PROTOCOL FOR MEASURING TRANS FATTY ACIDS IN FOODS

Contents

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1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

1.1 Clinical and Public Health Relevance

Trans fats or *trans*-fatty acids (TFA) are unsaturated fatty acids that contain at least one double bond in the *trans* configuration. The three-dimensional structure of TFA is more similar to saturated fatty acids than to regular unsaturated fatty acids, which have their double bond in the c*is* configuration. The *trans* configuration substantially alters the physical properties of the fatty acids and thus the properties of the oil containing these TFA for cooking and food manufacturing (1). Also, it substantially alters the biologic and health effects of the fatty acids when consumed (2,3).

A positive linear trend between TFA intake and total as well as LDL cholesterol concentration was established, which links elevated TFA in blood with increased risk of coronary heart diseases. International expert groups and public health authorities recommend consumption of TFA (artificial and ruminant) of less than 1% of total energy intake, which translates to 2.2 g/day with a 2,000-

calorie diet (4). Elimination of industrially-produced TFA from the global food supply by 2023 is a World Health Organization (WHO) priority programme (5,6).

Data on TFA content in foods is unknown in most low- and middle-income countries. Estimating TFA content in foods has been hampered by high associated costs and lacking knowledge to conduct laboratory testing. A global protocol for measuring TFA in foods addresses these challenges and enables the generation of accurate and globally comparable TFA data and tracking changes over time.

The method described in this procedure was adopted from the method used at Health Canada (7).

1.2 Test Principle

Fat is extracted from food by acidic hydrolysis (for all processed foods prepared from fats and oils containing only industrially-produced *trans* fats), a combination of alkaline-and acidic-hydrolysis (for all processed foods prepared from fats and oils containing both industrially produced *trans* and ruminant *trans* fats) or by solvent extraction (for spreads, margarines, vegetable ghee). Triacylglycerol (TAG), triheneicosanoin (21:0 TAG) is added as gas chromatographic (GC) internal standard. Extracted fat is converted to fatty acid methyl esters (FAMEs) via methylation using boron trifluoride (BF_3)-methanol reagent. FAMEs are separated by GC using a 100-meter fused silica capillary column coated either with SP-2560 or CP-Sil 88. The FAMEs are resolved according to their chain length, degree of unsaturation and geometric and position of the double bonds. GC FAME peaks are identified by their retention times and quantitatively measured against 21:0 FAME internal standard. Total fat is calculated as sum of individual fatty acids expressed as TAG equivalents. Individual fatty acids (including all *trans* fatty acid isomers) are expressed as % of total fatty acids and as g per 100g of food.

1.3 Scope

The analytical procedure described is designed to determine the fat content and the levels of all the individual fatty acids in processed foods by a single capillary GC procedure. The procedure is applicable to foods prepared by the food industry using fats and oils containing industrially produced (IP) *trans* fatty acids (TFA). IP-TFA containing fats and oils include crude, refined, partially or fully hydrogenated fats and oils.

Although ruminant milk, butter, chesses, other dairy products, and ruminant meat products, contain some naturally occurring TFA, these products need not be subject to TFA analysis since they do not contain IP-TFA. But processed foods containing TFA, from both industrial and ruminant sources, should be analyzed. TFA containing foods can be analyzed by the procedure described here. This document is not intended to provide information for distinguishing the amounts of ruminant TFA and IP-TFA in mixed foods. Currently, there are no official analytical procedures for distinguishing the amounts; however, an estimate could be made by measuring the ratio of the amount of butyric acid and total TFA in the mixed food as done in Denmark (8). This method (8) makes an assumption that all ruminant products contain 3.6 g butyric acid and 6 g total TFA per

100 g fat. However, analysts should be aware that this assumption is not valid universally because both butyric acid and TFA amounts in ruminant products often vary with geographical location and environment factors (9). This means that the application of the described method (8) to mixed products –those prepared using ruminant fat which contains amounts other than 3.6 g butyric acid and 6 g total TFA per 100 g fat –can result in inaccurate data pertaining to the amounts of ruminant TFA and IP-TFA if not adapted for local conditions. Further, because of the relatively high water solubility and volatility of butyric acid relative to other common dietary fats, reliable measurement of butyric acid (as methyl ester) by GC may be difficult. However, the presence of butyric acid does indicate presence of TFA of ruminant origin.

The capillary GC procedure described in this method provides resolution of all the fatty acids with minimum overlaps of *cis* and *trans* isomers. The procedure measures reliably fatty acids present at levels as low as 0.1% (w/w) of the total fatty acids. This method, therefore, is capable of providing information on the percent and gram levels of all the component fatty acids present at $\geq$ 0.1% of total fatty acids in processed foods, including total TFA, *trans*-monounsaturated fatty acids (t-MUFA), *trans*-polyunsaturated fatty acids (t-PUFA), saturated fatty acids (SFA), *cis*-monounsaturated fatty acids (c-MUFA), and *cis*- polyunsaturated (c-PUFA) fatty acids. The fatty acids generally encountered in processed foods are listed in Table 1.

The fat content and fatty acid data generated using this method can support surveillance, monitoring, or compliance efforts. The data can also be used for conservative assessment of per capita consumption of TFA. For the per capita assessment, however, national data on the sale of all major brands in each food category are required.

In this document, the naming of all fatty acids follows the Δ naming convention. For naturally occurring methylene-interrupted PUFAs, however, it is convenient to use the n minus system.

2. SAMPLE COLLECTION AND PREPARATION

2.1 Collection of Samples for Analysis

Fats and oils available to consumers and commercial producers: Collect from major grocery stores. Collect three consumer-sized packages of fats and oils samples (e.g., cooking, salad oils and ghee) from the same lot. Each consumer-sized package should weight at least 100g. If the weight of each package is less than 100g, collect more packages so that the total weight of all packages is 300g or more.

*Packaged foods from grocery store*s: Collect from major grocery stores. Collect three consumer-sized packages from the same lot of a given brand. Each consumer-sized package should weigh at least 100 g. If the weight of each package is less than 100 g, collect more packages so that the total weight of all packages of a given brand is 300 g or more.

Ready to eat food samples: Collect from restaurants, street vendors, grocery stores, corner stores and other medium and small food outlets. Collect three 100 g portions of food samples, ideally from different lots to take into account, especially in the case of street foods, possible variability between lots. In addition, in the case of traditional recipes for which there are geographical differences, samples should ideally be taken from different geographical areas. These samples should be analysed separately (no composite should be created by mixing of foods from different geographical areas).

For an ideal GC run, 100mg of fat is needed for FAME analysis. That means the amount of sample collected should be sufficient to produce 100 mg fat. Thus, those food samples known to contain very little fat (say less than 1% of weight food sample), collect more than 100 g portions.

2.2 Preparation of Samples for Analysis

Immediately after purchasing, place perishable food items (especially ready to eat foods) in containers (preferably glass or food-grade plastic containers) with ice packs. Transport, without delay, all the samples to the analytical laboratory. Record the name and address of the grocery store/ready to eat food outlets, where the food item was purchased, date purchased, date manufactured, food category (e.g., cookies), the sample size, brand name, name of the manufacturer, and the lot number.

For identification purposes, give a unique code number to each of the food items. The code number should give information pertaining as to the food category (e.g., B for bread, M for margarine etc.), region/city/town/village where the sample was purchased (for example G for Geneva), and the year the food item was purchased (e.g., 18 for 2018). These pieces of information should be followed by three digit numbers (e.g., 001). For instance, if the food item is bread, the full code could be BG18-001. Use this code number for labelling the food items and recording all the data with respect to each of the collected food items.

If the laboratory is not ready to analyse the collected food samples immediately, store the samples in a refrigerator (2-8 ^{0}C, for all packaged foods, margarines, spreads, shortenings, salad oils, cooking oils, and vegetable ghee), freezer (-17 ^{0}C for perishable food samples, specially ready to eat food items) or follow established food storage techniques. This is to avoid deterioration of the samples. Analyse the food samples within 4 months of purchase.

When the laboratory is ready to commence the analysis, take the entire contents from each of the three packages and combine the contents to make a composite. Do not create composites of different brands by mixing the contents together, as the goal is to identify the food brands with higher TFA content and follow their evolution over time.

The composite needs to grind and homogenize thoroughly to ensure that the composite is a true representation of the three packages of a given lot. Dipping the food sample in liquid nitrogen (liquid nitrogen can be placed in a ceramic bowl) for a few minutes would facilitate grinding and homogenizing.

Take a subsample from the resulting composite for fat analysis. The size of the subsample depends on the fat content. Ideally the subsample should contain 200 mg of fat. Place the remainder of the composite in a clean opaque bottle (with a tight cap), label the bottle with the code number, and store it in a refrigerator (all fats and oil samples, and packaged foods) or freezer (ready to eat food samples). These samples are for future use in case the fat analysis needs to be repeated. However, analysis should be completed within one month of preparing the composite sample.

3. ANALYTICAL METHODOLOGY

3.1 Safety Precautions

3.1.1 General Safety: General laboratory precautions should be observed: protective gloves, laboratory coats, and safety glasses must be worn at all times during all steps of this method.

3.*1.2 Chemical Hazards*: All acids and bases, reagents and organic solvents, used in this analytical procedure must be handled with extreme care. These chemicals/reagents are toxic, and or flammable-must be handled only in well-ventilated areas or as required under a fume hood. Before handling chemicals and reagents, safety information such as Safety Data Sheets should be obtained (i.e., at http://www.ilpi.com/msds/index.html) and reviewed.

Hydrochloric acid: Handle with extreme care. Concentrated HCl is corrosive. Avoid breathing vapors and avoid contact with skin and eyes. Handle only inside a fume hood.

Ammonium Hydroxide: Handle 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.

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

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

Toluene: Irritating to eyes, respiratory system and skin. Flammable and harmful. Avoid contact with skin and eyes. Keep container in a cool, well ventilated area.

Chloroform: Handle with care. Chloroform is highly toxic and is a probable human carcinogen. Avoid contact with eye and skin. Exposure to chloroform over a long period of time may damage

liver and kidneys. Large amounts of chloroform can cause sores when chloroform touches the skin. Handle only inside a fume hood.

Methylene chloride: Higher levels of dichloromethane inhalation can lead to headaches, mental confusion, nausea, vomiting, dizziness and fatigue. Redness and irritation may occur if skin comes in contact with liquid dichloromethane. Handle only inside a fume hood.

Boron trifluoride-Methanol Reagent: Avoid contact with eyes and skin. Toxic if swallowed.

3.2 Apparatus

1. *Gas chromatograph (GC)*: Suitable for use with a capillary column, equipped with hydrogen flame ionization detector (FID), temperature controlled split mode injector unit, oven chamber for the capillary column capable of maintaining the desired temperature to $\pm 1^0$C , monitor, and chromatographic software capable of manipulating the GC controls (gas flow, split ratio, oven, injector and FID temperatures, recording the GC-FID profile, GC peak areas, retention times, data handling, and data storage).

2. *Capillary column*: Fused silica capillary column 100 m, and 0.25 mm internal diameter, coated with SP-2560, CP-Sil 88, (100% cyanopropylsilicone stationary phase), to a thickness of 0.20 μm. Commercially prepared columns are available from: SP-2560 (part # 24056, Millipore-Sigma Canada Co., Oakville, ON, Canada) and CP-Sil 88 (part # CP7489, Varian, Walnut Creek, CA, USA; Chrompack, Middelburg, The Netherlands). Capillary columns providing resolution of FAMEs similar to those of SP-2560 and CP-Sil 88 may also be used.

3. *Microsyringe* for GC-10 μL delivery, with a hardened needle or an equivalent.

4. *Carrier gas*: Hydrogen or helium, 99.999% pure or better, GC quality, dried, and oxygen removed by suitable filters.

5. *Flame ionization detector gases*: Hydrogen and air, GC quality

6. *Injection port split liner*: 4 mm internal diameter, 6.3 mm outer diameter x 78.5 mm split liner with glass wool (e.g., Agilent part #5183-4647 or equivalent).

7. *Standard laboratory glassware, equipment*: Volumetric flasks, glass volumetric pipettes, disposable pipettes, heating block (hot water bath for methylation), glass test tubes, glass vials, electronic weighing scale for measuring mg quantities of material, liquid nitrogen, nitrogen cylinder, Teflon-lined screw caps, vortex mixer, rotor-evaporator for removing solvents, etc.

3.3 Reagents

3.3.1 General laboratory reagents

Use only recognized analytical grade reagents.

a) Hydrochloric acid: 12M and 8.3M. To make 8.3M HCl, add carefully and slowly 250 mL 12 ML to 110 mL distilled water. Mix well. Store at room temperature (20-25^{0}C).
b) Ammonium hydroxide: 58% (w/w), analytical grade
c) Hexane, analytical grade
d) Diethyl ether; anhydrous, purity $\geq$ 99.7%, containing 1 ppm BHT inhibitor (packed in aluminum containers)
e) Ethanol: 95% (v/v)
f) Toluene-Nanograde
g) Chloroform or methylene chloride-Reagent grade (see section 3.3.2 for additional considerations for use of these two solvents)
h) Sodium sulfate: anhydrous
i) Boron trifluoride-methanol reagent: 7% BF_3 (w/w) in methanol, made from commercially available 14% BF_3-Methanol. Prepare in the fume hood. 14% BF_3-Methanol is available from Millipore-Sigma Canada Co., Oakville, ON, Canada and other chemical suppliers.

   Store the boron trifluoride-methanol reagent in a fridge. Always try to use freshly purchased reagents. Do not use expired reagents. The reagent has a limited shelf-life and should be kept refrigerated. Aged reagents can form artifacts with unsaturated fatty acids during methylation (10). Therefore, it is recommended that each new batch purchased should be tested before use and tested periodically during its lifetime. The test can be performed by preparing methyl ester of pure oleic acid. If any extra, unexpected peaks appear in the in the GC analysis, the reagent should be rejected.

3.3.2 Preparation of standard solution of triacylglycerol (TAG) internal standard (IS): (for calculating fatty acid data as g per 100 g oil or 100 g food sample): Triheneicosanoin (21:0 TAG) (purity >99%), 5.0 mg/mL in chloroform.

Accurately weigh 2.5 g 21:0 TAG into a 500 mL volumetric flask. Add 400 mL chloroform, mix until dissolved. Dilute to volume with chloroform. Invert flask at least ten additional times. Store the solution in the refrigerator (2-8°C). The 21:0 TAG internal standard stock solution is generally stable up to one month, but precautions should be taken to eliminate the loss of chloroform and therefore the concentration of the IS. Pure 21:0 TAG is available on the market (e.g., from Nu-Chek-Prep). Purity of the IS should be confirmed by thin-layer chromatography or high-performance liquid chromatography, and gas chromatography analysis (after converting 21:0 TAG to 21:0 FAME via methylation) or by any other appropriate technique.

Note: In some laboratories, use of chloroform is prohibited because of its potential carcinogenicity. Methylene chloride may be substituted for chloroform. The main disadvantage of this approach is that methylene chloride is more volatile (bp 39.6°C) than chloroform (bp 61.2°C) so that the 21:0 TAG internal solution in methylene chloride is less stable than in chloroform. This stability problem, however, could be overcome by using the methylene chloride solution immediately after its preparation. This requires that the methylene chloride internal standard solution is prepared freshly. This could be very costly.

3.3.3 Reference fatty acid methyl esters for identification of GC FAME profile of test food sample

Identify GC peaks by comparing their retention times with FAME standards.

Wide ranges of highly purified (>99%) FAME standards, both as individual standards and as model mixtures are readily available from a number of commercial sources (e.g., from Alltech Associates, Inc., Deerfield, IL, Nu-Chek-Prep, Elysian, MN, Supelco Inc., Bellefonte, PA and Sigma Chemical Co., St. Louis, MO, MilliporeSigma Canada Co., Oakville, ON, Canada).

The commercially-available FAME standards include saturated fatty acid series (chain length ranging from C3:0 to C24:0), *cis* and *trans* isomers of octadecenoic (18:1), *trans* isomers of linoleic (9c,12t-18:2; 9t,12c-18:2; and 9t,12t-18:2), *trans* isomers of α-linolenic (9-*trans*,12-*cis*,15-*cis*-18:3; 9-*cis*,12-*trans*,15-*cis*-18:3; 9-*cis*,12-*cis*,15-*trans*-18:3 and 9-*trans*,12-*cis*,15-*trans*-18:3), and n-6 and n-3 *cis*-polyunsaturated fatty acids.

Samples of *trans* fatty acid containing quality reference materials (QRMs) (QRMs series include non-hydrogenated soybean oil and hydrogenated soybean oil) are also available from the American Oil Chemists' Society (AOCS Urbana IL, USA; email: general@aocs.org).

For identification of GC fatty acids peaks from processed foods containing both IP-TFA and ruminant TFA, use a butter sample. Extract the fat from the butter sample, as outlined in the procedure margarines (Section 3.4.3). Convert the extracted fat to FAME as outlined in Section 3.5.

3.4 Extraction of Fat

3.4.1 Packaged foods and ready to eat foods containing fats and oils from non-ruminant sources

The procedure has been adopted from AOAC Official Method 996.06, revised 2001 (11) with some minor modifications.

a) Finely grind and homogenize food samples prior to extraction of fat. Dipping the food sample in liquid nitrogen placed in a ceramic bowl for 3-4 minutes would facilitate grinding and homogenizing.

b) Take a subsample from the composite food sample (see Section 2.1 for the preparation of the composite) and weigh accurately. Ideally, the weight of the food sample should be such that it yields 200 mg of fat. A prior knowledge of the fat content of the food sample would be helpful in deciding the amount that should be taken for fat extraction. Information on fat content in many foods can be found on the internet by searching nutrient file data bases (e.g., Canadian Nutrient File is available from the website; food-nutrition.canada.ca/cnf-fce/index-eng.jsp). As an example, a food sample that contains 2% fat requires about 10 g of test portion to yield 200 mg of fat.
c) Place the food sample (subsample) in a Teflon®-lined screw-capped glass test tube (30 mL capacity). Add, using a micropipette, exactly 2 mL of the 21:0 TAG internal standard solution. Slowly evaporate chloroform on a water bath, using a slow stream of nitrogen to aid in evaporation. Make sure to evaporate chloroform completely.
d) Add 2 mL ethanol, 10 mL 8.3 M HCl and mix well until the product has dispersed.
e) Heat the test tube in an electric heating block or a water bath for 60 min at 80°C. Every 10 min using a vortex mixer, thoroughly mix the content of the test tube to incorporate into solution any particulates on the walls of the test tube.
f) After 60 min of heating, remove the test tube, allow to cool to room temperature and mix with 2.0 mL ethanol and 5.0 mL diethyl ether.
g) Transfer the contents of the tube to a 500 mL separatory funnel. To ensure quantitative transfer, rinse the tube with a mixture of 10 mL diethyl ether and 10 mL hexane. Repeat the rinse with the 10 mL diethyl ether and 10 mL hexane mixture one more time. Transfer these rinses to the separatory funnel and mix briefly.
h) Add 50 mL diethyl ether and 50 mL hexane to the separatory funnel and mix briefly.
i) Allow the layers to separate. Allow the contents to settle at least 1 h until the upper layer is clear. Slowly decant the upper layer (organic) layer to a 500 mL Erlenmeyer flask with a glass stopper.
j) Add 25 mL diethyl ether and 25 mL hexane to the 500 mL separatory funnel containing the bottom layer (aqueous layer). Mix the contents thoroughly and allow the layers to settle. Slowly decant the top layer (organic layer) to the 500 mL Erlenmeyer flask containing the previously collected organic layer. Discard the aqueous layer.
k) Dry the combined organic layer with anhydrous sodium sulphate.
l) Filter the dried organic layer into a round bottom flask and slowly evaporate the solvent (diethyl ether + hexane), using a rotor-evaporator or hot-water bath, under a nitrogen stream to aid in evaporation. Residue remaining in the round bottom flask contains extracted fat (including 21:0 TAG internal standard).

3.4.2 *Packaged and ready-to-eat foods containing a mix of fats and oils from non-ruminant and ruminant sources.*

The procedure has been adopted from AOAC Official Method 996.06, revised 2001 (11) with some minor modifications

a) Finely grind and homogenize food samples prior to extraction of fat. Dipping the food sample in liquid nitrogen placed in a ceramic bowl for 3-4 minutes would facilitate grinding and homogenizing.
b) Take a subsample from the composite food sample (see Section 2.1 for the preparation of the composite) and weigh accurately. Ideally, the weight of the food sample should be such that it yields 200 mg of fat. A prior knowledge of the fat content of the food sample would be helpful in deciding the amount that should be taken for fat extraction. Information on fat content in many foods can be found on the internet by searching nutrient file data bases (e.g., Canadian Nutrient File is available from the website; food-nutrition.canada.ca/cnf-fce/index-eng.jsp). As an example, a food sample that contains 2% fat requires about 10 g of test portion to yield 200 mg of fat.
c) Place the food sample (subsample) in a Teflon®-lined screw-capped glass test tube (30 mL capacity). Add, using a micropipette, exactly 2 mL of the 21:0 TAG internal standard solution. Slowly evaporate chloroform on a water bath, using a slow stream of nitrogen to aid in evaporation. Make sure to evaporate chloroform completely.
d) Add 2 mL ethanol, 4 mL water, and 2 mL NH_4OH and mix well.
e) Place the test tube in shaking water bath at 70-80 ^{0}C set at moderate agitation speed. Shake for 20 min.
f) Mix contents of flask every 6-7 min on Vortex to incorporate particulates adhering to sides of the test tube.
g) Add 10 mL 12 M HCl and place test tube in a boiling steam bath or block heater and maintain at 100 ^{0}C for 20 min. Every 6-7 min, Vortex the test tube to incorporate particulates adhering to the side of the test tube.
h) Remove the test tube and allow to cool to room temperature (20-25 ^{0}C). Then mix with 2.0 mL ethanol and 5.0 mL diethyl ether.
m) Transfer the contents of the tube to a 500 mL separatory funnel. To ensure quantitative transfer, rinse the tube with a mixture of 10 mL diethyl ether and 10 mL hexane. Repeat the rinse with the 10 mL diethyl ether and 10 mL hexane mixture one more time. Transfer these rinses to the separatory funnel and mix briefly.
n) Add 50 mL diethyl ether and 50 mL hexane to the separatory funnel and mix briefly.
o) Allow the layers to separate. Allow the contents to settle at least 1 h until the upper layer is clear. Slowly decant the upper layer (organic) layer to a 500 mL Erlenmeyer flask with a glass stopper.
p) Add 25 mL diethyl ether and 25 mL hexane to the 500 mL separatory funnel containing the bottom layer (aqueous layer). Mix the contents thoroughly and allow the layers to settle. Slowly decant the top layer (organic layer) to the 500 mL Erlenmeyer flask containing the previously collected organic layer. Discard the aqueous layer.
q) Dry the combined organic layer with anhydrous sodium sulphate. Filter the dried organic layer into a round bottom flask and slowly evaporate the solvent (diethyl ether + hexane), using a rotor-evaporator or hot-water bath, under a nitrogen stream to aid in evaporation. Residue remaining in the round bottom flask contains extracted fat (including 21:0 TAG internal standard).

3.4.3 Margarine and other spreads

a) Take 250 mg (accurately weighed) subsample from the composite (see Section 2.1 for the preparation of the composite).
b) Place the subsample (250 mg) in a separatory funnel (ideally 250 mL capacity).
c) Add 50 mL hexane, 2 mL 21:0 TAG stock internal standard solution and mix thoroughly to dissolve the test sample (composite subsample).
d) Add 50 mL distilled water. Shake gently. Allow the layers to separate. The organic layer (top layer) contains the extracted fat.
e) Collect the top layer (by first draining out the bottom aqueous layer) into an Erlenmeyer flask. Dry for about 15 minutes using anhydrous sodium sulphate. Filter and collect the organic extract into a suitable glass flask (round bottom flask). Slowly evaporate the solvent (hexane/chloroform) using a nitrogen stream to aid in evaporation. Residue remaining in the flask contains extracted fat (including 21:0 TAG internal standard).

3.4.4 Fats and oils (salad oils, cooking oils and vegetable Ghee)

a) Take 200 mg oil (accurately weighed) from the composite oil sample.
b) Add 2 mL 21:0 TAG stock solution, mix thoroughly and remove chloroform by evaporating in a 40°C water bath under a nitrogen stream. Make sure that no residue chloroform is left in the oil.
c) Since these are 100% pure oils, there is no need to extract oil.

3.5 Methylation of Extracted Fat

The procedure described below is based on the AOAC Official Method 996.06 revised 2001 (11). However, alternative standard methods are also available; for example, AOCS Official Method Ce 2-66 (12) and ISO 12966-2 (13).

a) Dissolve the extracted fat or the oil samples in 2 mL toluene.
b) Transfer the contents to a Teflon®-lined screw-capped glass test tube (20 mL capacity).
c) Add 2 mL 7% BF_3-methanol reagent (see Section 3.4.1.i for precautions using this reagent). Seal the glass with the Teflon®-lined screw-cap.
d) Heat the tube at 100°C for 45 min in a heating block, oven or hot water bath. Gently shake the tube every 10 min (note: evaporation of solvent from tubes indicates inadequate seals. If this occurs, discard solution and repeat the entire methylation procedure).
e) Remove the tube from the heating block (oven or hot water bath) and allow to cool to room temperature.
f) Add 5 mL distilled water, 2 mL hexane, and 1 g sodium sulphate. Cap the tube and shake the tube (note: Top layer contains FAME including 21:0 FAME of TAG internal standard). After 10 min, collect the FAME hexane solution into a small sealable glass vial. Flush with nitrogen and place the cap on top.
g) Analyse the sample immediately on the GC.

3.6 Gas Chromatography Analysis

a) *FAME sample concentration*: For best GC results, the concentration of FAME reference standards and FAME from the test food samples should be approximately 20 mg/mL hexane. Inject into the GC, 1 μL hexane solution, which is approximately equivalent to 20 μg FAME.

b) GC operating parameters for food samples containing fats and oils from non-ruminant sources (adopted from AOCS Official Method Ce 1h-05 Revised 2017 (14))

Injection port temperature	250°C
Detector temperature	250°C
Oven temperature	180°C

Carrier gas: Hydrogen; column head pressure 170 kPa (25 psi); flow rate, 1.0 mL/min; linear velocity, 26 cm/s; split ratio 100:1
Carrier gas: Helium; column head pressure 286 kPa (41 psi); flow rate, 1.0 mL/min; linear velocity, 19 cm/s; split ratio 100:1

c) GC operating parameters containing mix of fats and oils from non-ruminant and ruminant sources (adopted from AOCS Official Method Ce 1j-75 2017 (15))

Injection port temperature	235°C
Detector temperature	325°C
Oven temperature program	Isothermal at 180°C (32 min), ramped at 20^0C /min to 215^0C (hold 31.25 min)

Carrier gas: Hydrogen; column head pressure 170 kPa (25 psi); flow rate, 1.0 mL/min; linear velocity, 26 cm/s; split ratio 100:1
Carrier gas: Helium; column head pressure 286 kPa (41 psi); flow rate, 1.0 mL/min; linear velocity, 19 cm/s; split ratio 100:1

d) *Peak identification*: Food samples containing TFA (arising from both IP oils and ruminant fats) will yield complicated GC profiles because of the presence of an assortment of *cis* and *trans*-isomers of oleic, linoleic and linolenic acids. Nevertheless, almost all the individual FAME peaks can be identified by their retention times, and by comparison of the FAME profile with the reference standards (individual FAME, mix t*rans* FAME, AOCS QRMs non-hydrogenated soybean oil and hydrogenated soybean oil and butter FAME). Identifications can be further established by comparison of the FAME GC profile with those published in the literature (16-18) or in the AOCS Official Methods Ce 1h-05 (14) and Ce 1j-07 (15). See also Figures 1-10 for examples of quality FAME GC profiles.

When peaks of unknown identity are observed, attempt to identify such peaks using appropriate procedures that involve GC-MS, silver-ion chromatography, and classical chemical methods (19,20). Peaks of unknown identity should not be included in the

summation of peak areas when quantifying the concentrations of total fat and fatty acid composition, unless they have been confirmed to be fatty acids.

e) *Performance check*: Check the column and GC performance using reference standards (individual FAME, mix trans FAME and AOCS QRMS).

Perform the following checks for deciding the acceptability of GC runs:

1) There is a baseline separation between 9c-18:1 and 11c-18:1 (as shown in Fig. 1A).

2) The *trans* isomers from 4t-18:1 to 12t-18:1 are readily separated from all the *cis*-18:1 isomers (as shown in Fig. 3C).

3) Good Partial separation 13t + 14t-18:1 and 9c-18:1 should be obtained (as shown in Fig. 3C). Unfortunately, complete baseline separation of these three fatty acids is not possible under any GC condition. However, a good partial separation is sufficient for peak area determinations for (13t +14t)-18:1 and 9t-18:1. Please note that 13t-18:1 and 14t-18:1 are not separable by GC and therefore, elute together.

4) Near baseline separation between 16t-18:1 from 13c-18:1 and 14c-18:1 (as shown in Figure 3C). 16t-18:1 should always elute between 13c-18;1 AND 14c-18:1.

5) Near baseline separation among 11c-20:1, 9c,12c,15c-18:3 and 9t,12c,15c-18:3 (as shown in Fig. 3C).

Any GC runs showing poor resolution of the above critical FAMEs are not acceptable.

If the resolutions are not ideal, adjust the following in small increments until best resolutions are obtained:

- sample size
- concentration
- oven temperature

If column oven temperature needs to be adjusted, it should be adjusted with small increments, preferably by steps of 1°C. This is because in SP-2560, CP-Sil 88 and other cyanopropylsilicone columns, slight changes in the column temperature has a profound effect on the elution order of critical FAMEs (Reference 19).

3.7 Calculations: Total Fat (g /100 g food) and Fatty Acids (% and g /100 g food)

Calculate individual fatty acids (g) and total fat per 100 g of test *Total fat and fatty acids of test food samples.*

Total fat is the sum of all the correctly identified individual fatty acids, expressed as triacylglycerol (TAG) equivalents. Expression of measured fatty acids as TAG equivalents requires construction of a TAG molecule with each fatty acid. Construction needs 3 fatty acid molecules and 1 glycerol molecule. Essentially, 2 methylene groups and 1 methine group are added to every three 3 fatty acid molecules. Furthermore, since the peak areas of fatty acids by GC are measured as their methyl esters, the FAME peak areas have to convert to fatty acid peak areas. Table 3 shows the required correction factors for converting FAME peak areas to fatty acid and TAG equivalents.

1) Calculate the amount of individual fatty acids (g), expressed as FAME (W_{FAMEx}) in test sample as follows:

$$W_{FAMEx} = (A_X \times W_{IS} \times 1.0040 \times R_X) / A_{IS}$$

Where:

A_x = peak area counts for FAME x in test sample

W_{is} = weight of C21:0 TAG internal standard (in g) added to the test sample
Note: If procedure is followed exactly, W_{is} should be 0.010 g)

1.0040 = conversion of 21:0 TAG internal standard from TAG to FAME;

A_{is} = peak area counts of the C21:0 FAME internal standard added to the test sample as 21:0 TAG.

R_x = theoretical flame ionization detector response factor (TCF) for FAMEs relative to 21:0 FAME internal standard.

Note: TCF should be applied to the analytical data for optimum accuracy. The FID responds to only active carbon atoms (CH, CH_2 and CH_3), and not to carbonyl carbon. Therefore, the short chain fatty acids have a lower response on FID compared to longer chain fatty acids. This discrepancy must be corrected by applying the TCF. The TCF of a number of FAME which are commonly encountered in dietary fats are listed in Table 3.

2) Calculate the amount (in g) of individual fatty acids expressed as TAG equivalents W_{TAGx} in the test sample as follows:

$$W_{TAGx} = W_{FAMEx} \times F_{TAGx}$$

Where F_{TAGx} = Conversion factor for conversion of FAME x in test sample to its TAG equivalent (see Table 4 for a list of conversion factors)

3) Calculate the amount of TAG equivalents for each individual fatty acid in g per 100 g of test sample as follows:

$$W_{TAGx} \text{ (g per 100 g test sample)} = (\sum W_{TAGx} / W_{TS}) \times 100$$

Where:

W_{TS} = Weight of test sample in g

4) Calculate the total in g per 100 g of test sample (sum of all fatty acids, expressed as TAG equivalents as follows:

$$\text{Total fat (g/100 g test sample)} = \sum W_{TAGx} \text{ (g per 100 g test sample)}$$

5) Calculate the weight (in g) of each individual fatty acid (W_x) expressed as fatty acid itself (i.e., in the RCOOH form or unesterified form) in the test sample as follows:

$$W_x = W_{FAMEx} \times F_{Fax}$$

Where:
F_{Fax} = conversion factor for conversion of FAME x in test sample to its corresponding fatty acid (see Table 4 for a list of conversion factors)

6) Calculate the weight of each g of fatty acid x in 100 g of test sample as follows:

$$\text{Fatty acid } x \text{ (g /100 g test sample)} = (W_x / W_{TS}) \times 100$$

7) Calculate the weight of saturated fatty acids (in g; sum of all saturated fatty acids) in 100 g test sample as follows:

$$\text{Saturated fatty acids (g /100 g test sample)} = (\sum \text{Saturated } W_x / W_{TS}) \times 100$$

Saturated fatty acids generally include the following: 8:0, 10:0, 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 24:0.

8) Calculate the weight total *trans*-monounsaturated fatty acids (t-MUFA) (in g; sum of all t-MUFA) in 100 g test sample as follows:

$$\text{t-MUFA (g/100 g test portion)} = (\sum W_{t\text{-}MUFAx} / W_{TS}) \times 100$$

Where $W_{t\text{-}MUFAx}$ = Weight in g of individual t-MUFA

t-MUFA generally includes 5t-14:1, 9t-16;1, 4t-18:1, 5t-18:1, (6t+7t+8t)-18:1, 9t-18:1,10t-18:1, 13t+14t-18:1 and 16t-18:1. Since trans-18:1 isomers from 6t to 14t are eluting as a group with no clear baseline separations (see Fig 3C), it is practical to lump their peak areas together and express as (6t-14t)-18:1.

9) Calculate the weight of total *trans*-polyunsaturated fatty acids (t-PUFA) (in g; sum of all t-PUFA) in 100 g of test portion as follows:

$$\text{t-PUFA (g /100 g test sample)} = (\sum W_{t\text{-}PUFAx} / W_{TS}) \times 100$$

Where $W_{t\text{-}PUFAx}$ = Weight of individual t-PUFA

t-PUFA generally includes tt-18:2, 9t,12t-18:2, 9c,13t-18:2, 9c,12t:18:2, 9t,12c-18:2, (9t,15c + 10t,15c)-18:2, 9c,12c,15t-18:3, 9c,12t,15c-18:3, and 9t,12c,15c-18:3. Do not include the naturally occurring CLA isomers originated from ruminant fats in the calculation for t-PUFA. The CLA isomers include 9c,11t-18:2; 10t,12c-18:2; 11t,13t-18:2, 8t,10t-18:2, 9t,11t-18:2 and 10t,12t-18:2 (see Figures 5-10 for their GC profiles).

10) Calculate total *trans* fatty acids (TFA) (in g; sum of all *trans* fatty acids) in 100 g of test portion as follows:

    Total *trans* fatty acids (g / 100 g test portion) = (g t-MUFA + g t-PUFA) in 100 g test portion

11) Calculate total *cis*-monounsaturated fatty acids (c-MUFA) (in g; sum of all *cis-monounsaturated* fatty acids) in 100 g test portion as follows:

    Total c-MUFA (g/100 g test portion = $(\sum W_{c\text{-}MUFAx} / W_{TS})$ X 100

    Where:
    $W_{c\text{-}MUFAx}$ = Weight of individual c-MUFA

cis-MUFA generally includes the following: 5c-14:1, 9c-15:1, 9c-16:1, 9c-17:1, 7c-18:1, 9c-18:1, 10c-18:1, 11c-18:1, 12c-18:1, 13c-18:1, 14c-18:1, 15c-18:1, 16c-18:1, 11c-20;1, 13c-22:1, and 15c-24:1.

12) Calculate total cis-polyunsaturated fatty acids (c-PUFA) (in g; sum of all n-6 and n-3 polyunsaturated fatty acids) in 100 g test portion as follows:

    Total *cis*-PUFA = $(\sum W_{c\text{-}PUFAx} / W_{TS})$ X 100

    Where:
    $W_{c\text{-}PUFAx}$ = Weight of individual c-PUFA

    n-6 and n-3 polyunsaturated fatty acids include:

18:2n-6 (9c,12c-18:2)	18:3n-3 (9c,12c,15c-18:3)
18:3n-6 (6c,9c,12c-18:3)	18:4n-3 (6c,9c,12c,15c-18:4)
20:3n-6 (8c,11c,14c-20:3)	20:4n-3 (8c,11c,14c,17c-20:4)
20:4n-6 (5c,8c,11c,14c-20:4)	20:5n-3 (5c,8c,11c,14c,17c-20:5)
22:4n-6 (7c,10c,13c,16c-22:4)	22:5n-3 (7c,10c,13c,16c,19c-22:5)
22:5n-6 (4c,7c,10c,13c,16c-22:5)	22:6n-3 (4c,7c,10c,13c,16c,19c-22:6)

13) Calculate the fatty acid composition (i.e., % of each individual fatty acid per 100 g of total fatty acids) as follows:

    Fatty acid x, % (wt.% of total fatty acids) = $(W_x / \sum W_x) \times 100$

Where:
$\sum W_x$ = Sum of all individual fatty acids in g

3.8 Spread Sheet for Calculating Fat (g /100 g food) and Fatty Acids (% and g /100 g food)

A spreadsheet for calculating the total fat content and fatty acid data outlined above has been developed. A copy of the spread sheet is attached as a separate file to this document.
For calculating the fat and fatty acid data, the analyst has to input the relevant experimental derived data into the shaded cells in the spreadsheet. The data to be input are:

- sample ID
- date sample analysed by GC
- weight of test food sample used for analysis (in g; WTS)
- weight of 21:0 TAG IS added to the test food sample (in g, WIS)
- GC peak area counts of 21:0 FAME (derived from 21:0 TAG IS; AIS) and
- GC peak area counts of all the individual fatty acids in the test food sample (A_x).

3.9 Reporting Data

Report in tabulated form the following data:

- total fat (g per 100 g of test sample)
- all the positively identified individual fatty acids of concentration $\geq$ 0.1% of total fatty acids, expressed as both percent of total fatty acids (% w/w; i.e., the fatty acid composition) and g per 100 g of food.
- the levels of total SFA, t-MUFA, t-PUFA, total TFA, c-MUFA and c-PUFA expressed as both percent of total fatty acids and g per 100 g food.

4.0 GC-FID RESPONSE, LINEARIRTY, SENSITIVITY, METHOD PRECISION, AND ACCURACY

The FID response in GC is always linear; this is a known characteristic of GC. GC is highly sensitive; it can measure PPM levels of any analyte. In FAME analysis, individual FAMEs present at levels as low as 0.1% of the total fatty acids can be detected and measured accurately. Inter- and Intra-laboratory variation measuring 0.1% levels is usually less than 10%. For levels below <0.1 %, the variations are much higher (could be around 20%).

Table 5 shows precision data (intra- and inter-laboratory standard deviations, coefficient of variations and correlation coefficients) for total fat, c-MUFA, c-PUFA and total TFA for 3 partially hydrogenated fat samples from a collaborative study among 13 laboratories. The collaborative study was performed by AOCS as a component of AOCS Official Method Ce 1h-05 (Reference 17). The GC procedure used in the AOCS collaborative study was similar to that described here. The

precision data shows that total fat, c-MUFA, c-PUFA, and total TFA can be measured accurately with little intra- and inter-laboratory variation for repeated sample analysis. In particular, the data demonstrate total TFA ranging from 1 to 45 g per 100 g oil can be measured accurately.

5.0 QUALITY ASSURANCE AND CONTROL

5.1 Training of Analysts

For obtaining reliable data, the analysis should be conducted by experienced and well-trained analysts.

Analysts performing this analytical procedure must successfully complete:

- General Laboratory Safety Trainings
- Hazardous Chemical Waste Management Training
- Records Management Training

Further, the analyst must have received training on the specific GC instrumentation and software (such as Agilent ChemStation) used with this measurement procedure from a qualified laboratory or from the instrument manufacturer. The analyst should undergo training to correctly calculate and interpret results obtained and transferring data to an appropriate database.

Analysts should also gain experience in the analytical procedure described here by participating in the analysis of standard reference materials (SRM) produced by reputed international organizations. For example, a meat homogenate (Standard Reference Material 1546a) is available from National Institute of Standards and Technology, Gaithersburg, MD 20899, USA. This SRM is intended primarily for validation of methods for determining fatty acids and several other nutrients. The American Oil Chemist's Society (AOCS, 2710 S. Boulder, Urbana, IL 61802-6996, USA; email general@aocs.org) also offers a series 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 and come with GC chromatograms.

5.2 Blank Sample

On GC, analyse a blank sample (hexane) before running a test sample FAME. No peaks (except for hexane) should be detected in the blank run. Repeat this test every 10n samples.

5.3 Reference Food Samples

Analyse Standard Reference Material 1546a (meat homogenate from the National Institute of Standards and Technology) once every 20 test samples. The results should closely match the data provided for the SRM. If not, review the analytical procedure and make appropriate changes. For mixed fats, use FAME derived from a butter sample as the reference for GC profile.

6.0 CRITERIA FOR SELECTING A LABORATORY TO CONDUCT TFA ANALYSIS

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

1) 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).

2) The laboratory is equipped with the following laboratory equipment, glassware, reagents, chemicals, authentic fatty acid standards and certified reference food materials. These are essential for analysis of food samples and obtaining reliable and accurate data on their fat content and fatty acid composition.

   - *Gas chromatograph (GC)*: Suitable for use with a capillary column, equipped with hydrogen flame ionization detector (FID), temperature controlled split mode injector unit, oven chamber for the capillary column capable of maintaining the desired temperature to $\pm 1^0$C , monitor, and chromatographic software capable of manipulating the GC controls.
   - *Capillary column*: Fused silica capillary column 100 m, and 0.25 mm internal diameter, coated with either SP-2560 or CP-Sil 88 stationary phase. Capillary columns providing resolution of fatty acids similar to those of SP-2560 and CP-Sil 88 may also be used.
   - *Microsyringe* for GC-10 μL delivery, with a hardened needle or an equivalent.
   - *Carrier gas*: Hydrogen or helium, 99.999% pure or better, GC quality, dried, and oxygen removed by suitable filters.
   - *Flame ionization detector gases*: Hydrogen and air, GC quality
   - *Standard laboratory glassware, equipment*: Volumetric flasks, glass volumetric pipettes, disposable pipettes, heating block (hot water bath for methylation), glass test tubes, glass vials, electronic weighing scale for measuring mg quantities of material, liquid nitrogen, nitrogen cylinder, Teflon-lined screw caps, vortex mixer, rotor-evaporator for removing solvents.
   - *Chemicals and reagents*: Hydrochloric acid (12 molar, analytical grade), ammonium hydroxide (58% (w/w), analytical grade), hexane (analytical grade), diethyl ether (anhydrous, purity $\geq$ 99.7%, containing 1 ppm BHT inhibitor, packed in aluminum containers), ethanol (95% (v/v)), toluene (nano-grade), chloroform or methylene chloride (reagent grade), sodium sulfate (anhydrous), 14% boron trifluoride-methanol reagent (14% BF_3-Methanol), acetone (reagent grade), ready supply of distilled water (very often prepared in the lab).
   - *Authentic fatty acid methyl ester reference standards*: Saturated fatty acids of different chain lengths, *cis*-monounsaturated fatty acids, *trans*-monounsaturated fatty acids, natural *cis*-polyunsaturated fatty acids (e.g., linoleic and alpha-linolenic acid), *trans*-

polyunsaturated fatty acids (especially *trans*-linoleic and *trans*-linolenic). These are required for identification of the fatty acid profile of dietary fats of food samples. Wide ranges of highly purified (>99%) FAME standards, both as individual standards and as model mixtures are readily available from a number of commercial sources (e.g., from Alltech Associates, Inc., Deerfield, IL, Nu-Chek-Prep, Elysian, MN, Supelco Inc., Bellefonte, PA and Sigma Chemical Co., St. Louis, MO, MilliporeSigma Canada Co., Oakville, ON, Canada). The American Oil Chemist's Society (AOCS, 2710 S. Boulder, Urbana, IL 61802-6996, USA; email general@aocs.org) also offers a series Quality Reference Materials (QRMs) for analysts to gain practice with calibrating equipment and testing new methods. The QRMs includes non-hydrogenated soybean oil and hydrogenated soybean oil for TFA determination and come with GC chromatograms.

- *Quality reference materials (QRM)*: Standard reference materials (SRM) produced by reputed international organizations. For example, a meat homogenate (Standard Reference Material 1546a) is available from National Institute of Standards and Technology, Gaithersburg, MD 20899, USA. This SRM is intended primarily for validation of methods or determining fatty acids and several other nutrients.

3) The laboratory team is composed of experienced technicians who are familiar with capillary GC procedures to analyse fatty acid composition of dietary fats. The laboratory has checked GC performance using reference standards and AOCS QRMS described above.

- There is a baseline separation between 9c-18:1 and 11c-18:1 (as shown in Fig. 1A).
- The *trans* isomers from 4t-18:1 to 12t-18:1 are readily separated from all the *cis*-18:1 isomers (as shown in see Fig. 3C).
- There is a good partial separation between 13t + 14t-18:1 and 9c-18:1 (as shown in Fig. 3C).
- There is a near baseline separation between 16t-18:1 from 13c-18:1 and 14c-18:1 (as shown in Figure 3C).
- There is near baseline separation among 11c-20:1, 9c,12c,15c-18:3 and 9t,12c,15c-18:3 (as shown in Fig. 3C).
- Any GC runs showing poor resolution of the above critical FAMEs are not acceptable.

4) The analysts performing the analysis demonstrates acquisition of previous experience and training related to the analysis of dietary fatty acid composition as outlined below:

- General Laboratory Safety Trainings
- Hazardous Chemical Waste Management Training
- Records Management Training
- Training on the specific GC instrumentation and software (such as Agilent ChemStation) used with this measurement procedure from a qualified laboratory or from the instrument manufacturer.
- Training to correctly calculate and interpret results obtained and transferring data to an appropriate database.

- Experience in the analytical procedure described here by participating in the analysis of standard reference materials (SRM) produced by reputed international organizations. (For example, Standard Reference Material 1546a from National Institute of Standards and Technology (see Section 6.2) and Quality Reference Materials (QRMs) produced by AOCS (see Section 6.2))

5) The analyst has the knowledge and skill to understand and successfully adapt standard analytical procedures, such as the TFA analysis procedure described here.

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Table 1: List of fatty acids expected to encounter in processed foods containing both natural fats and partially hydrogenated fats

Fatty acid short hand notation	Fatty acid name
Saturated Fatty Acids (SFA)	
4:0	Butryic
6:0	Caproic
8:0	Caprylic
10:0	Capric
12:0	Lauric
13:0	Tridecanoic
14:0	Myristic
15:0	Pentadecanoic
Iso 16:0	iso-Hexadecanoic (iso-Palmitic)
16:0	Palmitic
17:0	Heptadecanoic
18:0	Stearic
20:0	Arachidic
22:0	Behenic
23:0	Tricosanoic
24:0	Lignoceric
***Trans* Monounsaturated Fatty Acids (t-MUFA)**	
5t-14:1	5-*trans*-tetradecenoic
9t-16:1	9-*trans*-hexadecenoic
4t-18:1	4-*trans*-Octadecenoic
5t-18:1	5-*trans*-Octadecenoic
(6t-14t)-18:1	(6+7+8+9+10+11+12+13+14)-*trans*-Octadecenoic
9t-18:1	9-*trans*-Octadecenoic (elaidic)
10t-18:1	10-*trans*-Octadecenoic
11t-18:1	11-*trans*-Octadecenoic (vaccenic)
12t-18:1	12-*trans*-Octadecenoic
13t+14t-18:1	(13,14)-*trans*-Octadecenoic
16t-18:1	16-*trans*-Octadecenoic
***Cis* Monounsaturated Fatty Acids (c-MUFA)**	
5c-14:1	5-cis-Tetradecenoic
10c-15:1	10-*cis*-Pentadecenoic
7c-16:1	7-cis-Hexadecenoic
9c-16:1	9-*cis*-Hexadecenoic
9c-17:1	9-cis-Heptadecenoic
9c-18:1	9-*cis*-Octadecenoic (oleic)
10c-18:1	10-*cis*-Octadecenoic
11c-18:1	11-*cis*-Octadecenoic (*cis* vaccenic)
12c-18:1	12-*cis*-Octadecenoic
13c-18:1	13-*cis*-Octadecenoic
14c-18:1	14-*cis*-Octadecenoic
15c-18:1	15-*cis*-Octadecenoic
16c-18:1	16-*cis*-Octadecenoic

11c-20:1	11-*cis*-Eicosenoic
13c-22:1	13-*cis*-Docosenoic (Erucic)
15c-24:1	15-*cis*-tetracosanoic (Nervonic)
***Trans*-Polyunsaturated Fatty Acids (t-PUFA)**	
tt-18:2	*trans*,*trans*-Octadecadienoic
9t,12t-18:2	9-*trans*,12-*trans*-Octadecadienoic
9c,13t-18:2	9-*cis*,13-*trans*-Octadecadienoic
9c,12t-18:2	9-*cis*,12-*trans*-Octadecadienoic
9t,12c-18:2	9-*trans*,12-*cis*-Octadecadienoic
(9t,15c + 10t,15c)-18:2	(9-*trans*,15-*cis*- + 10-*trans*, 15-*cis*-) Octadecadienoic
9c,12c,15t-18:3	9-*cis*,12-*cis*,15-*trans*-Octadecatrienoic
9c,12t,15c-18:3	9-*cis*,12-*trans*,15-*cis*-Octadecatrienoic
9t,12c,15c-18:3	9-*trans*,12-*cis*,15-*cis*-Octadecatrienoic
n-6 Polyunsaturated Fatty Acids (n-6 PUFA)	
9c,12-18:2 (18:2n-6)	9-*cis*,12-*cis*-Octadecadienoic (linoleic)
6c,9c,12c-18:3 (!8:3n-6)	6-*cis*,9-*cis*,12-*cis*-Octadecatrienoic (α-linolenic)
8c,11c,14c-20:3 (20:3n-6)	8-*cis*,11-*cis*,14-cis-Eicosatrienoic acid
5c,8c,11c,14c-20:4 (20:4n-6)	5-*cis*,8-*cis*,11-*cis*,14-*cis*-Eicosatetraenoic acid (arachidonic acid)
7c,10c,13c,16c-22:4 (20:4n-6	7-*cis*,10-*cis*,13-*cis*, 16-*cis*-Docosatetraenoic acid
4c,7c,10c,13c,16c-22:5 (22:5n-6)	4-*cis*,7-*cis*,10-*cis*,13-*cis*,16-*cis*-Docosapentanoic acid
n-3 Polyunsaturated Fatty Acids (n-3 PUFA)	
9c,12c,15c-18:3 (18:3n3)	9-*cis*,12-cis,15-*cis*-Octadecatrienoic (α-linolenic)
6c,9c,12c,15c-18:4 (18:4n-3)	6-*cis*,9-*cis*,12-cis,15-*cis*-Octadecatetraenoic
5c,8c,11c,14c,17c-20:5 (20:5n-3)	5-*cis*, 8-*cis*,11-*cis*,14-cis,17-cis-Eicosapentaenoic acid (EPA)
7c,10c,13c,16c,19c-22:5 (22:5n-3)	7-cis,10-*cis*,13-*cis*,16-*cis*,19-cis-Docosapentaenoic (DPA)
4c,7c,10c,13c,16-c,9c-22:5 (22:6n-3)	7-*cis*,10-cis,13-*cis*,16-cis,19-cis-Docosahexaenoic (DHA)

Table 2: List of fatty acids and their levels reported for best-selling brands of hard margarines sold in Canada in 2005 (21). (w/w % of total fatty acids, n=3)

Fatty acid	Mean ± SD	Range
12:0	0.6 ± 0.6	0.1-1.2
14:0	0.3 ± 0.2	0.1-0.5
16:0	9.9 ± 0.6	9.3-10.5
18:0	6.8 ± 2.2	5.3-9.4
Total SFA	**18.5 ± 3.2**	**15.6-22.0**
4t-18:1	0.1 ± 0.0	0.0-0.1
5t-18:1	0.2 ± 0.1	0.1-0.2
(6t+7t+8t)-18:1	4.2 ± 0.4	3.9-4.6
9t-18:1	5.5 ± 1.2	4.4-6.7
10t-18:1	8.4 ± 0.7	7.7-9.0
11t-18:1	6.7 ± 0.4	6.5-7.2
12t-18:1	3.9 ± 0.5	3.4-4.4
13t+14t-18:1	5.0 ± 0.6	4.4-5.5
16t-18:1	0.6 ± 0.2	0.4-0.7
Total t-MUFA	**34.8 ± 3.7**	**31.0-38.4**
9c-18:1	15.7 ± 0.6	15.3-16.4
10c-18:1	7.0 ± 0.3	6.8-7.3
11c-18:1	5.6 ± 0.2	5.5-5.9
12c-18:1	2.7 ± 0.1	2.6-2.8
13c-18:1	1.7 ± 0.1	1.6-1.7
14c-18:1	0.8 ± 0.0	0.7-0.8
15c-18:1	0.6 ± 0.0	0.6-0.6
16c-18:1	0.4 ± 0.1	0.4-0.4
Total c-MUFA	**33.3 ± 1.3**	**32.5-34.8**
tt-18:2	0.4 ± 0.2	0.2-0.6
9t,12t-18:2	0.3 ± 0.1	0.2-0.3
9c,13t-18:2	1.4 ± 0.1	1.3-1.5
9c,12t-18:2	1.1 ± 0.2	1.0-1.4
9t,12c-18:2	0.9 ± 0.3	0.6-1.2
(9t,15c + 10t,15c)-18:2	0.4 ± 0.1	0.3-0.4
9c,12c,15t-18:3	0.1 ± 0.1	0.1-0.1
9c,12t,15c-18:3	0.0 ± 0.0	0.0-0.0
9t,12c,15c-18:3	0.0 ± 0.0	0.0-0.0
Total t-PUFA	**4.5 ± 0.4**	**4.1-4.9**
Total TFA	**39.3 ± 3.5**	**35.9 -42.9**
9c,12-18:2 (18:2n-6)	7.3 ± 1.8	7.3-1.8
9c,12c,15c-18:3 (18:3n3)	0.7 ± 0.4	0.3-1.1
Total c-PUFA	8.0 ± 1.5	5.9 – 9.5

Fatty acids at levels of <0.05% of total fatty acids were not reported

Table 3: Theoretical flame ionization detector correction factor (TCF) for FAME with respect to 21:0 FAME

FAME	TCF	FAME	TCF	FAME	TCF	FAME	TCF
6:0	1.3378	16:0	1.0422	20:0	1.0067	22:2	0.9825
8:0	1.2195	16:1	1.0345	20:1	1.0005	22:3	0.9769
10:0	1.1486	17:0	1.0318	20:2	0.9943	22:4	0.9713
12:0	1.1013	17:1	1.0244	20:3	0.9880	22:5	0.9655
13:0	1.0831	18:0	1.0225	20:4	0.9819	22:6	0.9599
14:0	1.0675	18:1	1.0155	20:5	0.9665	24:0	0.9830
14:1	1.0587	18:2	1.0087	21:0	1.000	24:1	0.9779
15:0	1.0540	18:3	1.0017	22:0	0.9939		
15:1	1.0457	18:4	0.9949	22:1	0.9881		

TCF were calculated using the following formula.

$TCF_x = MW_x / (N_x - 1)\ (AWC)\ (1.3503)$

Where:

TCF_x = theoretical flame ionization detector response factor for fatty acid x (as methyl ester) with respect to 21:0 FAME (internal standard)
MW_x = molecular weight of FAME x
N = number of carbon atoms in the FAME x
AWC = atomic weight of carbon (12.011)
1.3503 = TCF for 21:0 FAME

Atomic weights used in calculating the molecular weights of FAME: Carbon 12.011; hydrogen 1.0079; oxygen 15.994.

Table 4: Factors for conversion of FAME to fatty acids and TAG equivalents

Fatty Acid	F_{FAx}	F_{TAGi}	Fatty Acid	F_{FAx}	F_{TAGx}
4:0 Butryic	0.8627	0.9868	18:4 Octadecatetraenoic	0.9517	0.9954
6:0 Caproic	0.8923	0.9897	20:0 Arachidic	0.9570	0.9959
8:0 Caprylic	0.9114	0.9915	20:1 Eicosenoic	0.9568	0.9959
10:0 Capric	0.9247	0.9928	20:2 Eicosadienoic	0.9565	0.9958
11:0 Undecanoic	0.9300	0.9933	20:3 Eicosatrienoic	0.9562	0.9958
12:0 Lauric	0.9346	0.9937	20:4 Arachidonic	0.9560	0.9958
13:0 Tridecanoic	0.9386	0.9941	20:5 Eicosapentaenoic	0.9557	0.9958
14:0 Myristic	0.9421	0.9945	21:0 Heneicosanoic	0.9588	0.9961
14:1 Tetradecenoic	0.9417	0.9944	22:0 Behenic	0.9604	0.9962
15:0 Pentadecanoic	0.9453	0.9948	22:1 Docosaenoic	0.9602	0.9962
15:1 Pentadecenoic	0.9449	0.9947	22:2 Docosadienoic	0.9600	0.9962
16:0 Palmitic	0.9481	0.9950	22:3 Docosatrienoic	0.9538	0.9961
16:1 Hexadecenoic	0.9477	0.9950	22:4 Docosatetraenoic	0.9595	0.9961
17:0 Heptadecanoic	0.9507	0.9953	22:5 Docosapentaenoic	0.9593	0.9961
17:1 Heptadecenoic	0.9503	0.9952	22:6 Docosahexaenoic	0.9590	0.9961
18:0 Stearic	0.9530	0.9955	23:0 Tricosanoic	0.9620	0.9964
18:1 Oleic	0.9527	0.9955	24:0 Lignoceric	0.9633	0.9965
18:2 Linoleic	0.9524	0.9954	24:1 Nervonic	0.9632	0.9965
18:3 Linolenic	0.9520	0.9954			

F_{FAx} is the correction factor for conversion of FAMEs to corresponding fatty acids.
F_{FAGx} is the conversion factor for conversion of FAMEs to corresponding TAG equivalents.

Table 5: Compilation of precision of total fat (g/100g oil), total c-MUFA, total c-PUFA, total SFA and total TFA (expressed as TAG equivalents) for 3 partially hydrogenated fat samples from an AOCS collaborative study carried out among 13 laboratories.

Sample	Measure	Mean	S_r	RSD_r	r	S_R	RSD_R	R
A	Total Fat	97.39	0.541	0.560	1.516	3.256	3.340	9.116
B	Total Fat	96.74	1.241	1.290	3.474	2.984	3.090	8.354
C	Total Fat	95.46	2.718	2.850	7.610	4.076	2.540	6.921
A	c-MUFA	38.75	0.359	0.930	1.004	1.416	3.650	1.580
B	c-MUFA	21,29	0.312	1.470	0.874	0.553	3.964	1.030
C	c-MUFA	22.57	0.494	2.190	1.384	1.068	4.730	2.990
A	c-PUFA	12.85	0.172	1.340	0.482	0.499	3.890	1.398
B	c-PUFA	41.70	1.317	3.160	3.689	1.716	4.110	4.805
C	c-PUFA	0.49	0.067	13.720	0.187	0.112	23.110	0.315
A	SFA	40.27	0.165	0.410	0.463	1.684	4.180	4.714
B	SFA	17.75	0.191	1.080	0.534	0.783	4.140	2.191
C	SFA	23.30	0.141	0.610	0.395	1.170	5.020	3.275
A	TFA	1.00	0.100	10.060	0.281	0.216	21.640	0.605
B	TFA	17.75	0.191	1.080	0.534	0.783	4.410	2.191
C	TFA	45.01	0.656	1.460	1.838	2.049	4.550	5.738

Samples: A, Partially hydrogenated lard; B, Margarine oil; C, Vegetable shortening

S_r = intra-laboratory standard deviation

RSD_r = intra-laboratory coefficient of variation

r = intra-laboratory correlation coefficient

S_R = inter-laboratory standard deviation

RSD_R = inter-laboratory coefficient of variation

R = inter-laboratory correlation coefficient

Figure 1: A (top graph) and B (bottom graph): GC profile of some FAME standards on SP-2560 capillary column (180°C isothermal, H_2 at 1.0 mL/min). ((adopted from AOCS Ce 1h-05, 2017 revised (13))

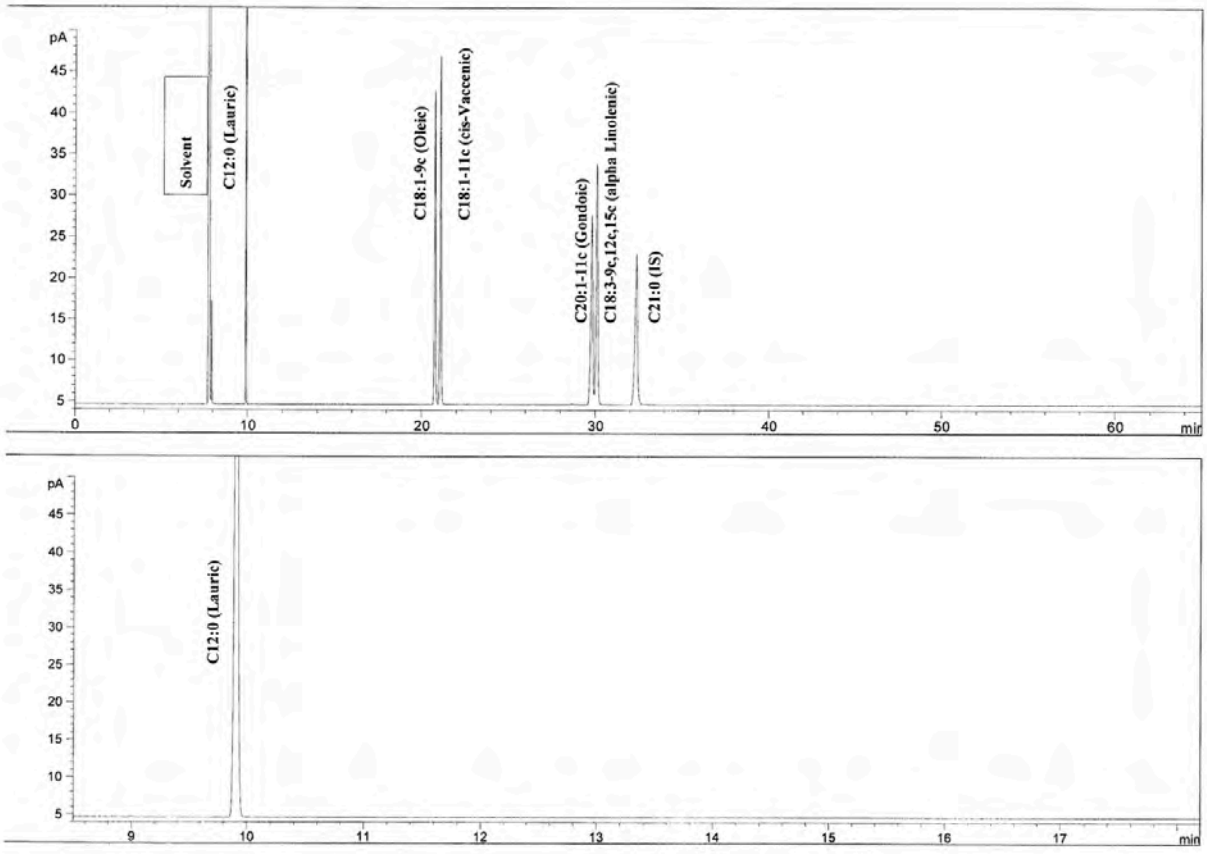

Figure 1 (contd.) C (top graph and D (bottom graph): GC profile of some FAME standards on SP-2560 capillary column (180°C isothermal, H_2 at 1.0 mL/min) ((adopted from AOCS Ce 1h-05, 2017 revised (13))

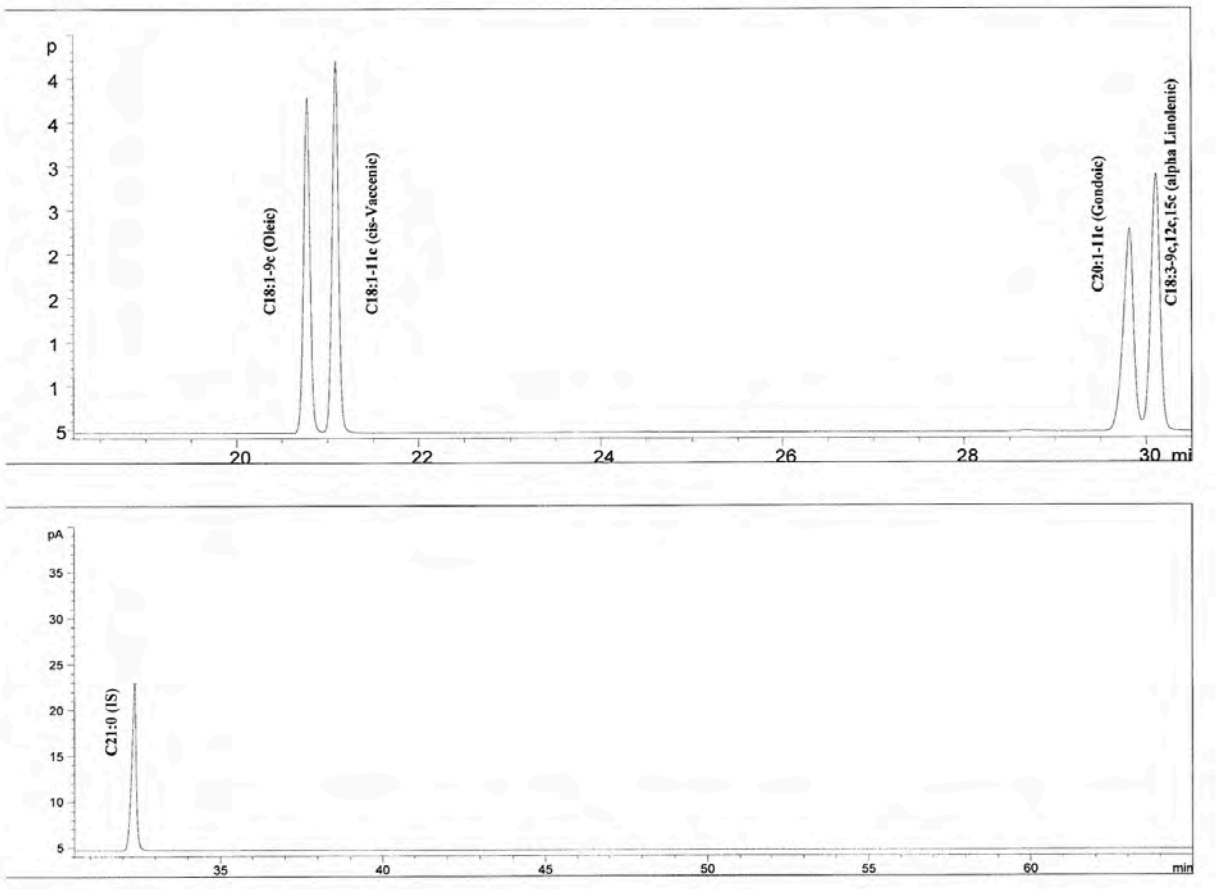

Figure 2: A (top graph) and B (bottom graph): GC profile of a salad oil (soybean) FAME: On SP-2560 100 m capillary column (180°C Isothermal, H_2 1.0 mL/min) (adopted from AOCS Ce 1h-05, 2017 revised (13))

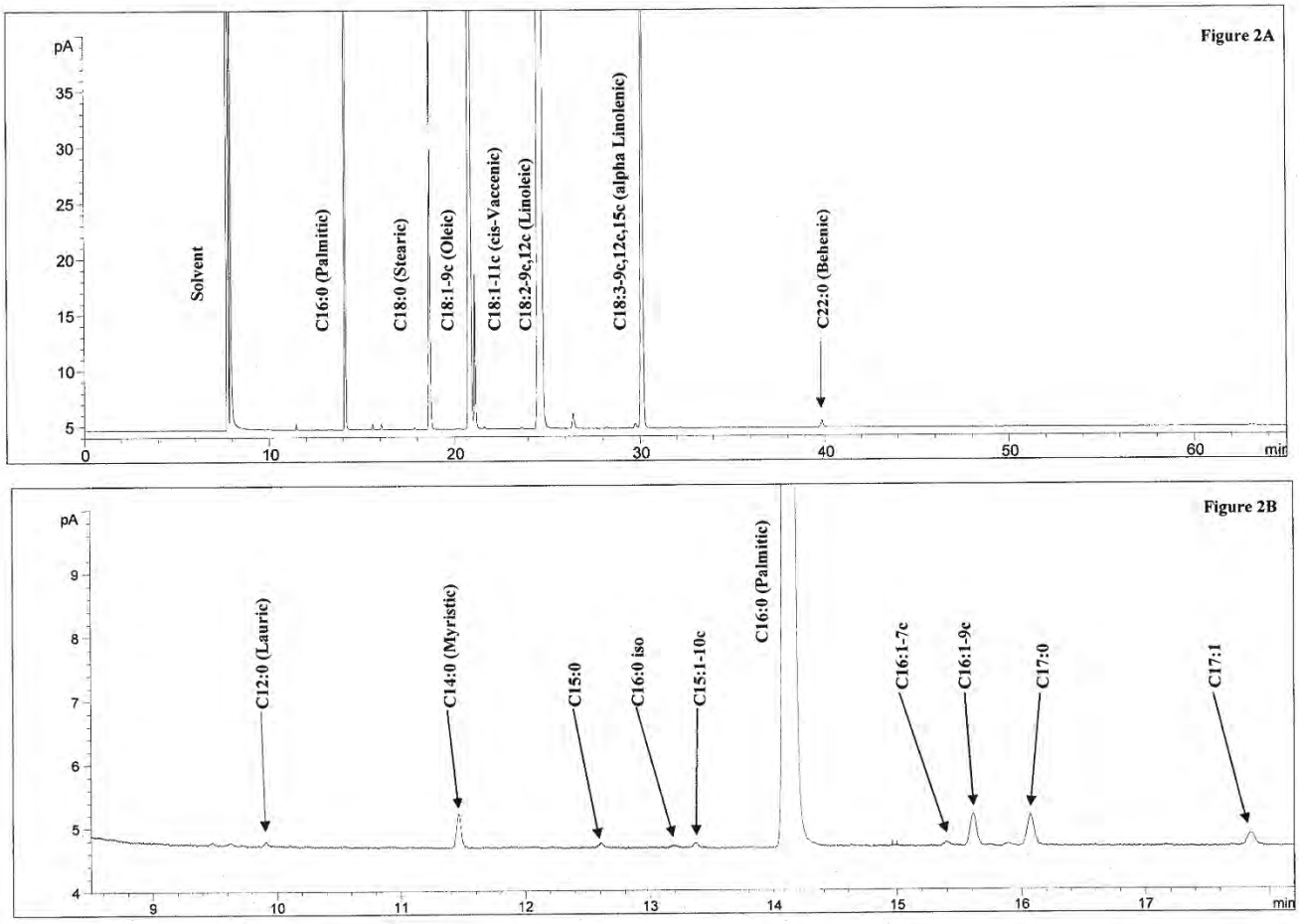

Figure 2 (contd.) C (top graph) and D (bottom graph): GC profile of a salad oil (soybean) FAME: On SP-2560 100 m capillary column (180°C Isothermal, H_2 1.0 mL/min) (adopted from AOCS Ce 1h-05, 2017 revised (13))

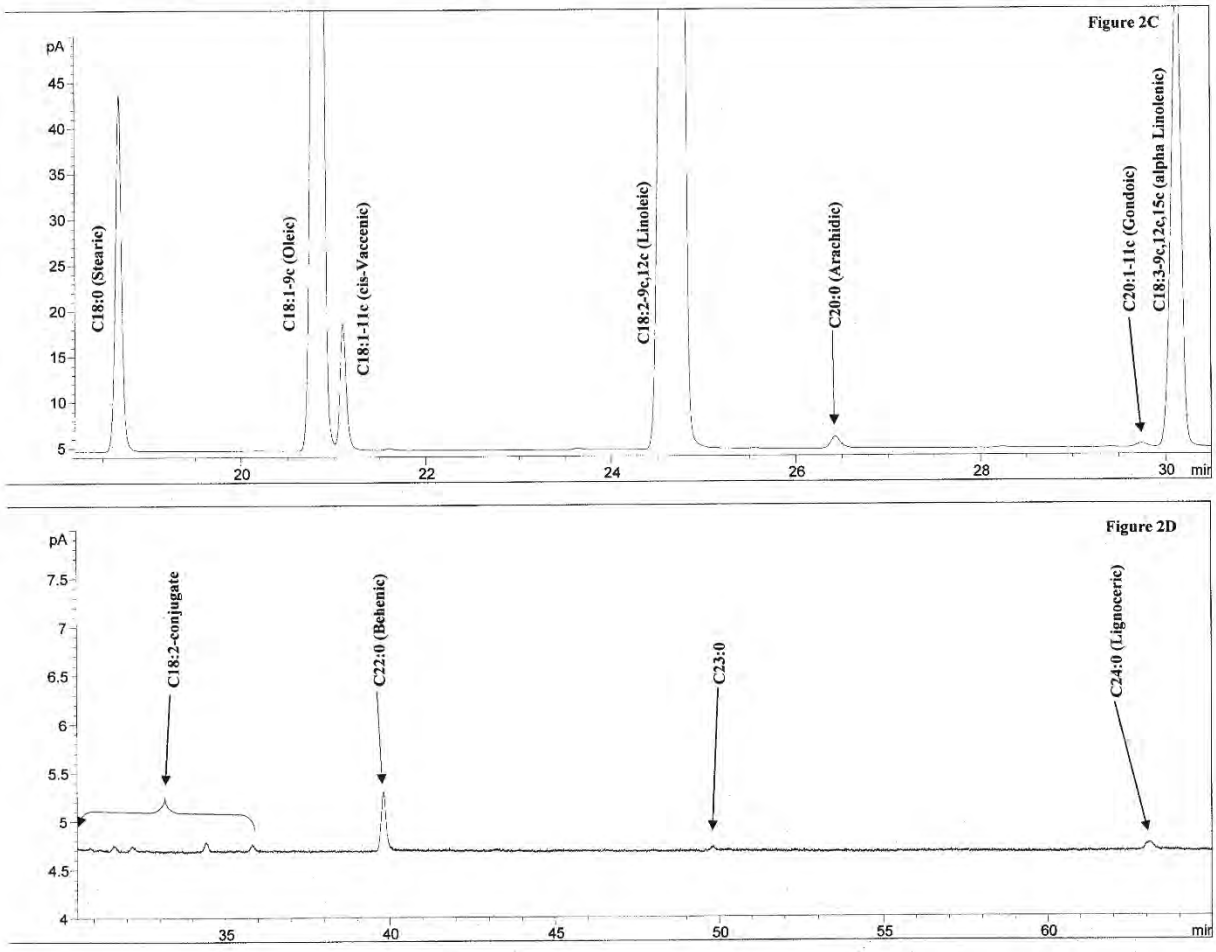

Figure 3: A (top graph) and B (bottom graph): GC profile of a low-*trans* margarine FAME: On SP-2560 100 m capillary column (180°C Isothermal, H_2 1.0 mL/min) (adopted from AOCS Ce 1h-05, 2017 revised (13))

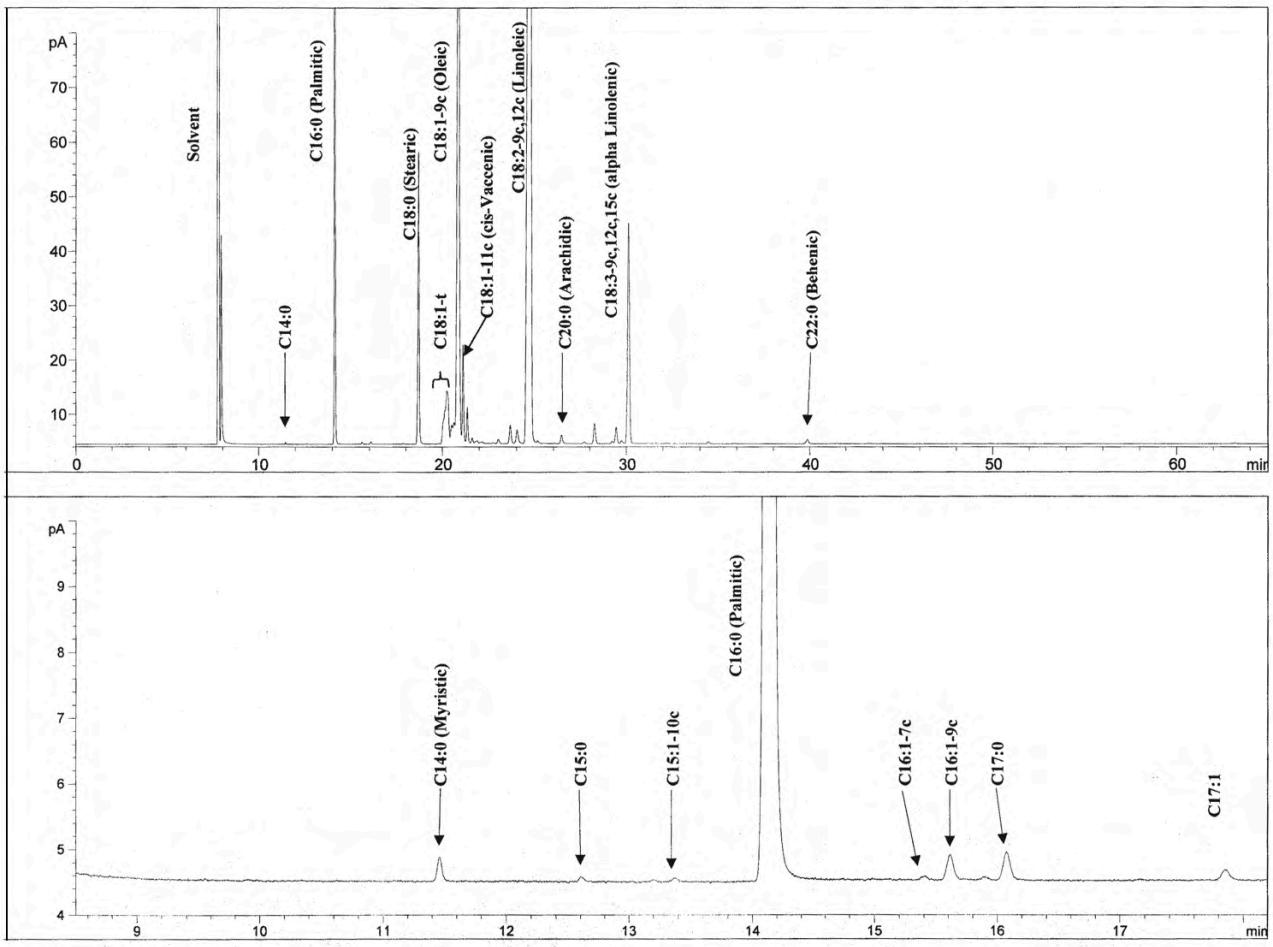

Figure 3 (contd.) C (top graph) and D (bottom graph): GC profile of a low-*trans* margarine FAME: On SP-2560 100 m capillary column (180°C Isothermal, H_2 1.0 mL/min) (adopted from AOCS Ce 1h-05, 2017 revised (13))

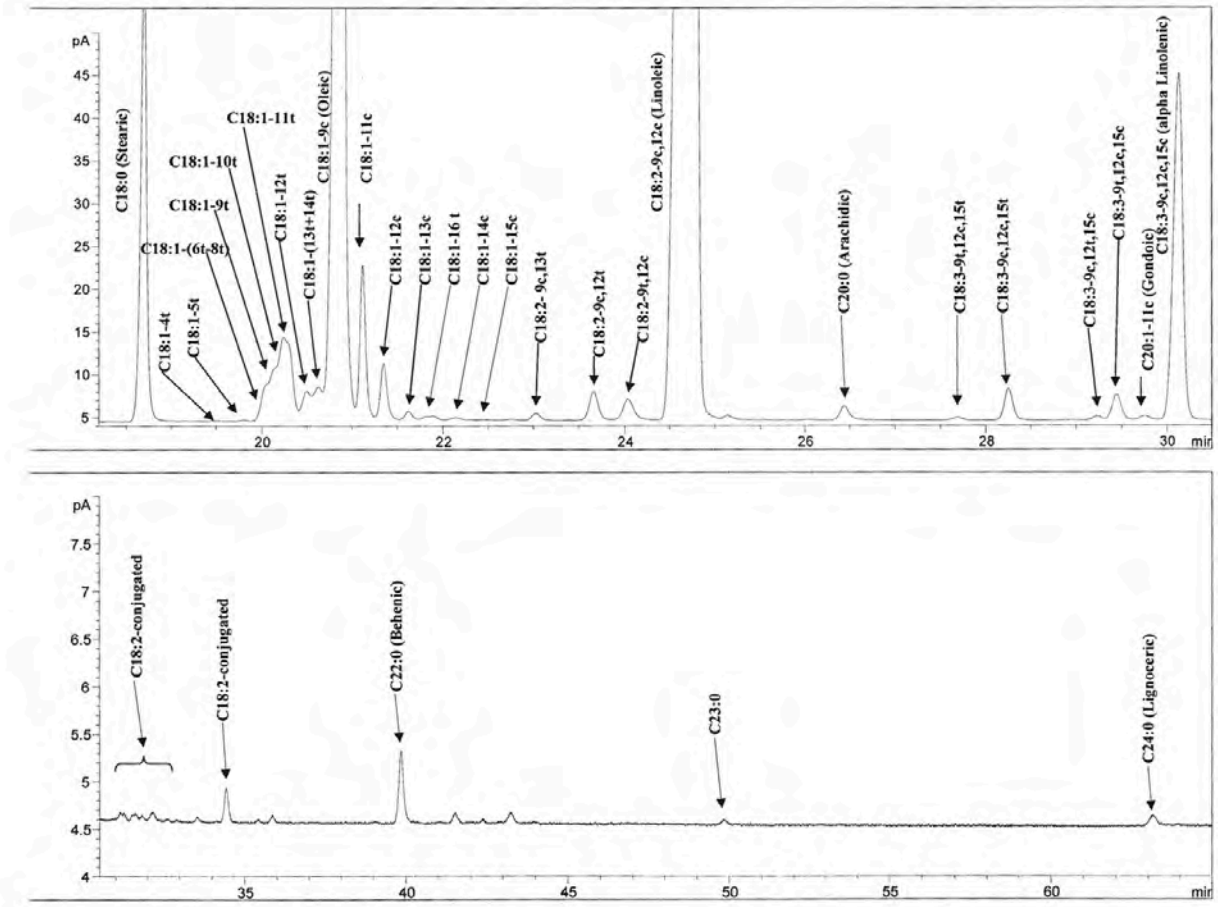

Figure 4: (top graph) A and B (bottom graph): GC profile of a high-*trans* PHO FAME: On SP-2560 100 m capillary column (180°C Isothermal, H_2 1.0 mL/min) (adopted from AOCS Ce 1h-05, 2017 revised (13))

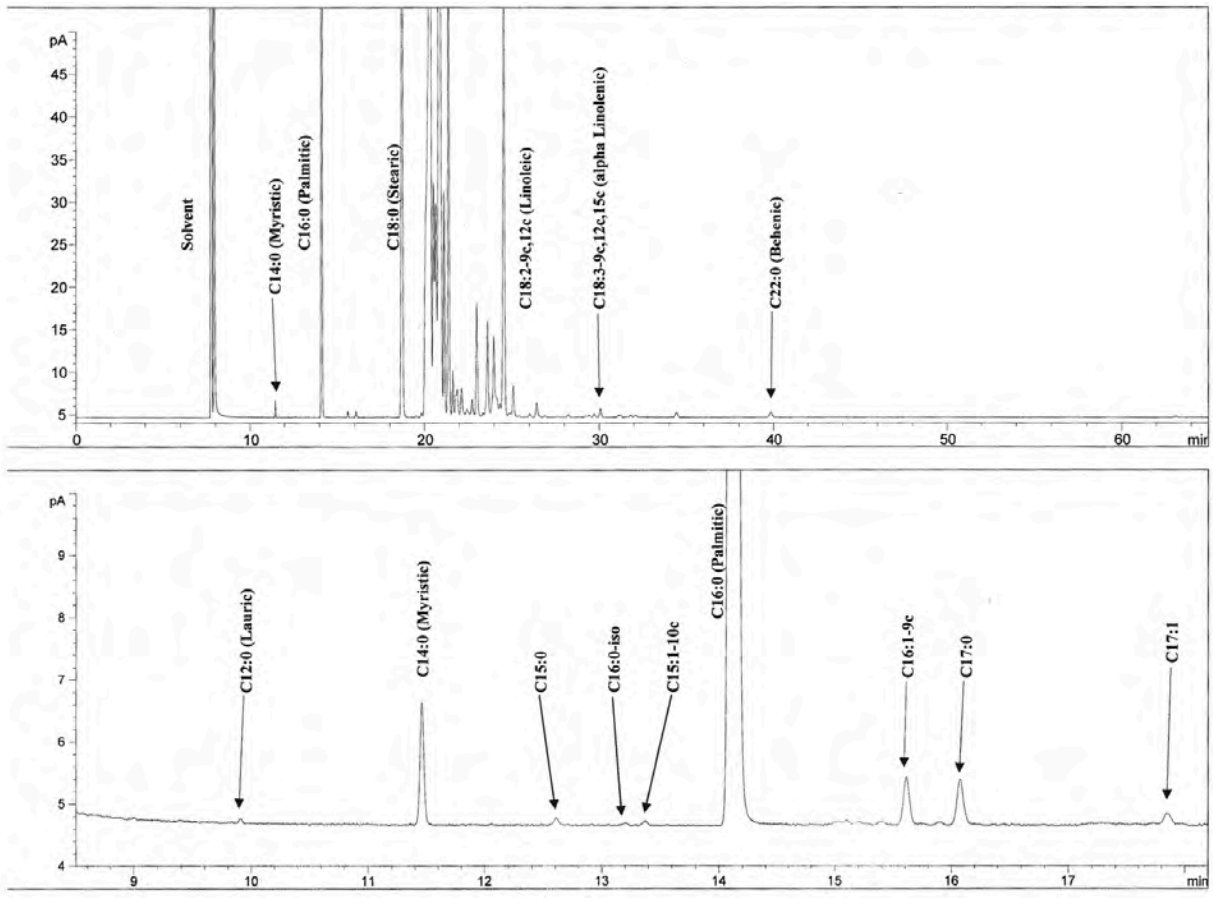

Figure 4: C(top graph) and D(bottom graph): GC profile of a high-*trans* PHO FAME: On SP-2560 100 m capillary column (180°C Isothermal, H_2 1.0 mL/min) (adopted from AOCS Ce 1h-05, 2017 revised (13))

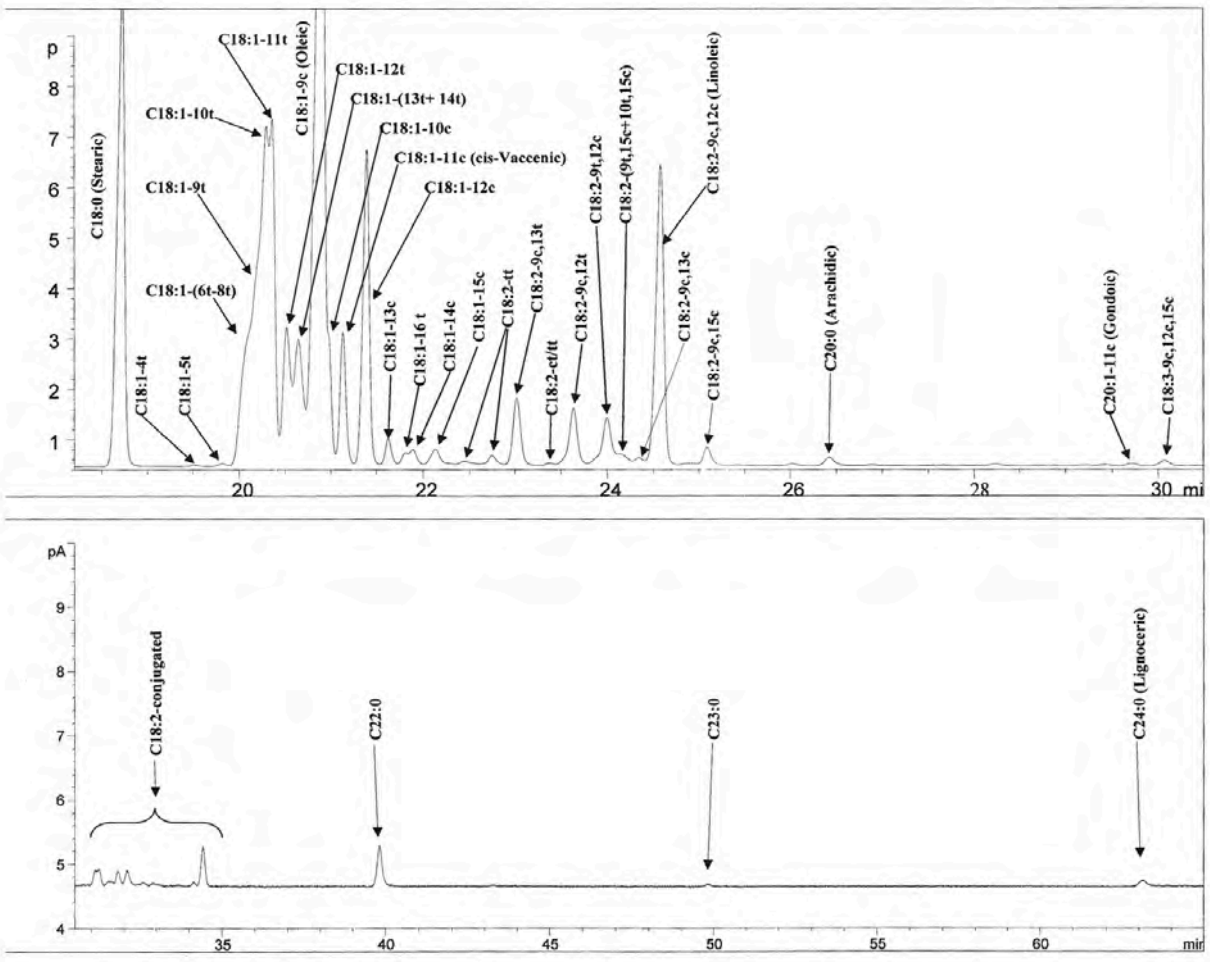

Figure 5: GC profile of a butter fat FAME. On SP-2560 100 m capillary column (180°C isothermal for 32 min, ramped to 215°C @ 20°C/min, hold 31.25 min (adopted from AOCS Official Method Ce 1j-07 (Reference 17).

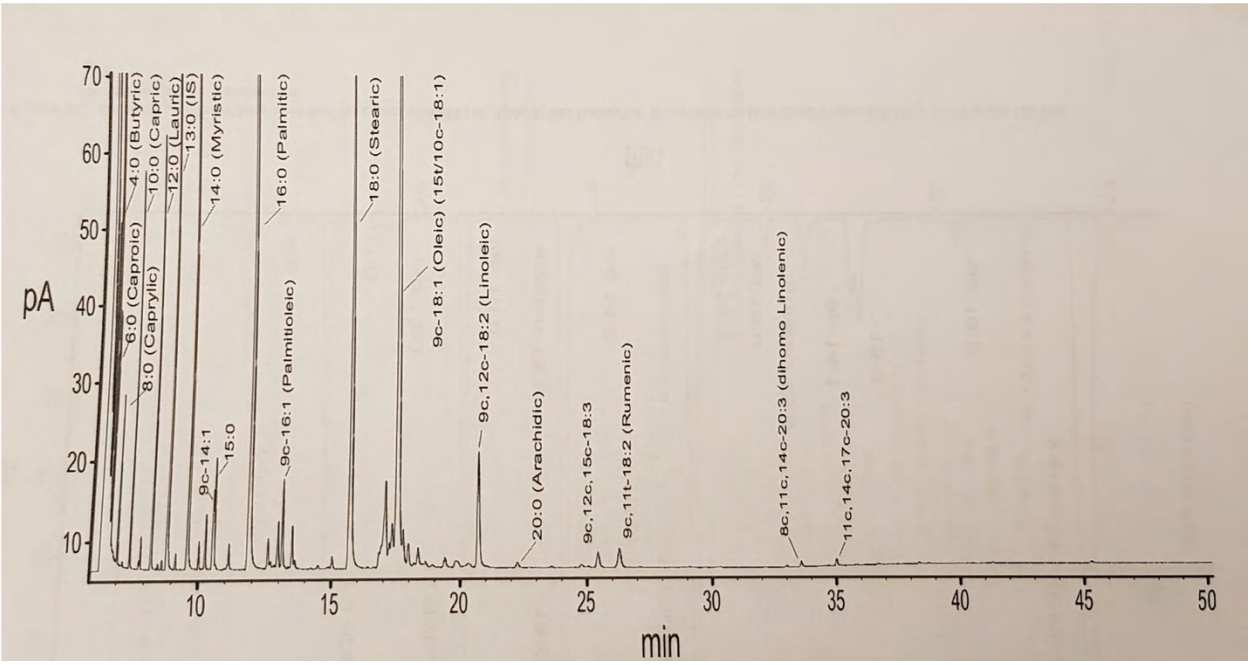

Figure 6: GC profile of a butter fat FAME. On SP-2560 100 m capillary column. Zoomed start to 16:0 (180°C isothermal for 32 min, ramped to 215°C @ 20°C/min, hold 31.25 min (adopted from AOCS Official Method Ce 1j-07 (Reference 17).

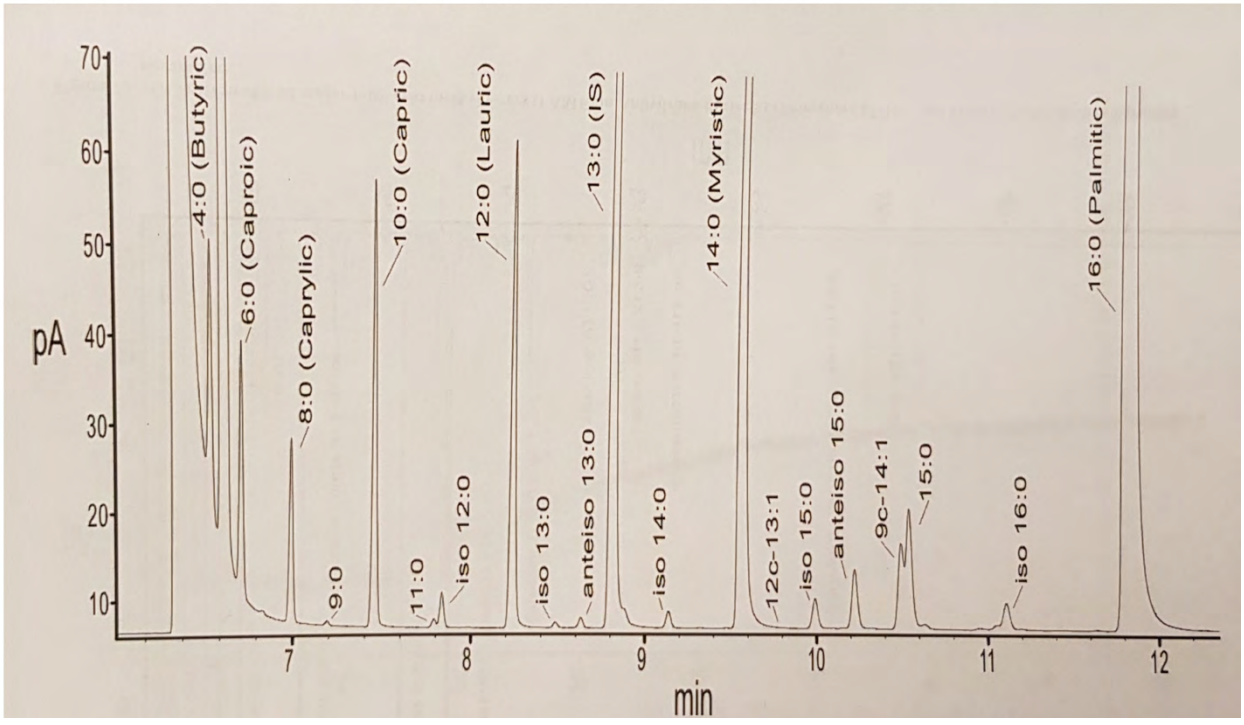

Figure 7: GC profile of a butter fat FAME. On SP-2560 100 m capillary column. Zoomed from 16:0 to 20:0 (180°C isothermal for 32 min, ramped to 215°C @ 20°C/min, hold 31.25 min (adopted from AOCS Official Method Ce 1j-07 (Reference 17).

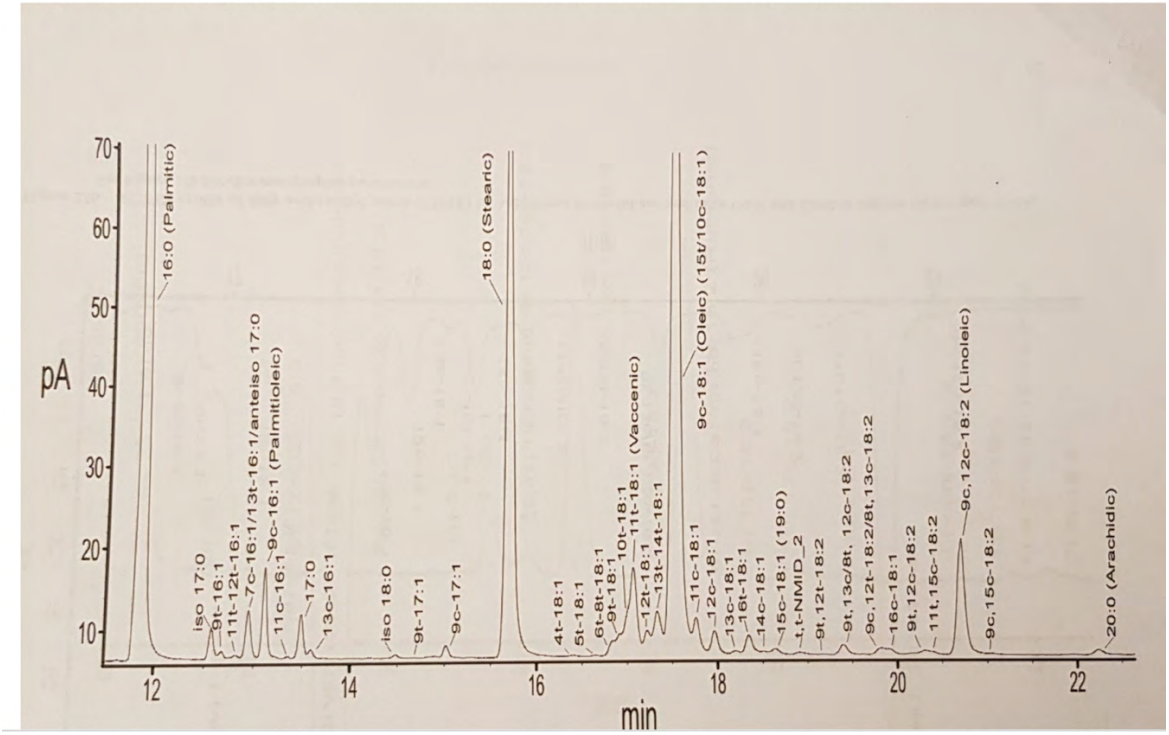

Figure 8: GC profile of a butter fat FAME. On SP-2560 100 m capillary column. Zoomed from t-18:1 region to linoleic region (180°C isothermal for 32 min, ramped to 215°C @ 20°C/min, hold 31.25 min (adopted from AOCS Official Method Ce 1j-07 (Reference 17).

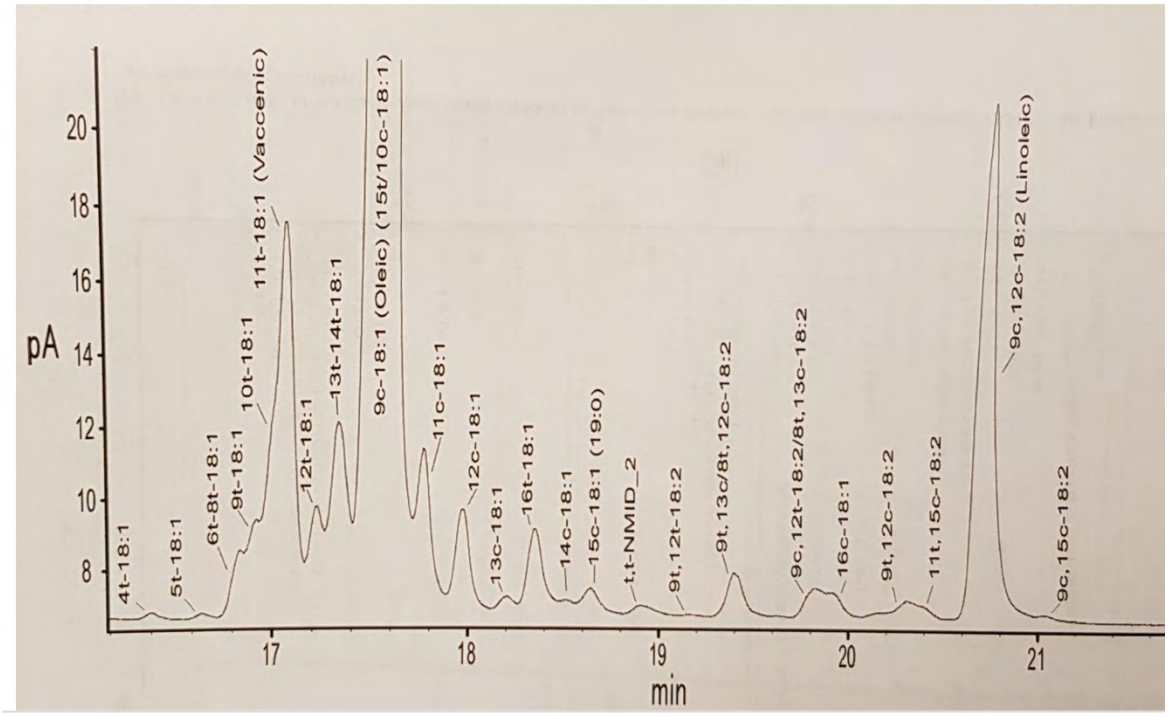

Figure 9: GC profile of a butter fat FAME. On SP-2560 100 m capillary column. Zoomed from 20:0 to CLA region (180°C isothermal for 32 min, ramped to 215°C @ 20°C/min, hold 31.25 min (adopted from AOCS Official Method Ce 1j-07 (Reference 17).

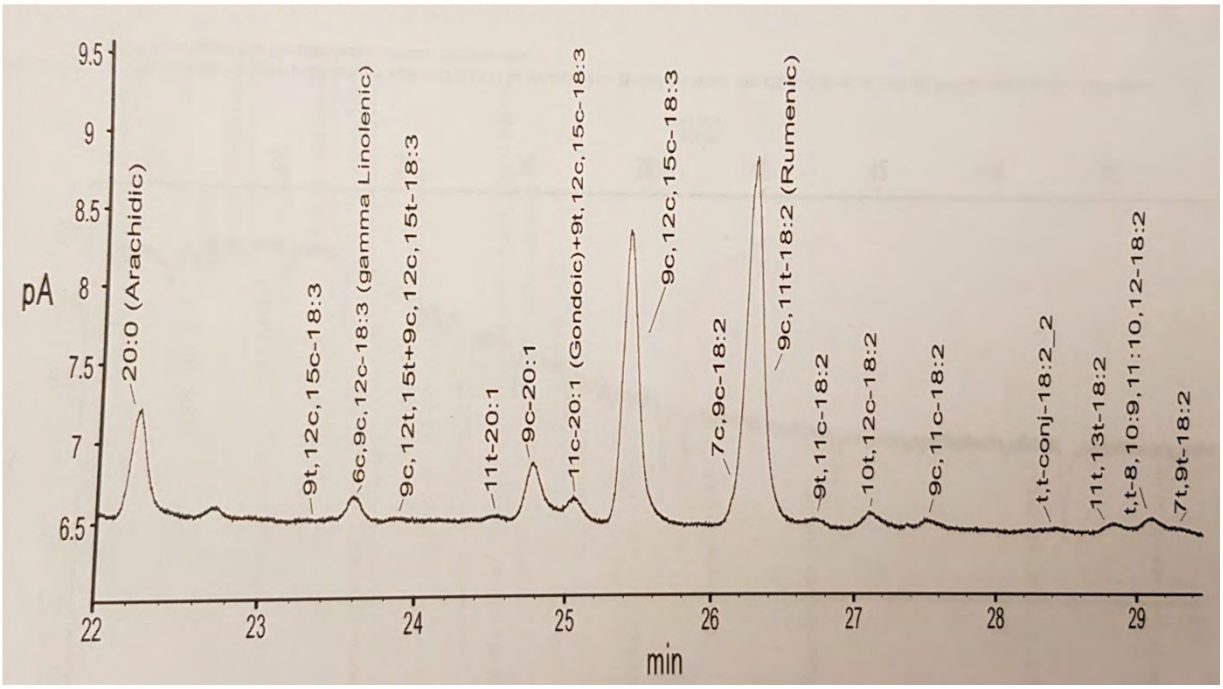

Figure 10: GC profile of a butter fat FAME. On SP-2560 100 m capillary column. Zoomed from CLA to end of the chromatogram (180°C isothermal for 32 min, ramped to 215°C @ 20°C/min, hold 31.25 min (adopted from AOCS Official Method Ce 1j-07 (Reference 17).