanoic	AI-C17:0
heptadecanoic	C17:0
octadecanoic (stearic)	C18:0
eicosanoic (arachidic)	C20:0
docosanoic (behenic)	C22:0
tetracosanoic (lignoceric)	C24:0
***trans*-monounsaturated fatty acids (t-MUFAs)**	
9-*trans*-tetradecenoic	9*t*-C14:1
9-*trans*-hexadecenoic	9*t*-C16:1
9-*trans*-heptadecenoic	9*t*-C17:1
(6+7+8+9+10+11+12+13+14)-*trans*-octadecenoic[c]	(4*t*-14*t*)-C18:1
16t-octadecenoic	16*t*-C18:1
***cis*-monounsaturated fatty acids (c-MUFAs)**	
9-*cis*-tetradecenoic	9*c*-C14:1
9-*cis*-pentadecenoic	9*c*-C15:1
7-*cis*-hexadecenoic	7*c*-C16:1
9-*cis*-Hexadecenoic	9*c*-C16:1
9-*cis*-heptadecenoic	9*c*-C17:1
(9-*cis*+10-*cis*)-octadecenoic[d]	(9*c*+10*c*)-C18:1
11-*cis*-octadecenoic (*cis* vaccenic)	11*c*-C18:1
12-*cis*-octadecenoic	12*c*-C18:1
13-*cis*-octadecenoic	13*c*-C18:1
14-*cis*-octadecenoic	14*c*-C18:1
15-*cis*-octadecenoic	15*c*-C18:1
16-*cis*-octadecenoic	16*c*-C18:1

IUPAC name (trivial name)	Shorthand notation ("n minus" notation)
11-*cis*-eicosenoic	11*c*-C20:1
13-*cis*-docosenoic (erucic)	13*c*-C20:1
15-*cis*-tetracosanoic (nervonic)	15*c*-C24:1
***trans*-polyunsaturated fatty acids (t-PUFAs)**	
trans, *trans*-octadecadienoic	*tt*-C18:2
9-*trans*,12-*trans*-octadecadienoic	9*t*,12*t*-C18:2
9-*cis*,13-*trans*-octadecadienoic	9*c*,13*t*-C18:2
9-*cis*,12-*trans*-octadecadienoic	9*c*,12*t*-C18:2
9-*trans*,12-*cis*-octadecadienoic	9*t*,12*c*-C18:2
(9-*trans*,15-*cis*- + 10-*trans*,15-*cis*-) octadecadienoic[e]	(9*t*,15*c*+10*t*,15*c*)-C18:2
9-*cis*,12-*cis*,15-*trans*-octadecatrienoic	9*c*,12*c*,15*t*-C18:3
9-*cis*,12-*trans*,15-*cis*-octadecatrienoic	9*c*,12*t*,15*c*-C18:3
9-*trans*,12-*cis*,15-*cis*-octadecatrienoic	9*t*,12*c*,15*c*-C18:3
n-6 polyunsaturated fatty acids (n-6 PUFAs)	
9-*cis*,12-*cis*-octadecadienoic (linoleic)	9*c*,12*c*-C18:2 (C18:2n-6)
6-*cis*,9-*cis*,12-*cis*-octadecatrienoic (γ-linolenic)	6*c*, 9*c*,12*c*-C18:3 (C18:3n-6)
8-*cis*,11-*cis*,14-*cis*-eicosatrienoic acid	8*c*,11*c*,14*c*-C20:3 (C20:3n-6)
5-*cis*,8-*cis*,11-*cis*,14-*cis*-eicosatetraenoic acid (arachidonic acid)	5*c*, 8*c*,11*c*,14*c*-C20:4 (C20:4n-6)
7-*cis*,10-*cis*,13-*cis*,16-*cis*-docosatetraenoic acid	7*c*,10*c*,13*c*,16*c*-C22:4 (C20:4n-6)
4-*cis*,7-*cis*,10-*cis*,13-*cis*,16-*cis*-docosapentanoic acid	4*c*, 7*c*,10*c*,13*c*,16*c*-C22:5 C(22:5n-6)
n-3 polyunsaturated fatty acids (n-3 PUFAs)	
9-*cis*,12-*cis*,15-*cis*-octadecatrienoic (α-linolenic)	9*c*,12*c*, 15*c*-C18:3 (C18:3n3)
6-*cis*,9-*cis*,12-*cis*,15-*cis*-octadecatetraenoic	6*c*, 9*c*,12*c*,15*c*-C18:4 (C18:4n-3)
5-*cis*,8-*cis*,11-*cis*,14-*cis*,17-*cis*-eicosapentaenoic acid (EPA)	5*c*, 8*c*,11*c*,14*c*,17*c*-C20:5 (C20:5n-3)
7-*cis*,10-*cis*,13-*cis*,16-*cis*,19-*cis*-docosapentaenoic (DPA)	7*c*,10*c*,13*c*,16*c*,19*c*-C22:5 (C22:5n-3)
7-*cis*,10-*cis*,13-*cis*,16-*cis*,19-*cis*-docosahexaenoic (DHA)	4*c*, 7*c*,10*c*,13*c*,16*c*, 9*c*-C22:5 (C22:6n-3)

[a] FAs 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] Note that *trans*-C18:1 isomers from 4*t* to 14*t* elute as a group (see Fig. 3c). Therefore, it is practical to group their peak areas together and express as (4*t*-14*t*)-C18:1

[d] Peak for 9*c*-C18:1 includes a small proportion of 10*c*-C18:1. Therefore, their peak areas are grouped together.

[e] Note that 9*t*,15*c*-C18:2 and 10*t*,15v-C18:2 elute together. Therefore, their peak areas are grouped together.

Table 3. Essential reference FAMEs for a laboratory

Saturated fatty acid as methyl ester	*cis*-unsaturated fatty acid as methyl ester	trans-unsaturated fatty acid as methyl ester
butyrate (C4:0)	9*c*-tetradecenoate (myristoleate; 9*c*-C14:1)	6*t*-octadecenoate (6*t*-C18:1)
hexanoate (C6:0)	9*c*-hexadecenoate (palmitoleate; 9*c*-C16:1)	8*t*-octadecenoate (8*t*-C18:1)
octanoate (C8:0)	6*c*-octadecenoate (6*c*-18:1)	9*t*-octadecenoate (elaidate; 9*t*-C18:1)
decanoate (C10:0)	8*c*-octadecenoate (8*c*-C18:1)	10*t*-octadecenoate (10*t*-C18:1)
dodecanoate (laurate; C12:0)	9*c*-octadecenoate (oleate; 9*c*-C18:1)	11*t*-Octadecenoate (vaccenoate; 11*t*-C18:1)
tetradecanoate (myristate; C14:0)	10*c*-octadecenoate (10*c*-C18:1)	12*t*-octadecenoate (12*t*-C18:1)
pentadecanoate (C15:0)	11*c*-octadecenoate (*cis*-vaccenoate, 11*c*-C18:1)	13*t*-octadecenoate (13*t*-C18:1)
hexadecanoate (palmitate; C16:0)	12*c*-octadecenoate (6*c*-C18:1)	14*t*-octadecenoate (14*t*-C18:1)
heptadecanoate (C17:0)	9*c*,12*c*-octadecadienoate (linoleoate; 9*c*,12*c*-C18:2)	15*t*-octadecenoate (15*t*-C18:1)
octadecanoate (stearate; C18:0)	9*c*,12*c*,15*c*-octadecatrienoate (linolenioate; 9*c*,12*c*,15v-C18:3)	9*t*,12*t*-octadecadienoate (linoelaidioate; 9*t*,12*t*-C18:2)
eicosanoate (arachidate; C20:0)	11*c*-eicosenoate (gondoleate; 11*c*-C20:1)	9*c*,12*t*-octadecadienoate (9*c*,12*t*-C18:2)
docosanoate (behenoate; C22:0)	5*c*, 8*c*, 11*c*,14v-eicosatetraenoate (arachidonate; 5*c*, 8*c*,11*c*, 14*c*-C20:4)	9*t*,12*c*-octadecadienoate (9*t*,12*c*-C18:2)
tetracosanoate (tetracosanoate; C24:0)	5*c*, 8*c*, 11*c*,14*c*,17*c*-eicosapentaenoate (EPA; 5*c*, 8*c*,11*c*,14*c*,17*c*-C20:5)	9*t*,12*c*,15*t*-octadecatrienoate (9*t*,12*c*,15*t*-C18:3)
	13*c*-docosaenoate (eruciate; 13*c*-C22:1)	9*c*,12*c*,15*t*-octadecatrienoate (9*c*,12*c*,15*t*-C18:3)
	4*c*,7*c*,10*c*,13*c*,16*c*,19*c*-docosahexaenoate (DHA; 4*c*,7*c*,10*cc*,13*c*,16*c*,19*c*-C22:6)	9*t*,12*c*,15*c*-octadecatrienoate (9*t*,12*c*,15*c*-C18:3)
	15*c*-tetracosenoate (nervonoate; 15*c*-C24:1)	

Table 4. Certified reference FAME standards available from commercial sources[a]

Supelco 37 component FAME mix solution in methylene chloride			
FAME	**Concentration**	**FAME**	**Concentration**
C4:0	400 µg/mL	9*t*,12*t*-C18:2	200 µg/mL
C6:0	400 µg/mL	9*c*,12*c*-C18:2	200 µg/mL
C8:0	400 µg/mL	6*c*,9*c*,12*c*-C18:3	200 µg/mL
C10:0	400 µg/mL	9*c*,12*c*,15*c*-C18:3	200 µg/mL
C11:0	200 µg/mL	C20:0	400 µg/mL
C12:0	400 µg/mL	11*c*-C20:1	≤200 µg/mL
C13:0	200 µg/mL	11*c*,14*c*-C20:2	200 µg/mL
C14:0	400 µg/mL	8*c*,11*c*,14*c*-C20:3	200 µg/mL
9*c*-C14:1	200 µg/mL	11*c*,14*c*,17*c*-C20:3	200 µg/mL
C15:0	200 µg/mL	5*c*,8*c*,11*c*,14*c*-C20:4	200 µg/mL
10*c*-15:1	200 µg/mL	5*c*,8*c*,11*c*,14*c*,17*c*-C20:5	200 µg/mL
C16:0	600 µg/mL	C21:0	200 µg/mL
9*c*-C16:1	200 µg/mL	C22:0	400 µg/mL
C17:0	200 µg/mL	13*c*-C22:1	200 µg/mL
10*c*-C17:1	200 µg/mL	13*c*,16*c*-C22:2	200 µg/mL
C18:0	400 µg/mL	4*c*,7*c*,10*c*,13*c*,16*c*,19*c*-C22:6	200 µg/mL
9*t*-C18:1	200 µg/mL	C23:0	200 µg/mL
9*c*-C18:1	400 µg/mL	C24:0	400 µg/mL
		15*c*-C24:1	200 µg/mL

Linoleic Acid Methyl Mix solution in methylene chloride			
9*c*,12*c*-C18:2	10% w/w	9*c*,12*t*-C18:2	20% w/w
9*t*,12*c*-C18:2	20% w/w	9*t*,12*t*-C18:2	50% w/w

Linolenic Acid Methyl Mix solution in methylene chloride			
9*t*,12*t*,15*t*-C18:3	30% w/w	9*c*,12*c*,15*t*-C18:3	7% w/w
9*t*,12*c*,15*t*-C18:3	15% w/w	9*c*,12*t*,15*c*-C18:3	7% w/w
9*t*,12*t*,15*c*-C18:3	15% w/w	9*c*,12*t*,15*t*-C18:3	15% w/w
9*t*,12*c*,15*c*-C18:3	7% w/w	9*c*,12*c*,15*c*-C18:3	3% w/w

[a] Supelco 37 component FAME mix (*Trace*CERT; catalogue number CRM47885), linoleic acid methyl mix (catalogue number CRM47791) and linolenic acid methyl ester mix (catalogue number L6031). All mixtures are available from MilliporeSigma.

Table 5. Resolution factors (R-value)[a] with different carrier gases (hydrogen and helium) at a flow rate of 1.0 mL/min at 180 °C isothermal on both 100 m CP-Sil 88 and SP-2560 capillary columns

R-value	CP-Sil 88 H_2	CP-Sil 88 He	SP-2560 H_2	SP-2560 He
(13*t*+14*t*)-C18:1 and 9*c*-C18:1	1.3	ND	1.1	1.0
9*c*-C18:1 and 11*c*-C18:1	2.0	2.0	2.4	2.3
16*t*-C18:1 and 14*c*-C18:1	0.5	ND	ND	0.3
11*c*-C20:1 and 9*c*,12*c*,15*c*-C18:3	2.3	1.9	1.3	1.0

ND: not determined

[a] Determined using the equation $R = 1.18 (t_{R2} - t_{R1})/(W_{0.5h1} + W_{0.5h2})$, where t_{R2} and t_{R1} = retention times (in minutes) of adjacent peaks 2 and 1, respectively (peak 1 elutes before peak 2), and $W_{0.5h1}$ and $W_{0.5h2}$ = width at half height of adjacent peaks 1 and 2, respectively

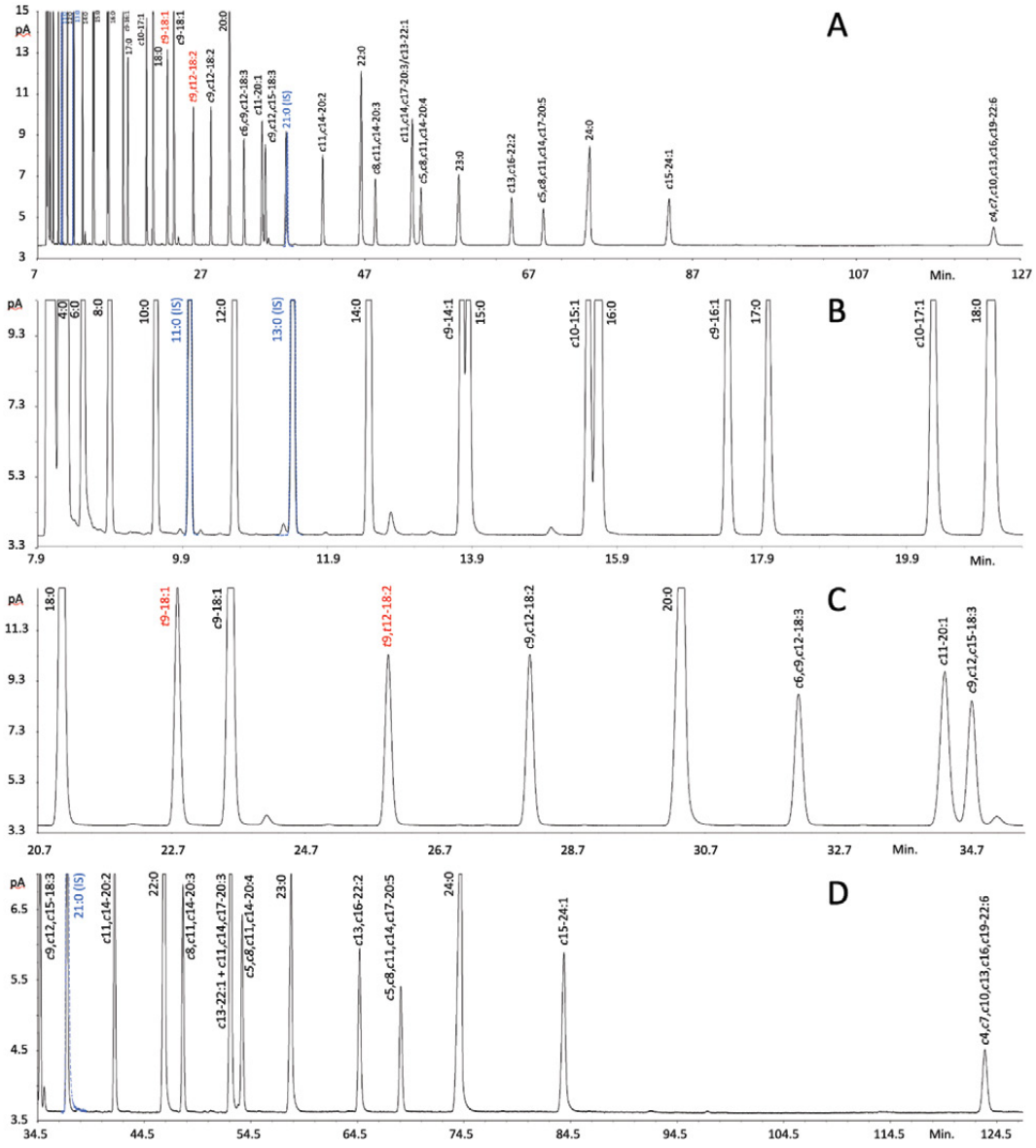
Source: Ratnayake, Hansen & Kennedy *(19)*

Table 6. Guide to test portion size and internal standard volume

Expected fat or oil concentration	Test partition amount (mg)	Fat or oil concentration (mg/mL)	Fat or oil amount (mg)	Amount of TAG IS added (mg)	13:0 TAG IS (mL)	21:0 TAG IS (mL)
90–100%	100	18–20	90–100	4.5–10	1–2	1–2
80–89%	100	16–18	80–90	4–9	1–2	1–2
70–79%	125	17.5–20	87.5–100	4.4–10	1–2	1–2
60–69%	125	15–17.5	75–87.5	3.8–8.8	1–2	1–2
50–59%	150	15–18	75–90	3.8–9	1–2	1–2
40–49%	200	16–20	80–100	4–10	1–2	1–2
30–39%	250	15–20	75–100	3.8–10	1–2	1–2
20–29%	325	13–19.5	65–97.5	3.3–9.8	1–2	1–2
10–19%	500	10–20	50–100	2.5–10	0.5–2	0.5–2
ND-9%	1000	<20	<100	<10	0.5–2	0.5–2

Source: AOCS Official Method Ce 2b-11 *(8)*

Fig. 1. GC chromatogram of Supelco 37 component FAME mix on SP-2560 capillary column (isothermal elution)



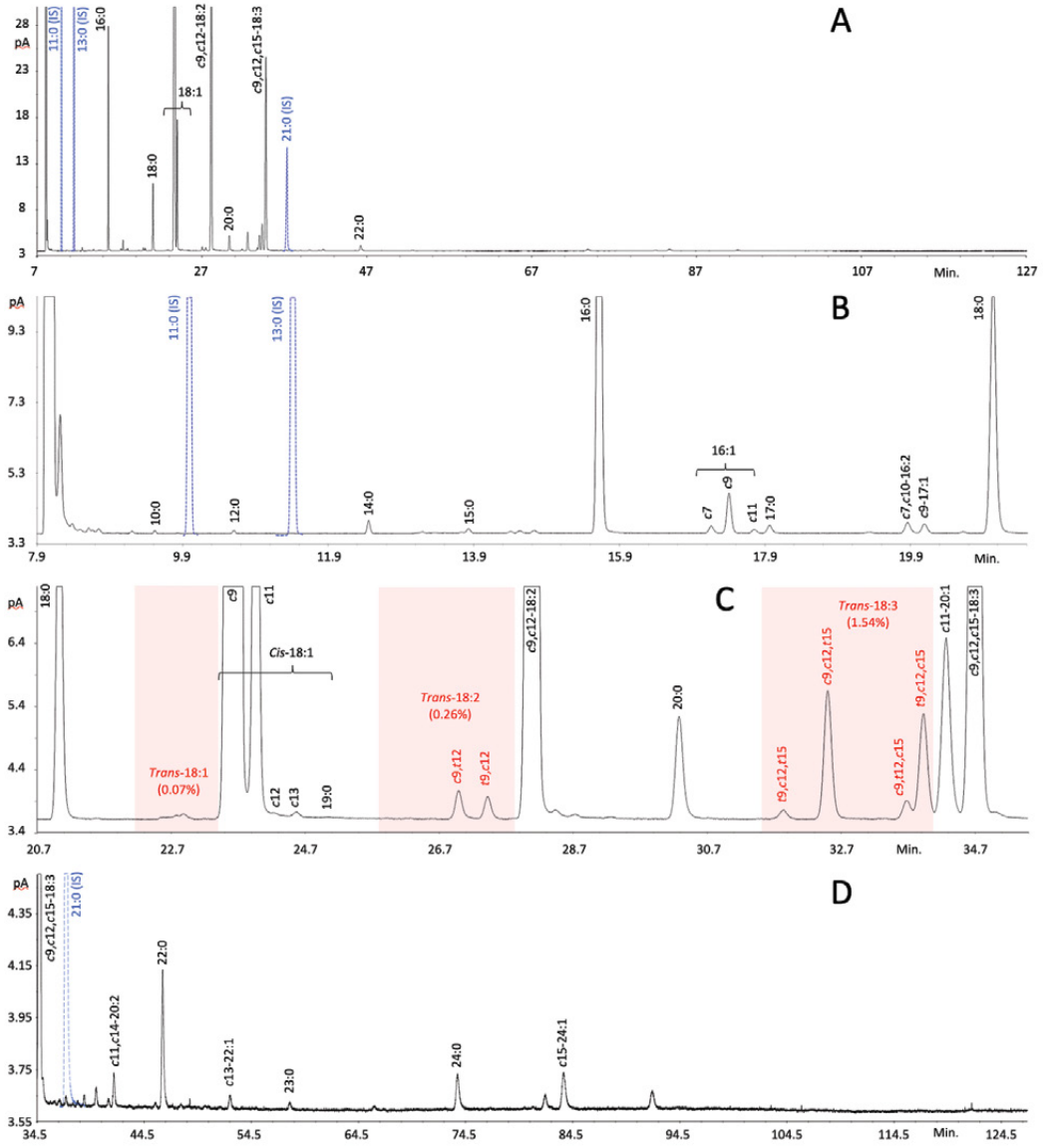
Notes:

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.

Plots are for 180 °C isothermal elution, hydrogen carrier at 1.0 mL/min constant flow.

Source: Dr Pierluigi Delmonte, Food and Drug Administration, United States of America (2023)

Fig. 2. GC chromatogram of canola oil on SP-2560 capillary column (isothermal elution)



Notes:

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.

Plots are for 180 °C isothermal elution, hydrogen carrier at 1.0 mL/min constant flow.

Source: Dr Pierluigi Delmonte, Food and Drug Administration, United States of America (2023)

Fig. 3. GC chromatogram of a PHO on SP-2560 capillary column (isothermal elution)

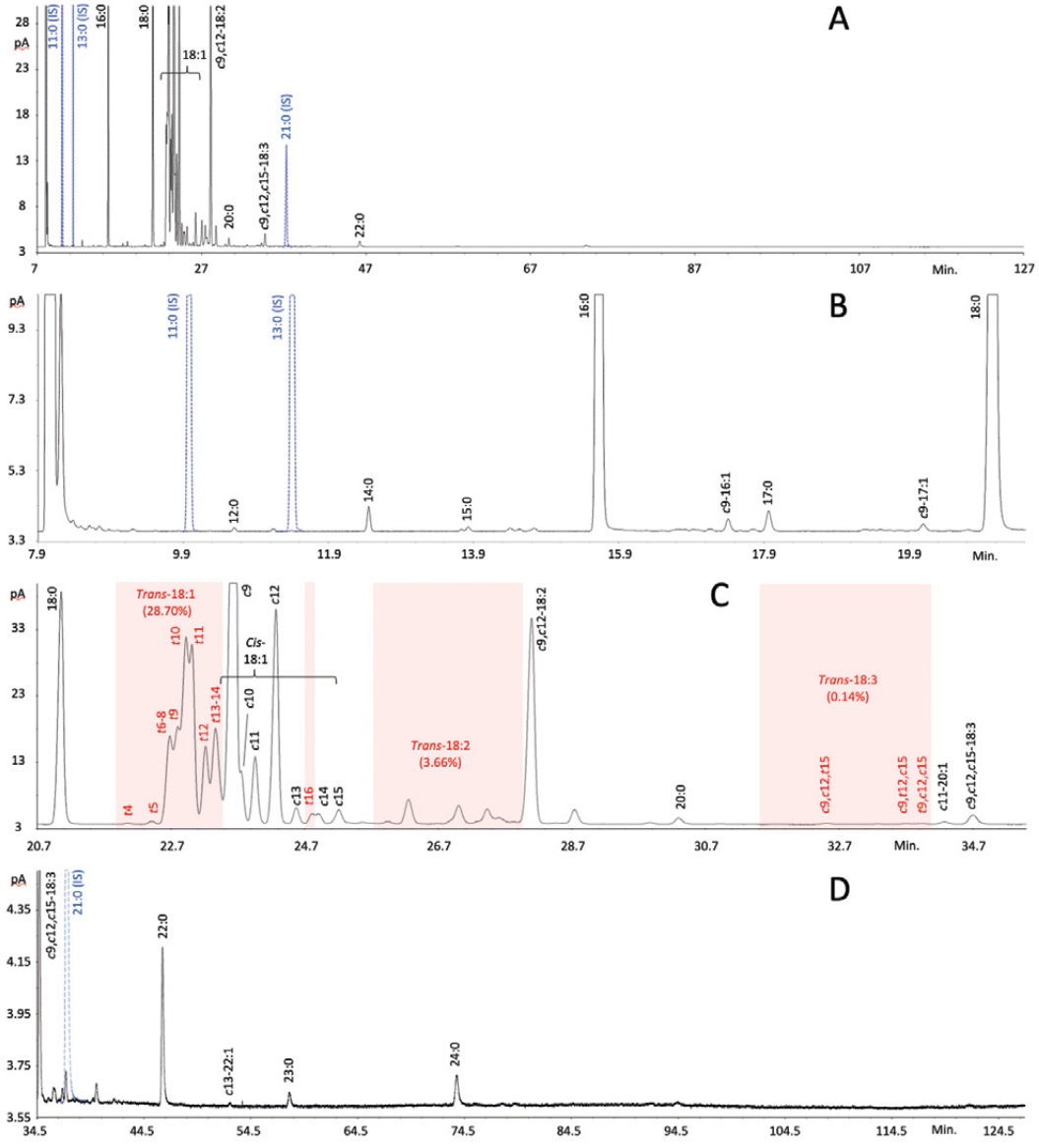
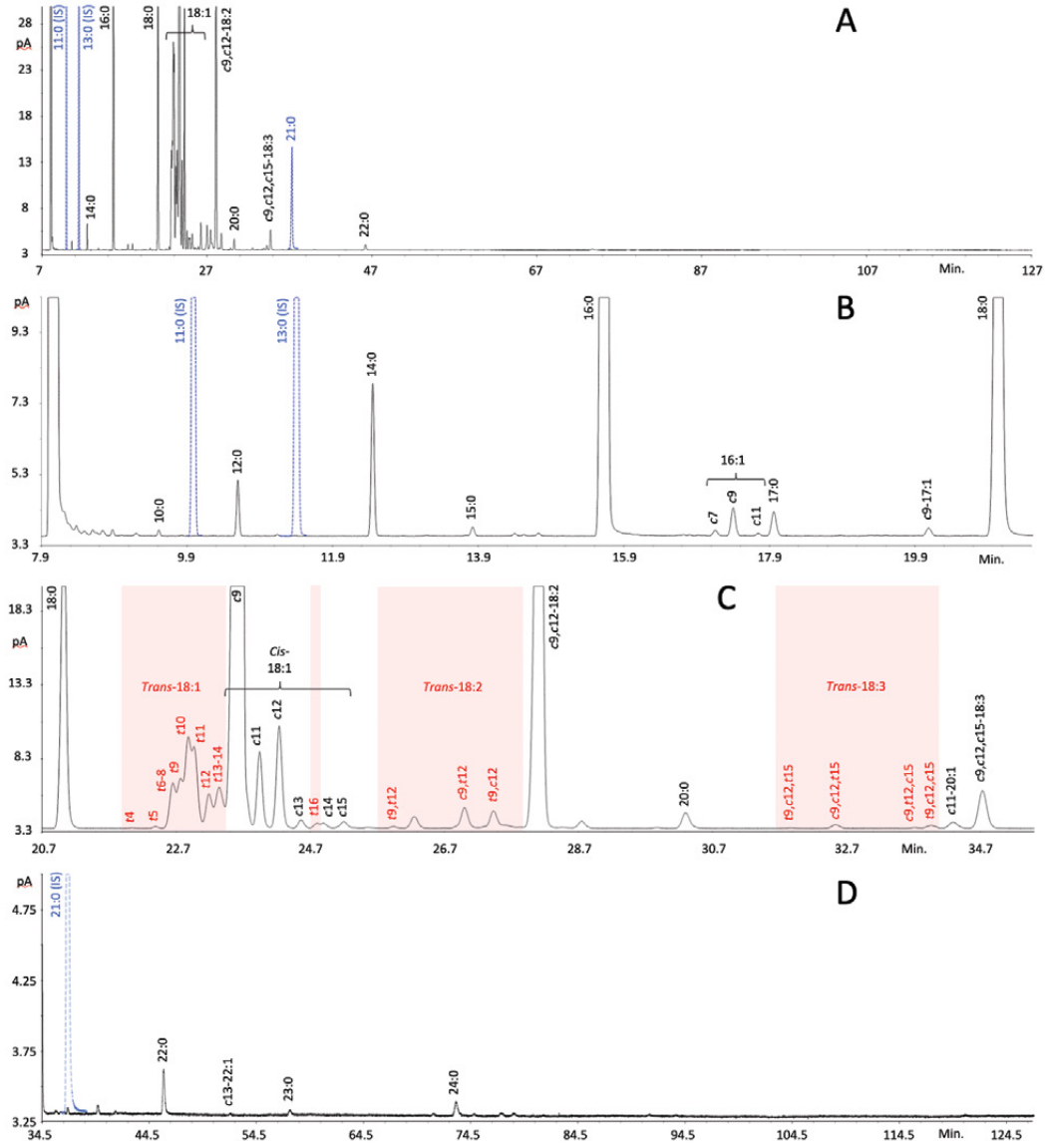


Fig. 3A: Entire GC chromatogram. Fig. 3B, 3C and 3D: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3n-3, and C18:3n-3 to 22:6n-3, respectively.

Plots are for 180 °C isothermal elution, hydrogen carrier at 1.0 mL/min constant flow.

Source: Dr Pierluigi Delmonte, Food and Drug Administration, United States of America (2023)

Fig. 4. GC chromatogram of a shortening blend on SP-2560 capillary column (isothermal elution)



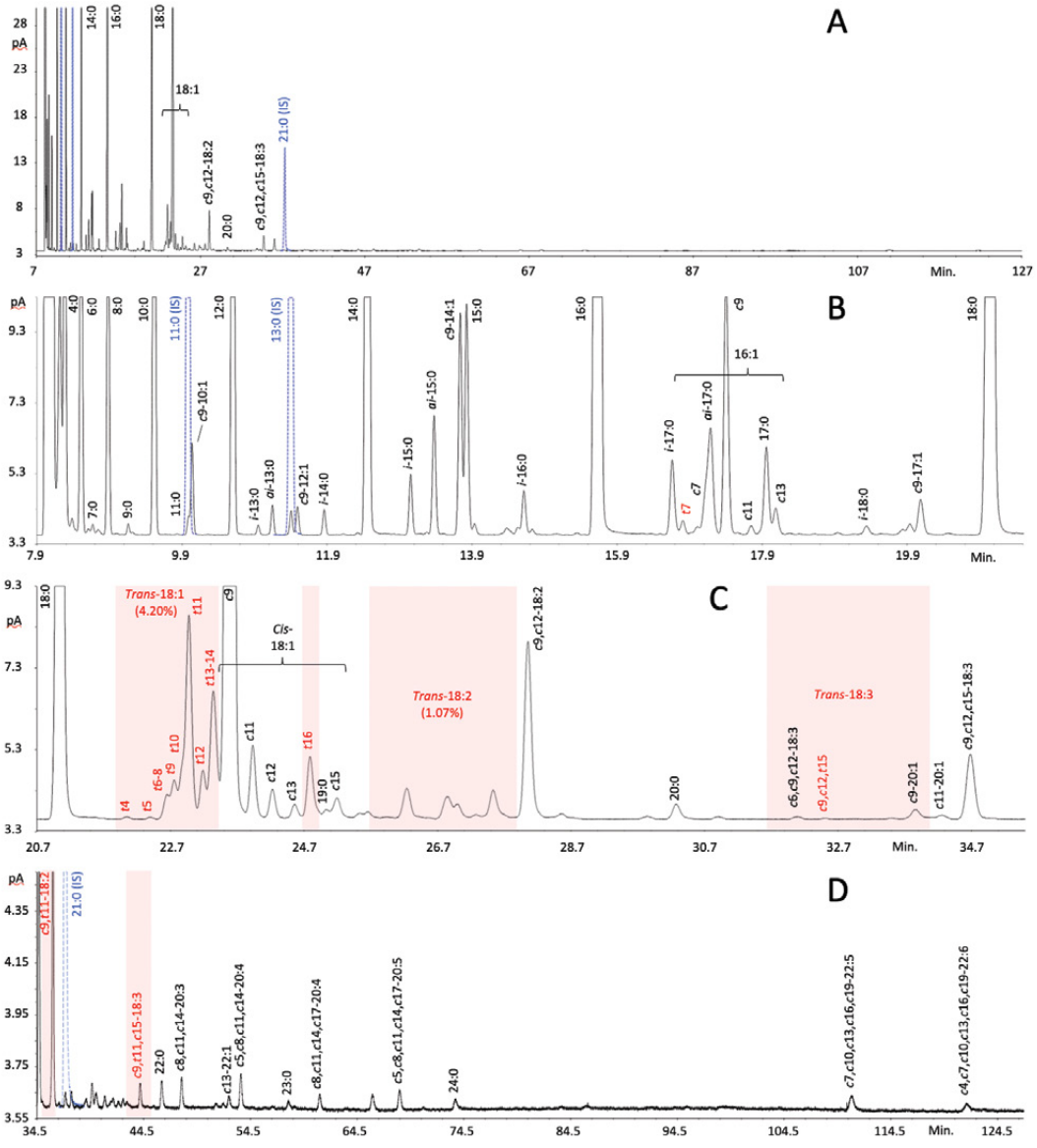
Notes:

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.

Plots are for 180 °C isothermal elution, hydrogen carrier at 1.0 mL/min constant flow.

Source: Dr Pierluigi Delmonte, Food and Drug Administration, United States of America (2023)

Fig. 5. GC chromatogram of a butter on SP-2560 capillary column (isothermal elution)



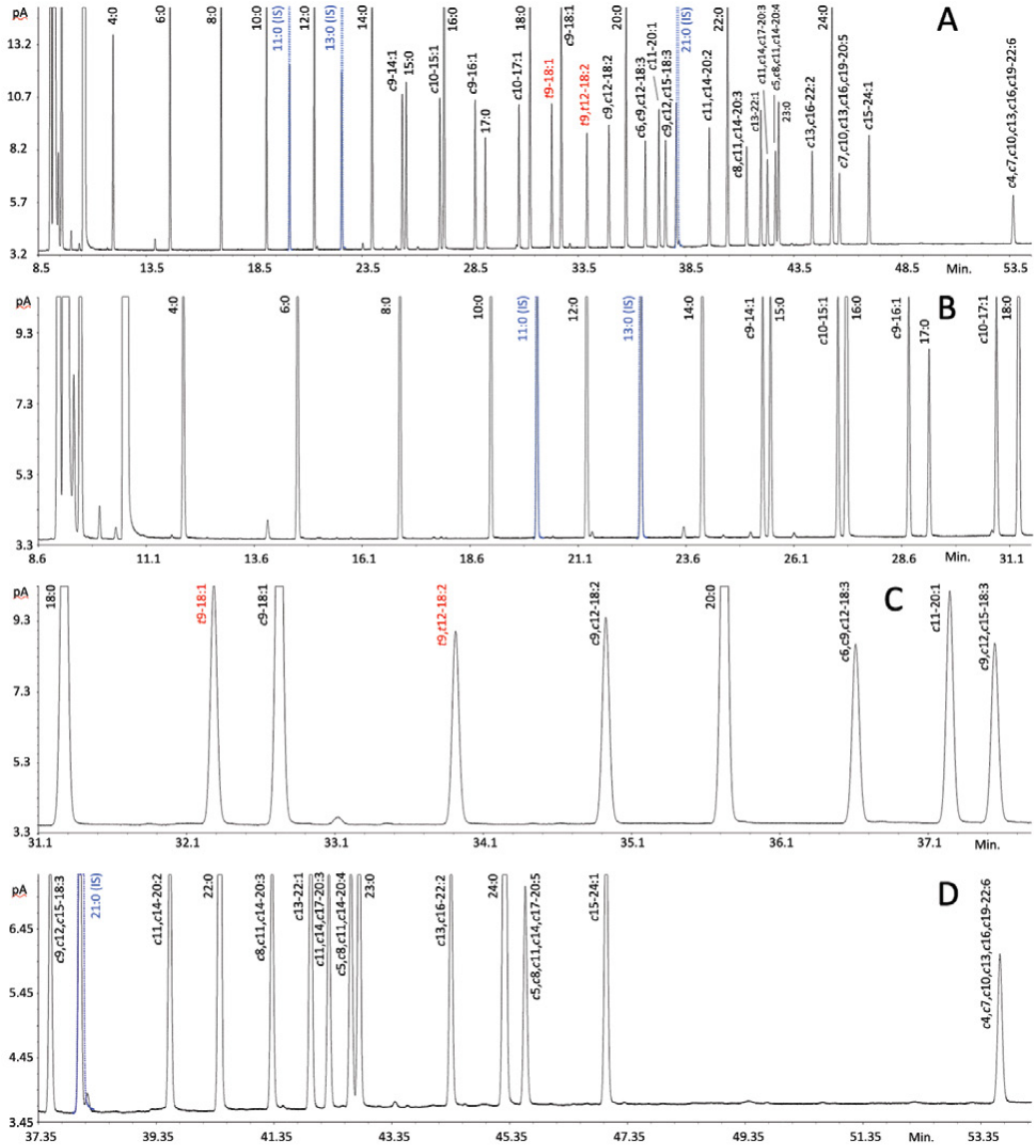
Notes:

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.

Plots are for 180 °C isothermal elution, hydrogen carrier at 1.0 mL/min constant flow.

Source: Dr Pierluigi Delmonte, Food and Drug Administration, United States of America (2023)

Fig. 6. GC chromatogram of Supelco 37 component FAME mix on SP-2560 capillary column (temperature programme)



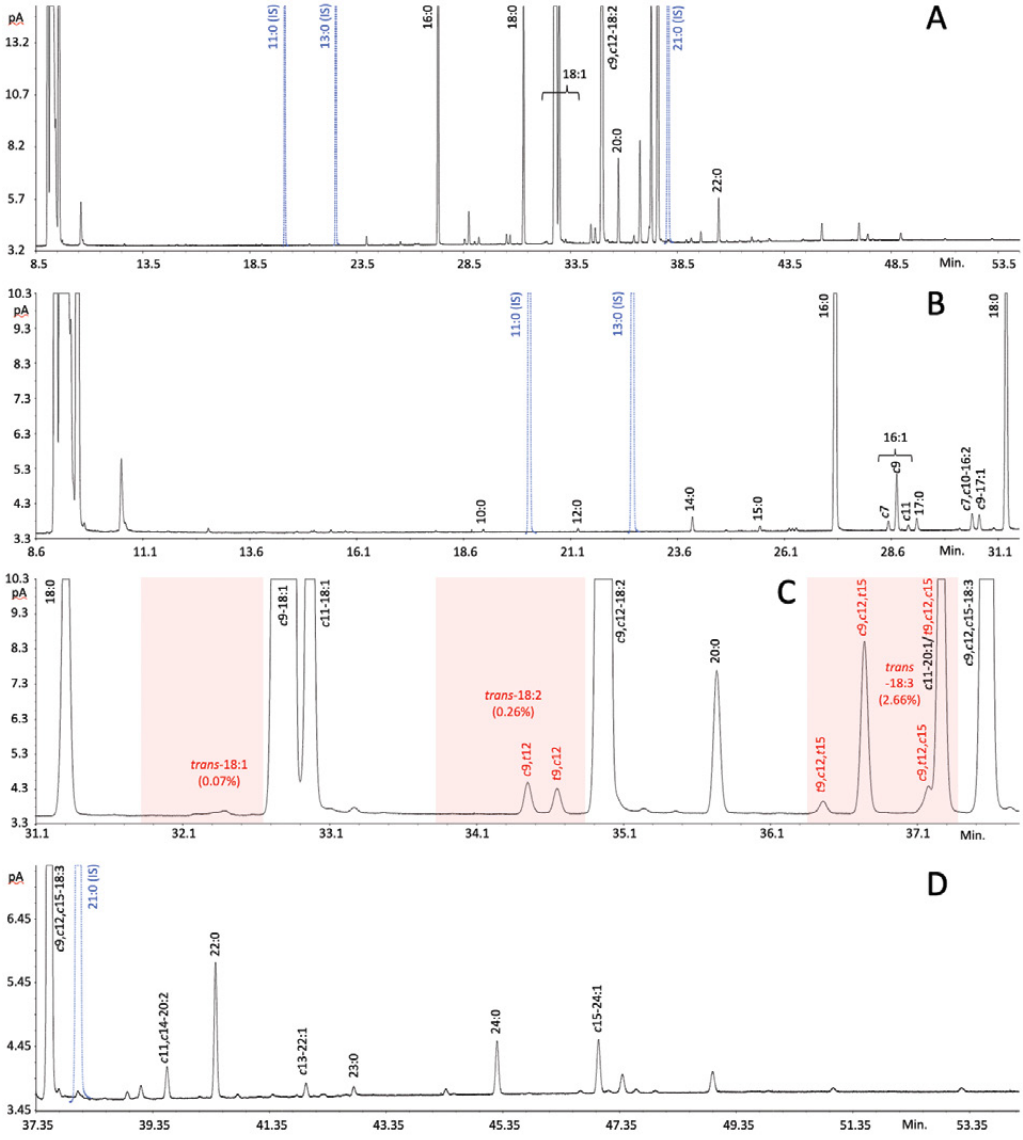
Notes:

Fig. 6A: Entire GC chromatogram. Fig. 6B, 6C and 6D: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3n-3, and C18:3n-3 to 22:6n-3, respectively.

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, Food and Drug Administration, United States of America (2023)

Fig. 7. GC chromatogram of canola oil on SP-2560 capillary column (temperature programme)



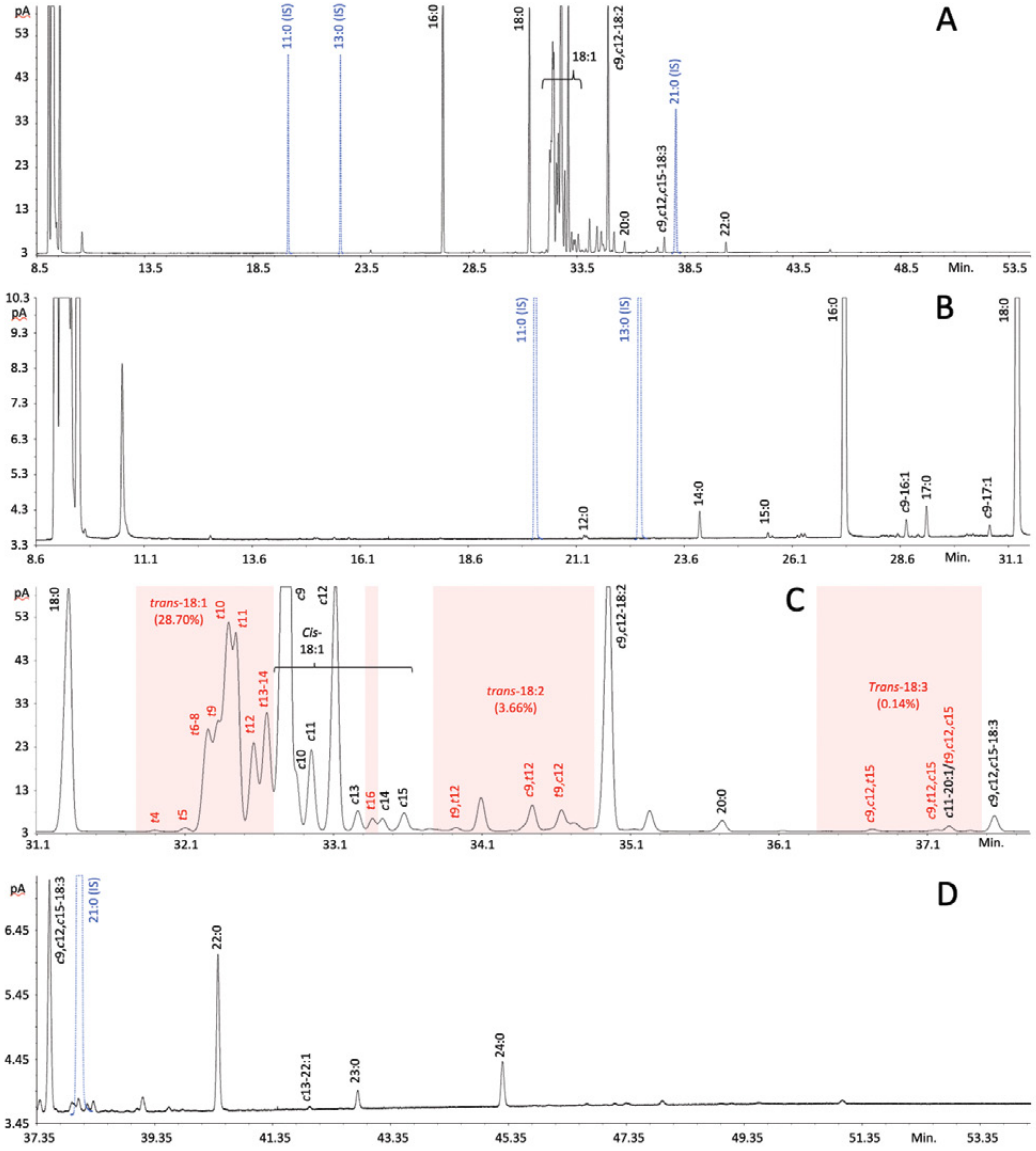
Notes:

Fig. 7A: Entire GC chromatogram. Fig. 7B, 7C and 7D: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3n-3, and C18:3n-3 to 22:6n-3, respectively.

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, Food and Drug Administration, United States of America (2023)

Fig. 8. GC chromatogram of a PHO on SP-2560 capillary column (temperature programme)



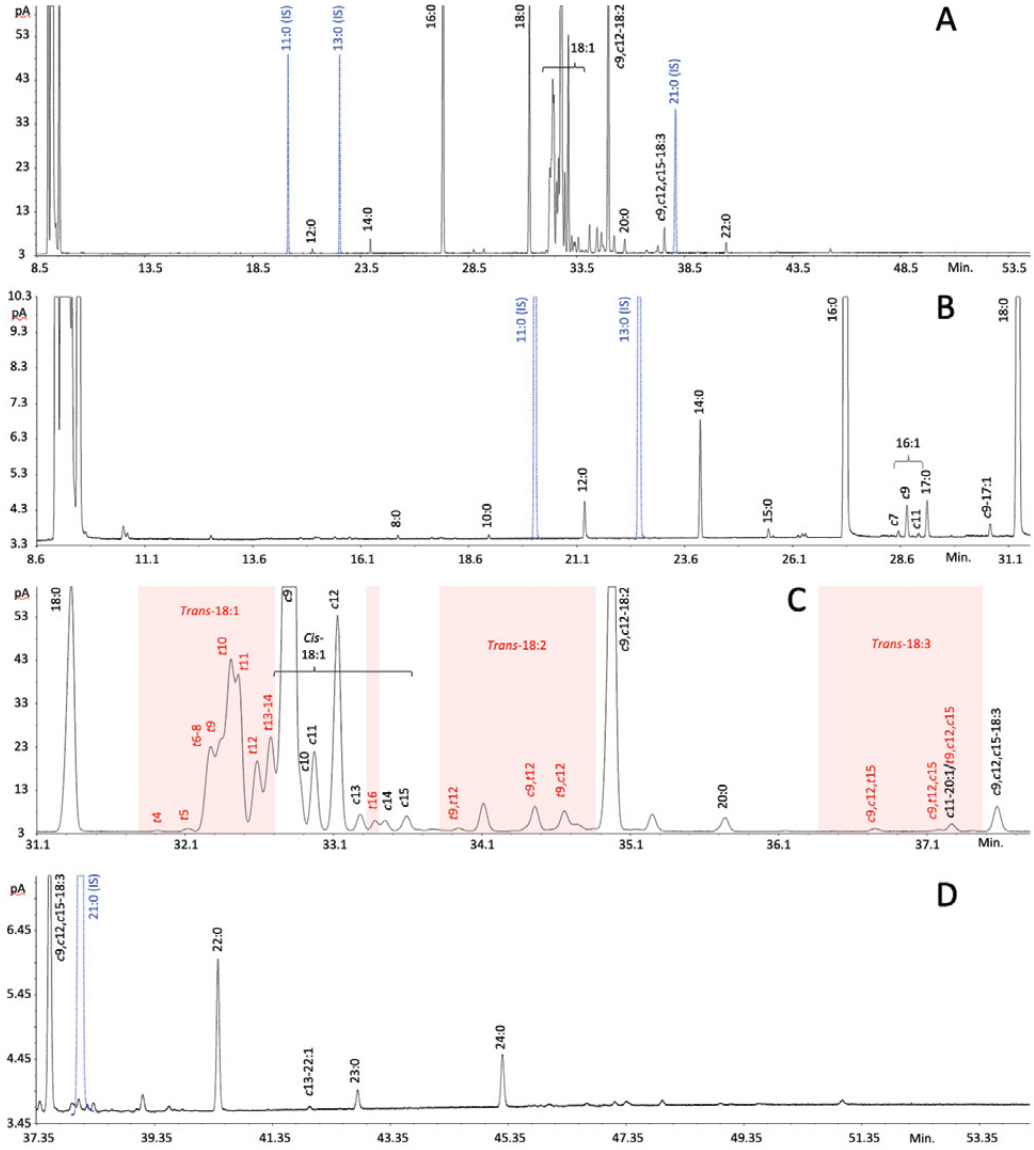
Notes:

Fig. 8A: Entire GC chromatogram. Fig. 8B, 8C and 8D: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3n-3, and C18:3n-3 to 22:6n-3, respectively.

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, Food and Drug Administration, United States of America (2023)

Fig. 9. GC chromatogram of a shortening blend on SP-2560 capillary column (temperature programme)



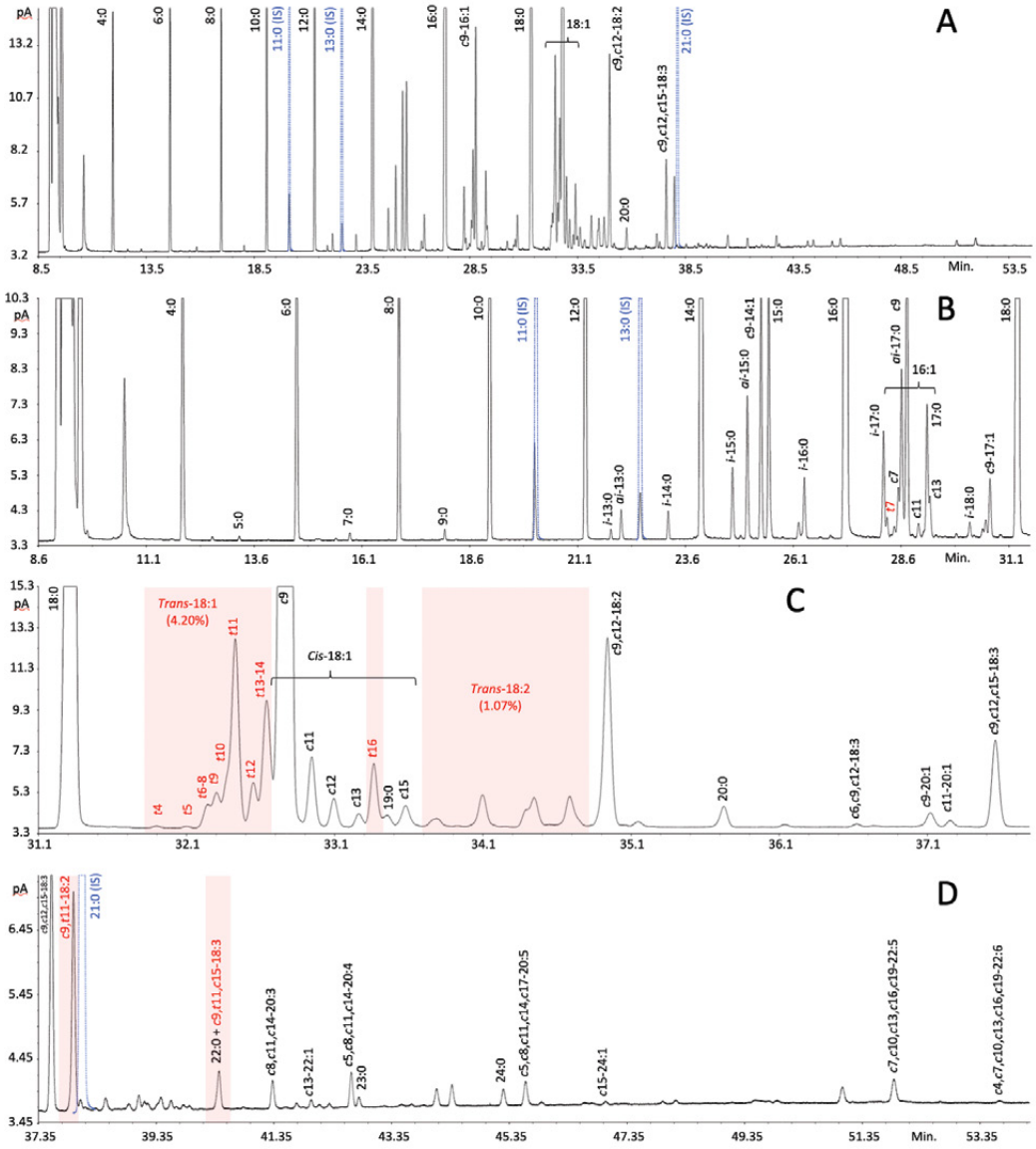
Notes:

Fig. 9A: Entire GC chromatogram. Fig. 9B, 9C and 9D: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3n-3, and C18:3n-3 to 22:6n-3, respectively.

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, Food and Drug Administration, United States of America (2023)

Fig. 10. GC chromatogram of a butter on SP-2560 capillary column (temperature programme)



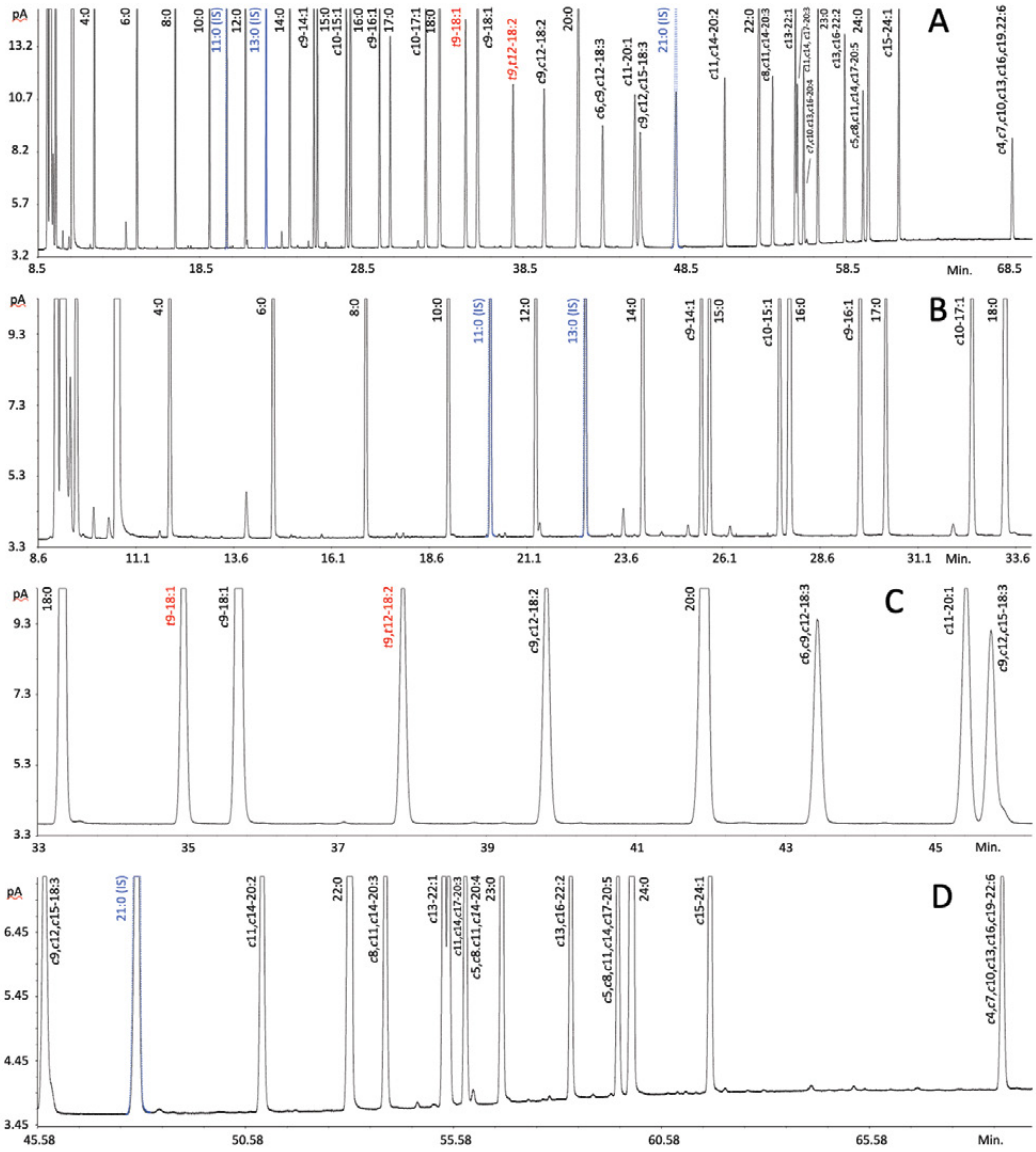
Notes:

Fig. 10A: Entire GC chromatogram. Fig. 10B, 10C and 10D: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3n-3, and C18:3n-3 to 22:6n-3, respectively.

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, Food and Drug Administration, United States of America (2023)

Fig. 11. GC chromatogram of Supelco 37 component FAME mix on SP-2560 capillary column (modified temperature programme)



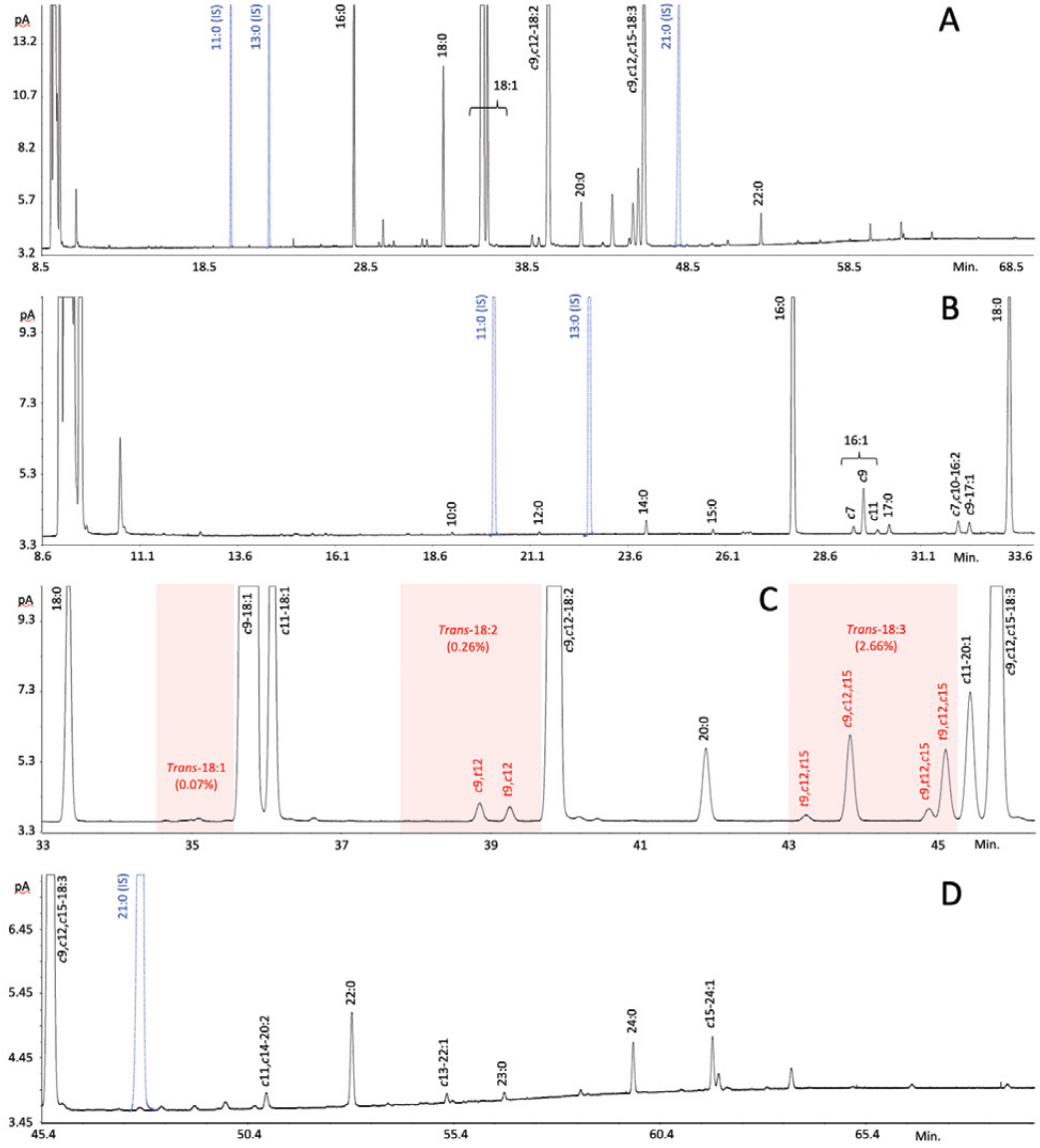
Notes:

Fig. 11A: Entire GC chromatogram. Fig. 11B, 11C and 11D: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3n-3, and C18:3n-3 to 22:6n-3, respectively.

Temperature programme: initial temperature 60 °C for 5 min, ramped to 165 °C at 15 °C/min, hold 1 min, ramped to 184 °C at 2 °C/min, hold 25 min, ramped to 225 °C at 4 °C/min, hold 25 min. Hydrogen carrier gas at 1.0 mL/min constant flow.

Source: Dr Pierluigi Delmonte, Food and Drug Administration, United States of America (2023)

Fig. 12. GC chromatogram of canola oil on SP-2560 capillary column (modified temperature programme)



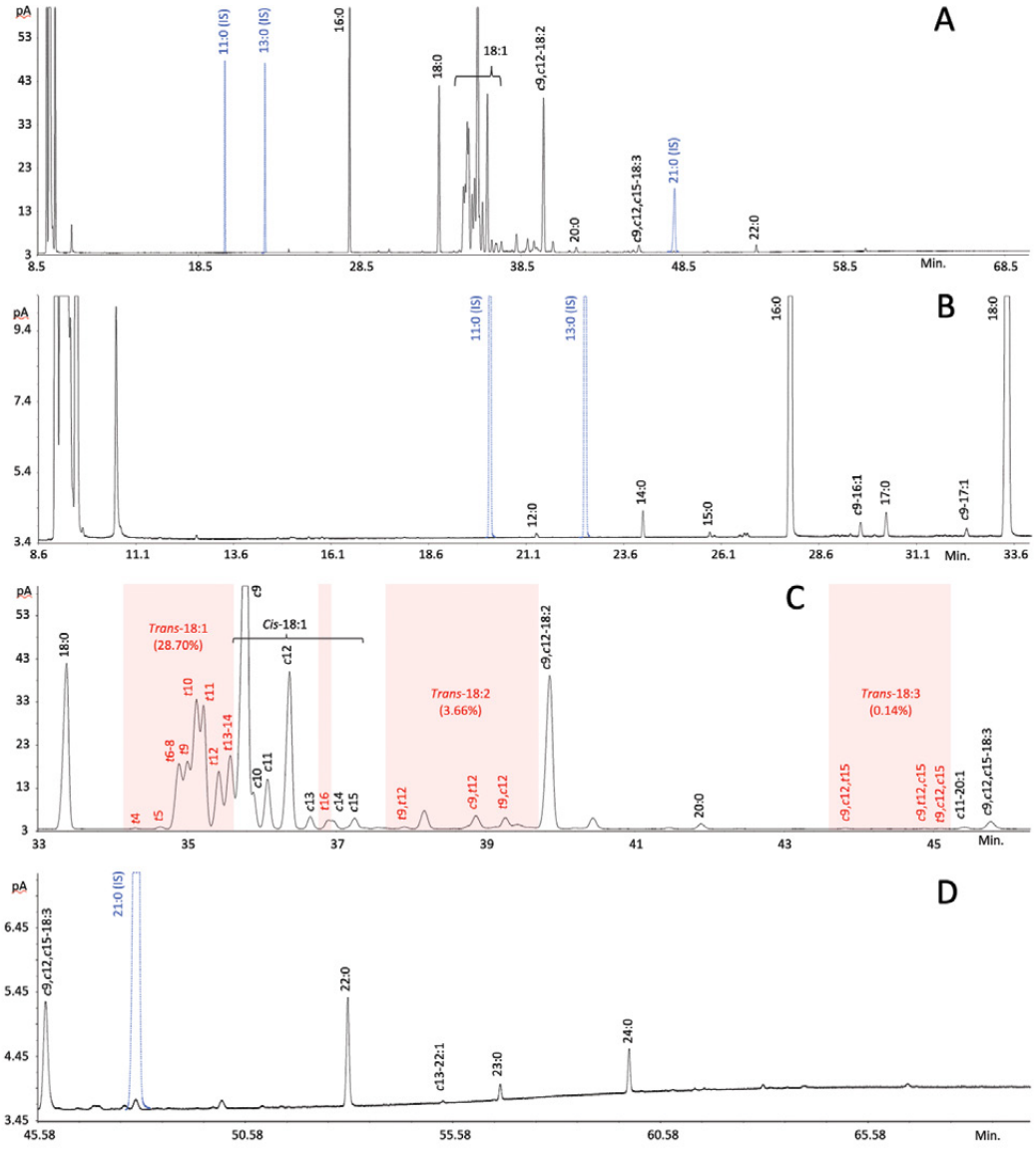
Notes:

Fig. 12A: Entire GC chromatogram. Fig. 12B, 12C and 12D: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3n-3, and C18:3n-3 to 22:6n-3, respectively.

Temperature programme: initial temperature 60 °C for 5 min, ramped to 165 °C at 15 °C/min, hold 1 min, ramped to 184 °C at 2 °C/min, hold 25 min, ramped to 225 °C at 4 °C/min, hold 25 min. Hydrogen carrier gas at 1.0 mL/min constant flow.

Source: Dr Pierluigi Delmonte, Food and Drug Administration, United States of America (2023)

Fig. 13. GC chromatogram of a PHO on SP-2560 capillary column (modified temperature programme)



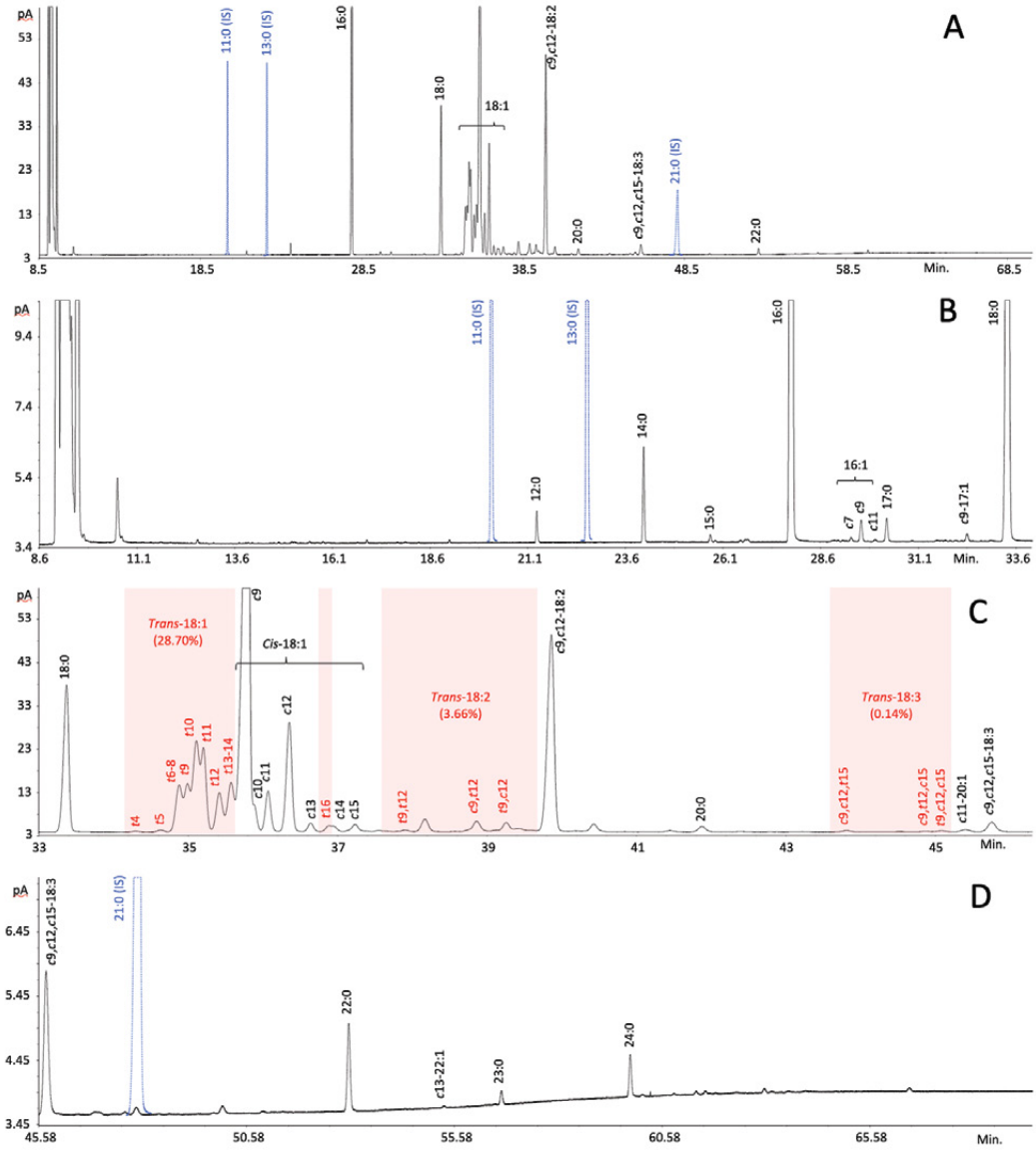
Notes:

Fig. 13A: Entire GC chromatogram. Fig. 13B, 13C and 13D: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3n-3, and C18:3n-3 to 22:6n-3, respectively.

Temperature programme: initial temperature 60 °C for 5 min, ramped to 165 °C at 15 °C/min, hold 1 min, ramped to 184 °C at 2 °C/min, hold 25 min, ramped to 225 °C at 4 °C/min, hold 25 min. Hydrogen carrier gas at 1.0 mL/min constant flow.

Source: Dr Pierluigi Delmonte, Food and Drug Administration, United States of America (2023)

Fig. 14. GC chromatogram of a shortening blend on SP-2560 capillary column (modified temperature programme)



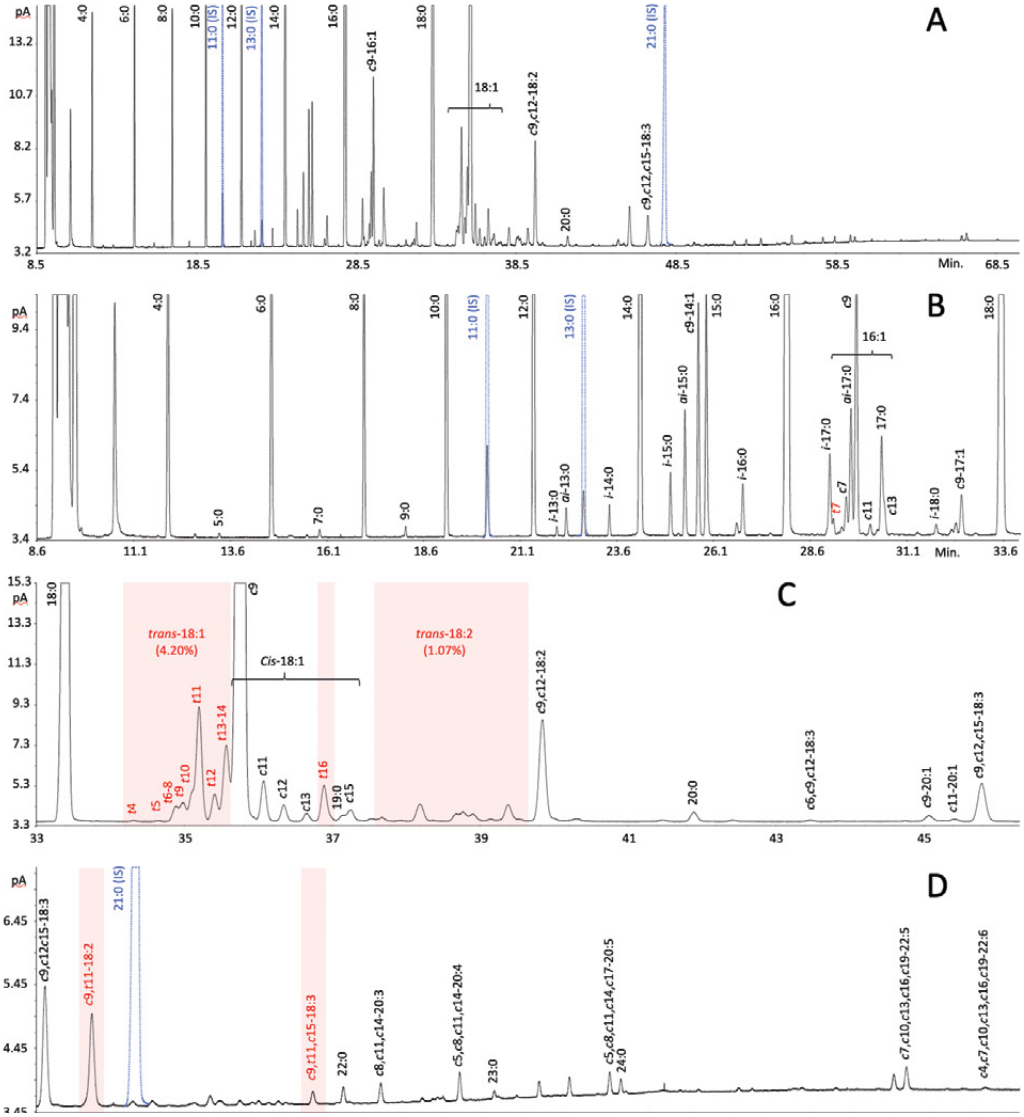
Notes:

Fig. 14A: Entire GC chromatogram. Fig. 14B, 14C and 14D: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3n-3, and C18:3n-3 to 22:6n-3, respectively.

Temperature programme: initial temperature 60 °C for 5 min, ramped to 165 °C at 15 °C/min, hold 1 min, ramped to 184 °C at 2 °C/min, hold 25 min, ramped to 225 °C at 4 °C/min, hold 25 min. Hydrogen carrier gas at 1.0 mL/min constant flow.

Source: Dr Pierluigi Delmonte, Food and Drug Administration, United States of America (2023)

Fig. 15. GC chromatogram of a butter on SP-2560 capillary column (modified temperature programme)



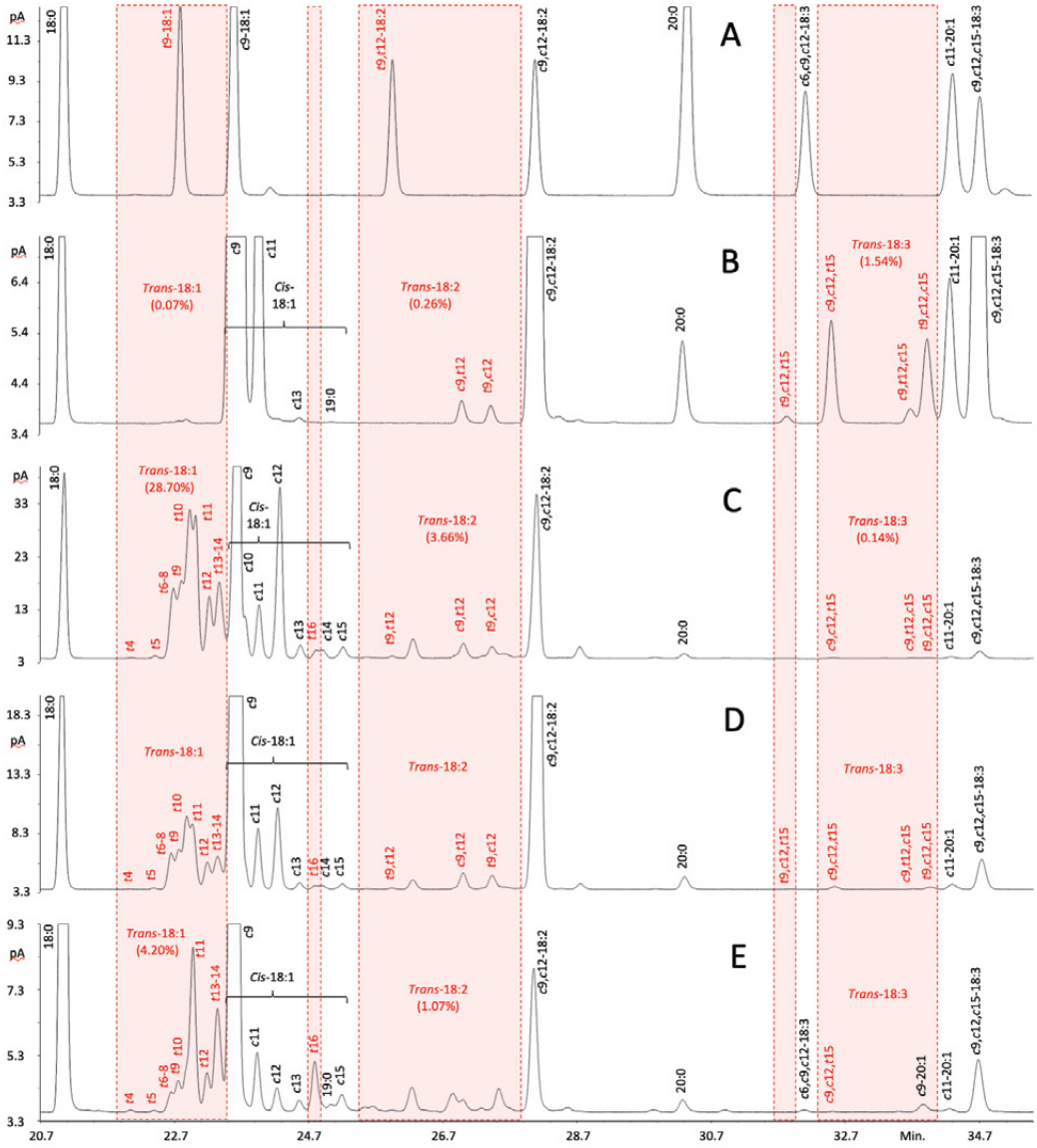
Notes:

Fig. 15A: Entire GC chromatogram. Fig. 15B, 15C and 15D: GC chromatogram segments from solvent front to C18:0, C18:0 to C18:3n-3, and C18:3n-3 to 22:6n-3, respectively.

Temperature programme: initial temperature 60 °C for 5 min, ramped to 165 °C at 15 °C/min, hold 1 min, ramped to 184 °C at 2 °C/min, hold 25 min, ramped to 225 °C at 4 °C/min, hold 25 min. Hydrogen carrier gas at 1.0 mL/min constant flow.

Source: Dr Pierluigi Delmonte, Food and Drug Administration, United States of America (2023)

Fig. 16. GC chromatogram of selected FAME samples on SP-2560 capillary column (isothermal elution)



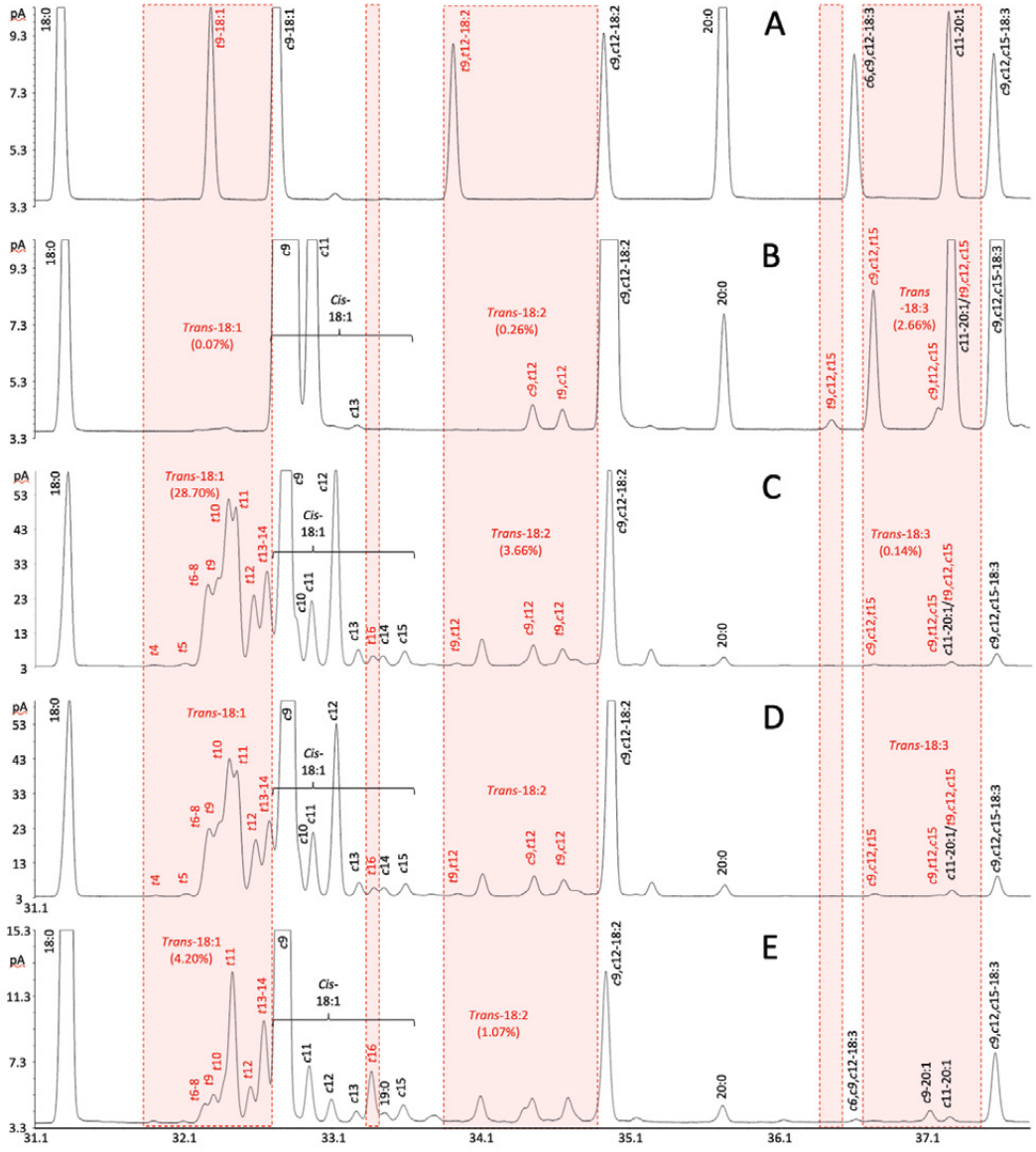
Notes:

Fig. 16A: Supelco 37 component FAME mix. Fig. 16B: canola oil. Fig. 16C: PHO. Fig. 16D: shortening blend. Fig. 16E: butter.

Plot is for 180 °C isothermal, hydrogen carrier at 1.0 mL/min constant flow.

Source: Dr Pierluigi Delmonte, Food and Drug Administration, United States of America (2023)

Fig. 17. GC chromatogram of selected FAME samples on SP-2560 capillary column (temperature programme)



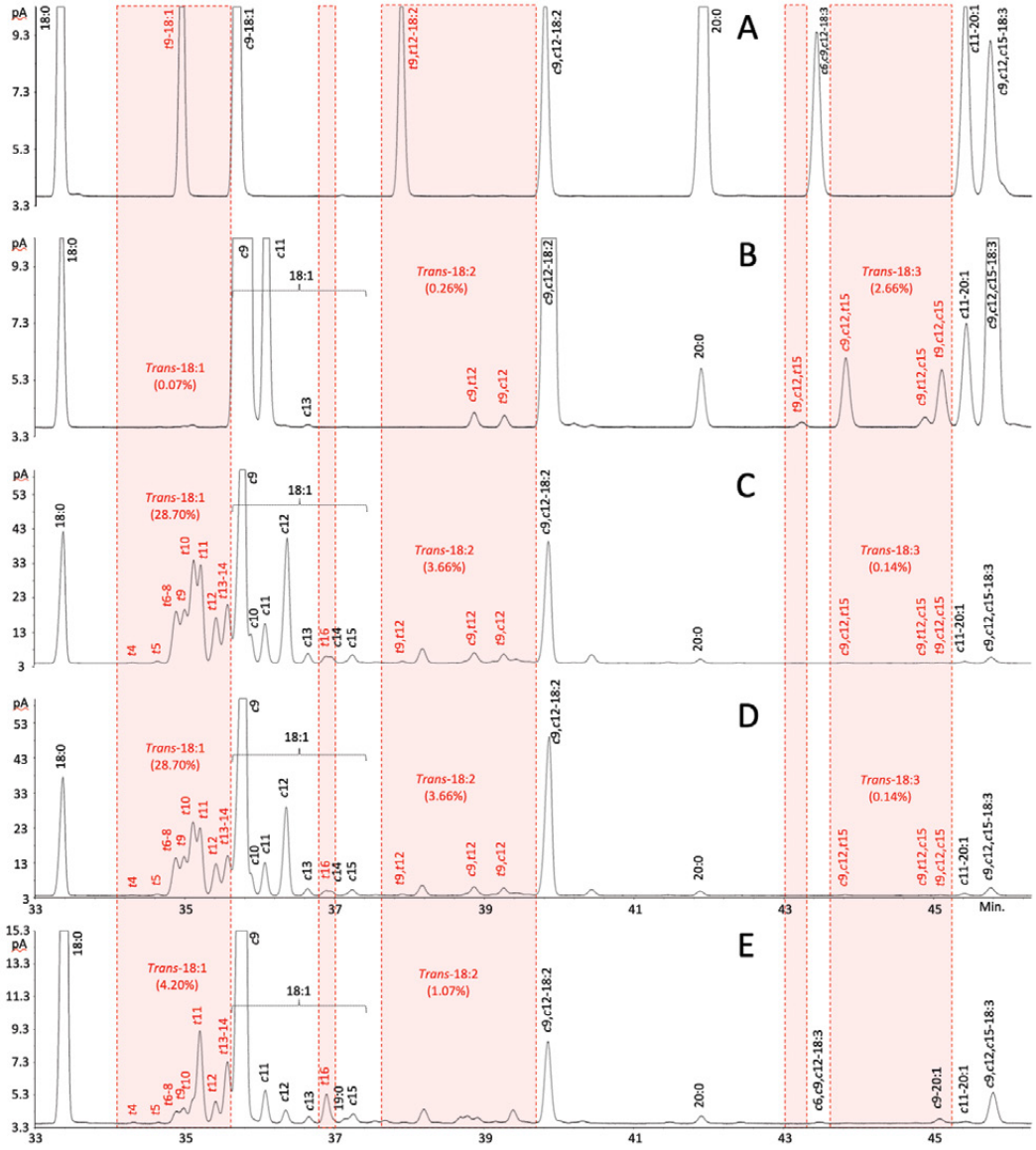
Notes:

Fig. 17A: Supelco 37 component FAME mix. Fig. 17B: canola oil. Fig. 17C: PHO. Fig. 17D: shortening blend. Fig. 17E: butter.

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, Food and Drug Administration, United States of America (2023)

Fig. 18. GC chromatogram of selected FAME samples on SP-2560 capillary column (modified temperature programme)

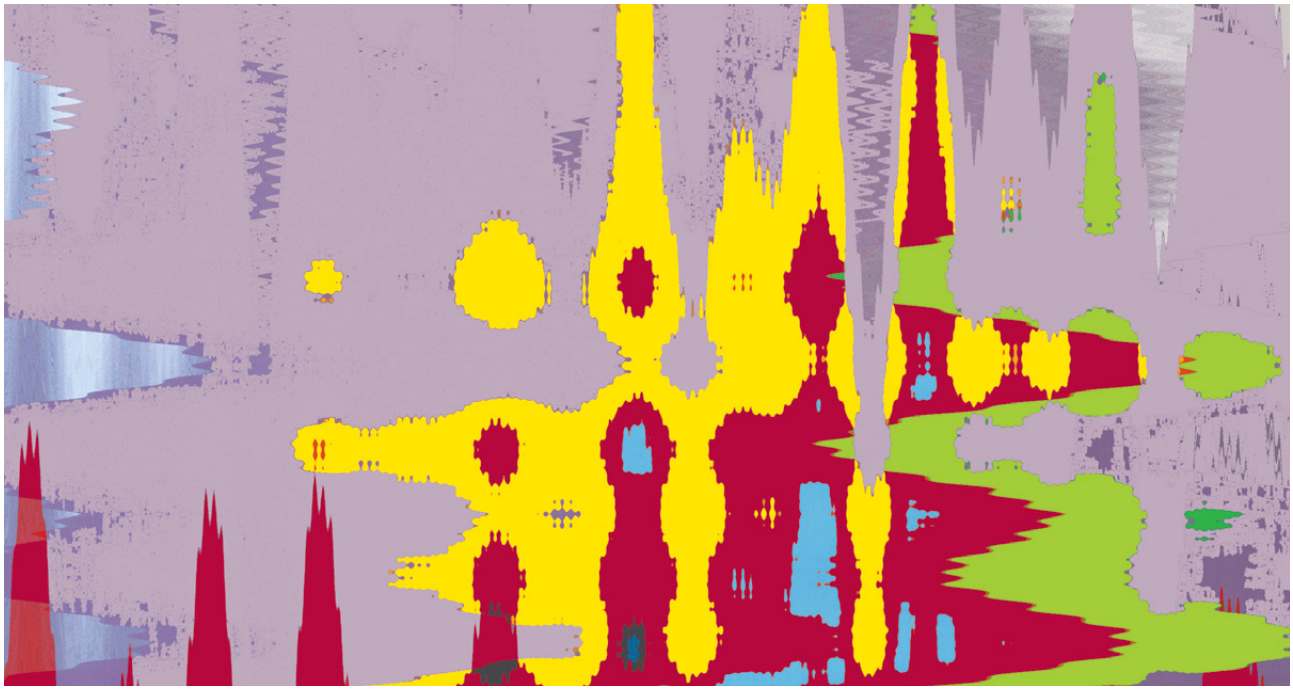


Notes:

Fig. 18A: Supelco 37 component FAME mix. Fig. 18B: canola oil. Fig. 18C: PHO. Fig. 18D: shortening blend. Fig. 18E: butter.

Temperature programme: initial temperature 60 °C for 5 min, ramped to 165 °C at 15 °C/min, hold 1 min, ramped to 184 °C at 2 °C/min, hold 25 min, ramped to 225 °C at 4 °C/min, hold 25 min. Hydrogen carrier gas at 1.0 mL/min constant flow.

Source: Dr Pierluigi Delmonte, Food and Drug Administration, United States of America (2023)



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