LABORATORY PROTOCOL

LABORATORY PROCEDURE MANUAL

Analytes: Fatty Acids

Matrix: Plasma and Serum

Method: Analysis of trans fatty acids in plasma and serum by GC-NCI-MS

Analytes:

***trans*-Vaccenic acid**	**C18:1n-7t**
Elaidic acid	**C18:1n-9t**
Palmitelaidic acid	**C16:1n-7t**
Linoelaidic acid	**C18:2n-6t,9t**
Myristic acid	**C14:0**
Myristoleic acid	**C14:1n-5c**
Palmitic acid	**C16:0**
Palmitoleic acid	**C16:1n-7c**
Stearic acid	**C18:0**
Oleic acid	**C18:1n-9c**
***cis*-Vaccenic acid**	**C18:1n-7c**
Linoleic acid	**C18:2n-6c,9c**
y-Linolenic acid	**C18:3n-6c, 9c,12c**
a-Linolenic acid	**C18:3n-3c,6c,9c**
Arachidic acid	**C20:0**
Gondoic acid	**C20:1n-9c**
Eicosadienoic acid	**C20:2n-6c,9c**
Dihomo-y-Linolenic acid	**C20:3n-6c,9c,12c**
Behenic acid	**C22:0**
Arachidonic acid	**C20:4n-6c,9c,12c,15c**
Eicosapentaenoic acid	**C20:5n-3c,6c,9c,12c,15c**
Lignoceric acid	**C24:0**
Docosatetraenoic acid	**C22:4n-6c,9c,12c,15c**
Nervonic acid	**C24:1n-9c**
Docosapentaenoic acid 6	**C22:5n-6c,9c,12c,15c,18c**
Docosapentaenoic acid 3	**C22:5n-3c,6c,9c,12c,15c**
Docosahexaenoic acid	**C22:6n-3c,6c,9c,12c,15c,18c**

Matrix: Plasma and Serum

Method: Analysis of trans fatty acids in plasma and serum by GC-NCI-MS

CONTENTS

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The following are the main technical contributors:

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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. Also, it substantially alters the biologic and health effects of the fatty acids when consumed [1].

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. Elimination of industrially-produced TFA from the global food supply by 2023 is a World Health Organization (WHO) flagship priority [2, 3].

Estimating the extent of TFA exposure in the population has been hampered by the lack of an accurate and comprehensive database on which to derive the data and the trend towards the reformulation of products over the past decade to reduce levels. This later issue complicates analysis of historical food intake data. Additionally, the variability in the TFA content of foods within a food category is extensive and can introduce substantial error when the calculations

are based on food frequency questionnaires that heavily rely on the grouping of similar foods. Furthermore, TFA intake data is unknown in most low- and middle-income countries, and the few surveys that exist are old and may not be nationally representative. The lack of such data and the uncertainties associated with indirect exposure assessments through questionnaires created the need for biomonitoring data that describe exposure of the population to TFA.

Over 10 different TFA were identified in foods such as milk and margarine, with monounsaturated octadecenoic acid (C18:1) isomers representing over 80% of total *trans* fats in ruminant fats and partially hydrogenated vegetable oils. Partially hydrogenated vegetable oils show a more even distribution of the different *trans* C18:1 isomers than dairy fats. In partially hydrogenated vegetable oils, elaidic acid (C18:1n-9t) is one major TFA while in dairy fats vaccenic acid (C18:1n-7t) is clearly the major isomer [4]. Another isomer reported in dairy fat and partially hydrogenated vegetable oils is palmitelaidic acid (C16:1n-7t) [5].

Fatty acid content in blood is reported either as concentration (i.e., in nmol/L) or as percent of total fatty acids. The latter requires measurement of regular fatty acids in blood.

The method described in this procedure was adopted from the method used at the Centers for Disease Control and Prevention (CDC), National Center for Environmental Health (NCEH) [6].

1.2 Test Principle

This measurement procedure determines the total (free and esterified) content of selected TFA in plasma and provides results in concentration units as well as percent units (TFA as percent of total fatty acids).

The fatty acids in plasma are converted into free fatty acids by subsequent acidic and alkaline hydrolysis. The free fatty acids are extracted from the sample solution using liquid-liquid extraction and derivatized with pentafluorobenzyl-bromide (PFB-Br) [7, 8]. The derivatized fatty acids are separated by capillary gas chromatography and detected by mass spectrometry using negative chemical ionization.

The fatty acids are identified based on their chromatographic retention time and on the specific mass to charge ratio of the carboxylate anion formed in the source. Retention times are compared against those obtained with known standards. Quantitation is performed with standard solutions using stable isotope-labeled fatty acids as internal standards.

To calculate TFA as percent of total fatty acids, 27 fatty acids are determined with this measurement procedure (for the names of the specific fatty acids determined in this procedure see Appendix 1). These fatty acids cover over 95% of all fatty acids reported in plasma [9]. This method determines the following four TFA: *trans*-9-hexadecenoic acid (palmitelaidic acid, C16:1n-7t), *trans*-9-octadecenoic acid (elaidic acid, C18:1n-9t), 1*trans*-11-octadecenoic acid (vaccenic acid, C18:1n-7t-), *trans*-9, *trans*-12-octadecadienoic acid (linolelaidic acid, C18:2n-6t,9t).

The procedure described in this document consists of 6 parts (see also flow chart in Appendix 2):

1. Preparation of the samples for analysis
2. Acidic and alkaline hydrolysis of the samples
3. Isolation of the free fatty acids by liquid-liquid extraction
4. Derivatization of fatty acids
5. Analysis of derivatized fatty acids by GC-MS
6. Data processing and calculations

Acid treatment hydrolyzes most lipids, but may lead to partial or complete decomposition of functional groups such as epoxy, hydroperoxy, cyclopropenyl, cyclopropyl and possibly hydroxyl and acetylenic fatty acids. It will also isomerize some *cis/trans* and *cis/cis* conjugated linoleic acid isomers to their *trans/trans* isomers [10]. Thus, this method is not suitable for measuring these particular fatty acids.

The method was developed for TFA in plasma but can also be used with serum and red blood cells.

1.3 Scope

The measurement procedure described in this document is intended for quantitatively measuring the fatty acids described in Section 1.2 in human serum or plasma for situations where limited specimen is available such as in human biomonitoring studies. It addresses all aspects related to the measurement process (specimen collection, storage, processing, analysis and reporting). Specific details related to equipment maintenance and operations are not addressed in this document and need to be created for each laboratory separately. Further, this document is not intended to provide information on data interpretation.

2 SAFETY PRECAUTIONS

2.1 General Safety

All plasma or serum specimens should be considered potentially positive for infectious agents including HIV and the hepatitis B virus. Hepatitis B vaccination series is required for all analysts performing this measurement procedure.

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

Disposable bench covers must be used during sample preparation and sample handling, and must be discarded after use. All work surfaces must be wiped with 10% bleach solution after work is finished.

2.2 Chemical Hazards

All acids, bases, and all the other reagents and organic solvents used in this measurement procedure must be handled with extreme care; they are caustic, flammable and toxic and they must be handled only in a well-ventilated area or, as required, under a chemical fume hood. Before handling chemicals and reagents described in this procedure, safety information such as Safety Data Sheets should be obtained (i.e., at http://www.ilpi.com/msds/index.html) and reviewed. Appropriate personal protective equipment (gloves, safety glasses and lab coats) must be worn at all times while handling the following chemicals:

Hydrochloric acid: Handle with extreme care. Concentrated HCl is corrosive. Avoid breathing vapors and avoid contact with skin and eyes. Handle only inside a properly operating chemical fume hood with the sash placed between the operator and the chemicals. Store container in the Acid/Corrosives cabinet.

Sodium hydroxide: Handle with extreme care. Sodium hydroxide is caustic and toxic. Avoid contact with skin and eyes. Eye contact may result in permanent eye damage, and contact with skin causes skin irritations. Store container in the designated Base cabinet.

Acetonitrile: May cause eye and skin irritation. May be harmful if swallowed, inhaled or absorbed through the skin. Keep from contact with oxidizing materials. Store in a tightly closed container in the designated flammable cabinet.

Toluene: Irritating to eyes, respiratory system and skin. Flammable and Harmful. Keep away from heat. Store in a flammable cabinet in a segregated and approved area. Keep container in a cool, well-ventilated area. Keep container tightly closed and sealed until ready for use. Avoid contact with skin and eyes. Keep away from incompatibles such as oxidizing agents.

Hexane: Irritating to eyes, respiratory system and skin. Flammable and Harmful. Avoid contact with skin or eyes. Store container in the designated flammable cabinet.

Methanol: Danger of permanent damage through inhalation, eye and skin contact and if swallowed. Flammable and Toxic. Avoid contact with skin or eyes. Store container in the designated flammable cabinet.

Pentafluorobenzyl bromide: PFB-Br is a lachrymator and is very damaging to eyes and mucous membranes. Always wear gloves, safety glasses or face shields, lab coat, and work only inside a properly operating chemical fume hood with the sash placed as far down as possible between the operator and the chemicals.

Triethylamine: Avoid contact with skin or eyes. It is corrosive. Always wear gloves, safety glasses or face shields, lab coat, and work only inside a properly operating fume hood with the sash placed as far down as possible between the operator and the chemicals. Store container in the Base cabinet.

CAUTION! Acetonitrile, toluene, methanol, and hexane are volatile organic compounds. Wear gloves, safety glasses, lab coat and/or apron, and work only inside a properly operating chemical fume hood. Keep container tightly closed and sealed in the designated flammable cabinet until ready for use.

CAUTION! Hydrogen gas used for analysis by GC-MS is categorized as a Hazardous Material Class 2, in the Compressed Gases category and is flammable. Laboratory staff should be trained appropriately before handling hydrogen gas.

2.3 Mechanical Hazards

There are only minimal mechanical hazards when performing this procedure using standard safety practices. Analysts must read and follow the manufacturer's information regarding safe operation of the equipment. Avoid direct contact with the mechanical and electronic components of analytical equipment and instrumentation unless all power is 'off'. Generally, mechanical and electronic maintenance and repair must only be performed by qualified technicians.

Follow the manufacturer's GC-MS operating instructions and hydrogen safety instructions located in the trans Fatty Acids area of the laboratory. Turn off the hydrogen at its source every time the GC or MSD are shut down or while the MSD is vented. Use leak-checking equipment to periodically check for hydrogen leaks.

2.4 Waste Disposal

All solid waste used in sample preparation process, (disposable plastic pipette tips, gloves, bench diapers, caps, etc.) as well as any residual sample material, needs to be placed into the appropriate biohazard autoclavable bags and waste pans until sealed and autoclaved.

All glass pipette tips and any sharps (i.e., broken glass) must be placed into the appropriate sharps containers and autoclaved.

All liquid waste disposal must be performed in compliance with local policies and regulations using the appropriate waste management and chemical tracking systems.

2.5 Training

Analysts performing this measurement procedure must successfully complete:

- Safety Trainings: General Laboratory Safety, Bloodborne Pathogens Safety
- Hazardous Chemical Waste Management Training
- Records Management Training

Further, the analyst must have received training on the specific instrumentation and software used with this measurement procedure from designated staff and from the instrument manufacturer as needed.

Analysts must be familiar with:

- Laboratory safety
- Biological exposure safety
- Chemical hazards including Safety Data Sheets

3 COMPUTERIZATION AND DATA-SYSTEM MANAGEMENT

3.1 Software and Knowledge Requirements

This measurement procedure requires work with different software operated instruments such as Agilent GC-MS (using MSD Chemstation™ Software version E.02.02 or higher and Mass Hunter Software version B.07 or higher). Specific training to operate this software is required to ensure appropriate and safe instrument function.

Further, calculations of results obtained with the GC-MS software are performed using calculation templates created with Microsoft Excel. The calculation results obtained with the Excel templates are transferred to a database that is created and maintained by the local organization. Assessment of bench Quality Control results is performed using a specific program and maintained by the organization.

The database activities and QC calculations are performed by dedicated and specially trained staff. Initial calculations using the Excel templates are performed by the analysts after receiving specific training from qualified laboratory staff.

3.2 Sample Information

All samples must have unique identifiers. Aliquots of the same sample need to be identified As such. No personal identifiers are used, and all samples are referenced to a blind coded sample identifier. For sample specimen handling procedures, see the flow chart in Appendix 2.

3.3 Data Maintenance

Information about samples and related analytical data are checked prior to being entered into the database for transcription errors and overall validity. Filing of electronic and physical files and their maintenance is the responsibility of designated staff in the laboratory. The database is maintained by laboratory staff and is routinely backed up by the organization.

3.4 Information Security

Information security is managed at multiple levels. The information management systems that contain the final reportable results are restricted through user ID and password security access. The computers and instrument systems that contain the raw and processed data files require specific knowledge of software manipulation techniques and physical location. Site security is provided through restricted access to the individual laboratories, buildings, and offices.

Confidentiality of results is protected by referencing results to blind coded sample IDs (no names or personal identifiers).

4 PROCEDURE FOR COLLECTING, STORING, AND HANDLING SPECIMENS; CRITERIA FOR SPECIMEN REJECTION

4.1 General Specimen Requirements

Specimens for TFA analysis may be fresh or frozen plasma, serum, or red blood cells. Gels used in serum separator tubes may contain fatty acid contaminations and should be tested for contamination before use. A minimum of 150 µL sample is needed; a 0.5-mL sample is preferable to allow for repeat analyses. A sample volume of 100 µL is required for analysis. Additional plasma sample volume may be needed if blood clots are present in the vial.

Fasting samples (i.e., samples collected in the morning after overnight fast) are recommended to minimize variability caused by recent food consumption. The specimen should be transported in 2.0-mL cryogenic vial with external screw-caps. These cryovials should be labeled uniquely as described in section 3.2.

Other specimen handling conditions or procedures used in the local laboratory may apply.

4.2 Specimen Storage

The specimens collected can be shipped frozen on dry ice. Specimens can be kept refrigerated for 3 days. For long-term storage, samples are stored at -70 °C. Studies have shown that storage of fatty acids in all lipid classes at -60 °C resulted in negligible changes in concentration [11]. Samples are stable for at least 5 years if stored at -70 °C [12]. Because of the potential for oxidation of polyunsaturated fatty acids, specimens that have been through more than five freeze-thaw cycles, been refrigerated for more than one week, or undergone hemolysis may give inaccurate results, and are not recommended for analysis.

4.3 Unacceptable Specimens

Specimens that do not meet the above mentioned criteria, were transported at room temperature, or have evidence of leakage are not acceptable.

5 PREPARATION FOR REAGENTS, CALIBRATION MATERIALS, CONTROL MATERIALS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION.

5.1 Equipment, Chemicals, and Consumables

The chemicals, equipment and other materials as described below or equivalents can be used in this measurement procedure. All materials used can contain contamination with fatty acids. Thus, batches of materials need to be screened for fatty acid contamination before use. Use of trade names and commercial sources is for identification only and does not constitute endorsement by WHO.

5.1.1 Equipment, Chemicals, and Consumables Used for Reagent Preparation

1. Various glass beakers (25 mL, 50 mL, 100 mL) (Fisher Scientific, Suwanee, GA)
2. 100 mL graduated cylinders (Fisher Scientific, Suwanee, GA)
3. Capped 250 mL bottles, class A glassware (Fisher Scientific, Suwanee, GA)
4. Sodium Hydroxide, 10N solution, Certified ACS/ASTM (Fisher Scientific, Suwanee, GA)
5. Acetonitrile, HPLC Grade Reagent (Fisher Scientific, Suwanee, GA)
6. Hydrochloric Acid, 6N Solution, Certified (Fisher Scientific, Suwanee, GA)
7. Methanol, 99.8+% A.C.S. (Fisher Scientific, Suwanee, GA)

5.1.2 Equipment, Chemicals, and Consumables Used for Sample Processing

1. Vortex: T Genie 2 (Scientific Industries, Inc., Bohemia, NY)
2. Fisher Multi-Tube Vortexer, 120 V, speed range 50-2500 rpm, (Fisher Scientific, Suwanee, GA)
3. GeneVac EZ-2 Evaporation System with side bridge holders and universal rotor (GeneVac Inc., Valley Cottage, NY)
4. Hamilton Microlab STARLet Liquid Handler (using Microlab Vector Software version 4.11.5878 (Hamilton Company, Reno, NV)
5. Freas Mechanial Convection Oven 625, 230 V, temperature range from up to 325°C (Thermo Scientific, Marietta, OH)
6. Eberbach Model E6010 Fixed-Speed Reciprocal Shaker (Elma Company, South Orange, NJ)
7. Eppendorf Centrifuge 5810 R V4.2 with A-4-62 rotor (GMI, Ramsey, MN)
8. Hand Held Scanner (Symbol Technologies Inc., Bohemia, NY)
9. Analytical Balance AX 205, with printer (Mettler-Toledo, Columbus, OH)
10. Brady Label Maker IP300 printer (Brady Worldwide Inc., Milwaukee, WI) Boekel Orbitron Platform Rotator I (Boekel Scientific, Feasterville, PA)
11. Gilson Positive Displacement Pipettes (Gilson, Inc., Middleton,WI)
    a. Gilson Microman M100 (10-100 µL)
    b. Gilson Microman M1000 (100-1000 µL)
12. Repeater Pipette Adapter (Fisher Scientific, Suwanee, GA)
13. Eppendorf Combitips (Eppendorf, Hauppauge, NY)
    a. Eppendorf Combitips, 5 mL

14. Conductive 300 µL Filter Tips for Hamilton (Hamilton Company, Reno, NV)
15. Conductive 1000 µL Filter Tips for Hamilton (Hamilton Company, Reno, NV)
16. Nalgene 2 mL cryovials with external thread (Fisher Scientific, Suwanee, GA)
17. Pyrex disposable glass culture tubes (threaded, 11.5 mL, 16x100 mm) (Corning Inc., Acton, MA)
18. Pyrex disposable glass culture tubes (extraction vials, rimless,11.5 mL,16x100 mm) (Corning Inc., Acton, MA)
19. Kimble black phenolic screw caps, PTFE-faced rubber liner (Kimble Chase, Vineland, NJ)
20. Disposable glass Pyrex Pasteur pipettes, 5 3/4 inch (Corning Inc., Acton, MA)
21. Pasteur pipette bulbs (Fisher Scientific, Suwanee, GA)
22. Kimble 5 mL disposable glass pipettes (Kimble Chase, Vineland, NJ)
23. Acetonitrile, HPLC Grade Reagent (Fisher Scientific, Suwanee, GA)
24. Pentafluorobenzyl Bromide (PFB-Br) (Fisher Scientific, Suwanee, GA)
25. Triethylamine (TEA) 99.7%, extra pure (Acros Organics, Morris Plains, NJ)
26. Hexane, Reagent Plus $\geq$ 99% (Sigma-Aldrich, St. Louis, MO)
27. Hydrochloric Acid, 6N Solution, Certified (Fisher Scientific, Suwanee, GA)

5.1.3 Equipment, Chemicals, and Consumables Used for Sample Measurement

1. Agilent GC-MSD 6890 Gas chromatograph and 5975B Mass selective detector for EI, PCI and NCI (Agilent Technologies, Wilmington, DE)
2. Data Processing Software: Agilent MSD ChemStation version E.02.02 or higher and Mass Hunter version B.07 or higher (Agilent Technologies, Wilmington, DE)
3. Gerstel Multipurpose Sampler (MPS 2) with Peltier Cooled drawer (4 ^{o} to 40 ^{o}C) (Gerstel Inc., Linthicum, MD)
4. Gerstel Maestro Basic Software version 1.1.1 or higher (Gerstel Inc., Linthicum, MD)
5. Hydrogen Generator Outlet, Flowrate, 500mL/min.; Purity, 99.9999%; Pressure, 10-100 psi (Fisher Scientific, Suwanee, GA)
6. Chemical Ionization Gas Purifier (Agilent Technologies, Wilmington, DE)
7. Non-stick Fluorocarbon Liner Viton O-ring (Agilent Technologies, Wilmington, DE)
8. Ultra Inert, Split, Low Pressure drop, Glasswool Liner (Agilent Technologies, Wilmington, DE)
9. Fixed Tapered Needle Syringe 10 µL (Agilent Technologies, Wilmington, DE)
10. Big Universal Trap 1/8” Fittings, Hydrogen (Agilent Technologies, Wilmington, DE)
11. Chemical Ionization Gas Regulator (Matheson TriGas, Montgomeryville, PA)
12. Advanced Green Non-stick 11mm Septa (Agilent Technologies, Wilmington, DE)
13. Capillary Column CP 7421 Select FAME 200 m x 250 µm x 0.25 µm (Agilent Technologies, Wilmington, DE)
14. Acetone, GC ResDlv (Fisher Scientific, Suwanee, GA)
15. N-Hexane, Reagent Plus $\geq$ 99% (Fisher Scientific, Suwanee, GA)
16. GC vials 1.5-mL, Footed, Amber Glass (Fisher Scientific, Suwanee, GA)
17. Caps with septa, blue PTFE/Silicone/PTFE (Fisher Scientific, Suwanee, GA)
18. Toluene, Certified ACS/ASTM (Fisher Scientific, Suwanee, GA)
19. Deionized water with resistance to at least 18 megaOhm-cm
20. Refer to Appendix 3 for calibrators and internal standards information

5.2 Preparation of Reagents Used For Sample Preparation

5.2.1 Preparation of 10% 6N HCl in Acetonitrile

This solution is used for the acidic hydrolysis step described in section 7.1.3. Prepare 100 mL of this solution by transferring 10 mL of 6N hydrochloric acid to a 100 mL graduated cylinder and adding acetonitrile up to the 100 mL mark. Transfer to the labeled 250 mL bottle for use in sample preparation. The solution can be prepared weekly and stored at room temperature.

5.2.2 Preparation of 10% 10N NaOH in Methanol

This solution is needed for the alkaline hydrolysis step as described in section 7.1.3. Prepare 100 mL of this solution by transferring 10 mL of 10N sodium hydroxide to a 100 mL graduated cylinder and adding methanol up to the 100 mL mark. Transfer to the labeled 250 mL bottle for use in sample preparation. The solution can be prepared weekly and stored at room temperature.

5.2.3 Preparation of 7% Pentafluorobenzyl bromide (PFB-Br) in Acetonitrile

This solution is needed to derivatize the fatty acids for GC-MS analysis as described in section 7.1.5. Using a positive displacement pipette, add 376 µL PFB-Br to 5 mL of acetonitrile in a threaded culture tube. 5 mL of this solution is sufficient for 49 samples. This solution is prepared on the day of experiment. Store at room temperature in the designated cabinet protected from light by covering the vial with aluminum foil.

5.3 Calibration Materials

5.3.1 Preparation of Calibrator Solutions

All fatty acid standards need to be analyzed for purity and integrity and results need to be compared with the certificate of analysis provided with the standard for consistency before use as calibrators.

Laboratories may have specific instruction for preparing calibrator solutions and for documenting the preparation of the solutions.

Once calibrator solutions are prepared, appropriate procedures need to be applied to verify the accuracy and consistency of the new calibrator solutions before they are used with study samples. For example, the new calibrator solutions can be treated as samples and analyzed with the old calibrator solutions, or new calibrator solutions are used to analyze certified reference materials, and results are compared with the assigned target values.

Calibrator concentrations provided herein are appropriate for analysis of fatty acids in the U.S. population. Adjustment to calibration levels may be required for other populations.

The standards described in Appendix 3 are used to create fatty acid stock solutions in 25 or 50 mL of toluene with the following concentrations:

Table 1: Fatty Acid Stock Solutions and concentrations (for Analyte codes see Appendix 3)

Analyte Code	Fatty Acid Concentration (mmol/L)	Analyte Code	Fatty Acid Concentration (mmol/L)	Analyte Code	Fatty Acid Concentration (mmol/L)	Analyte Code	Fatty Acid Concentration (mmol/L)
HDT	1	OD9	6	OD1	6	OTT	0.4
ALN	20	DP6	5	LG1	10	PL1	60
AR1	10	DTA	5	LNA	200	PM1	200
ARA	100	ED1	2.5	ML1	5	ST1	50
DA1	10	EN1	3	MR1	30	VC1	40
DE1	2.5	EPA	40	NR1	10		
DHA	50	GLA	10	OC6	12.5		
DP3	10	HGL	12.5	OL1	150		

1. Calculate the amount of fatty acid needed to create the target concentration stated in the table using the molecular weight of the standard.
2. Weigh the amount of fatty acid needed (+/- 15%) in a 25 mL or 50 mL (LNA, OL1, PM1, and ST1) volumetric flask using an analytical balance. Note the mass of fatty acid and use it to calculate the exact concentration of the fatty acid stock solution.
3. Fill the volumetric flask to just below the line and bring the flask to 20 ^{o}C in a water bath with shaking over 30 minutes. Fill to the mark with toluene.
4. Using the fatty acid stock solutions, 500 mL of a so called “level 40” (TFAC40) calibrator working solution is created using the amounts detailed in Table 3. The target concentration of each analyte in this working solution are as follows:

Table 2: Target Concentration (µmol/L) for the “level 40” Calibrator Working Solutions

Analyte Code	Fatty Acid Concentration (µmol/L)	Analyte Code	Fatty Acid Concentration (µmol/L)	Analyte Code	Fatty Acid Concentration (µmol/L)	Analyte Code	Fatty Acid Concentration (µmol/L)
HDT	25	OD9	125	OD1	125	OTT	7
ALN	400	DP6	100	LG1	200	PL1	1200
AR1	200	DTA	100	LNA	8000	PM1	8000
ARA	2000	ED1	50	ML1	100	ST1	2000
DA1	200	EN1	50	MR1	600	VC1	800
DE1	50	EPA	800	NR1	200		
DHA	1000	GLA	200	OC6	250		
DP3	200	HGL	250	OL1	6000		

5. Pipette from the individual fatty acid stock solutions the volumes listed in the table below in a 500 mL volumetric flask and fill the flask to just below the 500 mL mark with toluene. Bring the flask to 20 °C in a water bath with shaking over 30 minutes, then fill to the mark with toluene.

Table 3: Fatty Acid Stock Solution Needed for the "level 40" Calibrator Working Solutions

Analyte Code	Volume of Fatty Acid Stock Solution (mL)	Analyte Code	Volume of Fatty Acid Stock Solution (mL)	Analyte Code	Volume of Fatty Acid Stock Solution (mL)	Analyte Code	Volume of Fatty Acid Stock Solution (mL)
HDT	10	OD9	10	OD1	10	OTT	10
ALN	10	DP6	10	LG1	10	PL1	10
AR1	10	DTA	10	LNA	20	PM1	20
ARA	10	ED1	10	ML1	10	ST1	20
DA1	10	EN1	10	MR1	10	VC1	10
DE1	10	EPA	10	NR1	10		
DHA	10	GLA	10	OC6	10		
DP3	10	HGL	10	OL1	20		

6. Prepare four levels called "35", "30", "20", "10" calibrator working solutions in toluene using the dilution scheme described in the following table (use volumetric flasks and volumetric pipettes to prepare these solutions):

Table 4: Dilution Table for the Preparation of Level "35", "30", "20", "10" Calibrator Working Solutions

Calibrator working solution	Volume of Level 40 to use (mL)	Dilute to volume (mL)	Name of solution created
Level 40	100	200	Level 35 (TFAC35)
Level 40	50	200	Level 30 (TFAC30)
Level 40	20	200	Level 20 (TFAC20)
Level 40	10	250	Level 10 (TFAC10)

The target concentrations of the calibrators are listed in **Appendix 3.**
Calibrator working solutions are stable for at least two years when stored at -70 °C.

5.3.2 Preparation of Internal Standard Solutions

All fatty acid standards need to be analyzed for purity and integrity and results need to be compared with the certificate of analysis provided with the standard for consistency before use as calibrators.

Laboratories may have specific instructions for preparing internal standard solutions and for documenting the preparation of the solutions. If the calibrator concentrations have been changed, then the internal standard concentrations should be adjusted accordingly.

Once internal standard solutions are prepared, appropriate procedures need to be applied to verify the consistency of the new internal standard solutions before they are used with study samples.

The internal standards described in Appendix 3 are used to create internal standard fatty acid stock solutions in toluene with the concentrations listed in Table 5.

Table 5: Desired Internal Standard Fatty Acid Stock Solution Concentration

Analyte Code	Desired Internal Standard Fatty Acid Stock Solution Concentration (mg/mL)	Volume of Stock Solution used to Prepare Working Solution (mL)	Desired Internal Standard Fatty Acid Concentration of the Working Solution (µmol/L)
HDT_IS	0.26	20	10
OD9_IS	0.86	20	30
OD1_IS	0.86	20	30
OTT_IS	0.57	2	2
ALN_IS	5	20	200
AR1_IS	1.76	20	50
ARA_IS	10	50	800
DA1_IS	2.08	20	50
DHA_IS	10	20	300
EPA_IS	9.22	20	300
LG1_IS	1.92	20	50
LNA_IS	89.48	20	3,000
MR1_IS	5.11	20	200
OL1_IS	45.05	20	1,500
PL1_IS	10	20	500
PM1_IS	57.52	20	2,000
ST1_IS	15.99	20	500
VC1_IS	5.75	20	200

1. Calculate the amount of fatty acid needed to create the target concentration of the internal standard stock solution stated in the table using the molecular weight of the standard.
2. Weigh the amount of fatty acid needed (+/- 15%) in a 25 mL volumetric flask (5 mL flask for OTT_IS) using an analytical balance. Use a separate flask for each fatty acid. Note the mass of fatty acid and use it to calculate exact concentration of the fatty acid stock solution.
3. Fill the flask with toluene to the 25 mL (or 5 mL for OTT_IS) mark.
4. For each fatty acid solution, transfer the volume needed to prepare the working solution as stated in the table to a 2000 mL volumetric flask.
5. Fill the volumetric flask to just below the 2000 mL line and bring the flask to 20 ^{o}C in a water bath with shaking over 30 minutes. Fill to the 2000 mL mark with toluene.
6. Mix well.
7. Aliquot solution in a threaded glass tube with screw cap and store at -70 ^{o}C.

6 CALIBRATION AND CALIBRATION VERIFICATION

6.1 Calibration

6.1.1 Calibration of Instruments and Equipment

All volumetric pipettes are calibrated annually following procedures recommended by the manufacturers. Mass spectrometry instruments are calibrated for mass accuracy regularly as recommended by the manufacturer and following the manufacturer's procedures. Accuracy of other equipment such as pH-meters and oven temperatures are verified regularly according to the manufacturer's recommendation or using established references (i.e., commercial buffer solutions, external thermometers).

All calibration and calibration verification activities are documented appropriately.

6.1.2 Calibration of measurement

Calibrators used in this measurement procedure are traceable to commercial, pure compound standards (for details on pure compound specifications see Appendix 3). Calibration solutions are prepared starting with gravimetric measurements. For Metrological traceability according to ISO 17511 [13] see Appendix 4. Calibrators are analyzed together with each set of samples.

6.2 Calibration Verification

Calibration verification of equipment is performed 6 months after calibration was performed or earlier when recommended by the manufacturer.

With each set of samples, five levels of calibration material and duplicate low, mid, and high level quality control materials are analyzed. Possible shifts in calibration are assessed by comparing bench Quality Control material data against predefined acceptance limits using appropriate software programs (see also Section 8).

Calibration is further verified by analyzing commercial standards every 6 months and comparing the results obtained against predefined acceptance limits (+/- 15% from target value).

7 PROCEDURE OPERATION INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

All instruments are checked for correct function using the manufacturer's acceptance criteria. All automated steps in the sample preparation process may also be performed manually.

7.1.1 Specimen Storage and Handling during Testing

All vials are labeled as outlined in this document and according to the laboratory's specific policies and procedures and are scanned during the process of sample preparation, sample transfer and analysis in order to ensure that individual samples can be tracked throughout the process.

Specimens are allowed to reach room temperature for sample preparation. The unused portion of the patient specimen is returned to the freezer and stored at -70 ^{o}C. Samples ready for analysis by GC-MS are either stored at -70 ^{o}C in the freezer or at 10 °C +/-2 °C in the GC-MS instrument sample tray.

7.1.2 Preparation of Samples for Analysis

All samples are processed together with 1 reagent blank (toluene), 1 set calibrators (5 levels: TFAC10, TFAC20, TFAC30, TFAC35, TFAC40), and 6 bench QC samples (2 low, 2 medium and 2 high). Typically, 36 patient samples are processed in one batch (total number of samples per batch: 49 including the Retention Time Standard).

1. Assess all samples for acceptability using the criteria described in sections 4.2 and 4.3.
2. Thaw all specimens to room temperature before preparation. Frozen plasma samples, QC samples, Internal Standard (IS) solutions and calibrator solutions are allowed to reach ambient temperature and are homogenized by placing them on the rotator for approximately 30 minutes.
3. Place all patient samples, QC samples, calibrators and internal standard solutions on the Hamilton Microlab STARLet Liquid Handler instrument in the designated locations, according to the laboratory's specific policies and procedures, in a manner that allows the instrument's barcode reader to read all barcodes properly. Place all additional supplies on the instrument at the designated positions. The Hamilton instrument will complete the following steps:
4. Scan the barcodes of all coded vials and reagents.

   When a barcode cannot be read, the instrument software will prompt and will allow manual entering of the barcode information. After the scanning process is successfully completed, an excel file containing the barcode information, the location of the particular sample, calibrator and reagent on the Hamilton instrument and the current

date and time is automatically created on the Hamilton's computer. This file is transferred to a defined location according to the laboratory's specific policies and procedures and this information is copied in the calculation template.

5. After scanning the barcodes, remove samples from the Hamilton, and working in the hood transfer 100 µL of samples, QC samples, calibrators and the blank (toluene) to the labeled 16 x 100 mm Pyrex threaded culture tubes using a 100 µL positive displacement pipette.
6. After the transfer of all samples, the analyst must visually inspect all vials for potential blood clots. If blood clots are noted, the vial is discarded and the sample is manually pipetted using a positive displacement pipette into a new Pyrex threaded culture tube.
7. Transfer 100 µL of Internal Standard solution to all samples and calibrators using a 100 µL positive displacement pipette and visually verify successful transfer by checking the solution levels in all vials.
8. Recap sample vials and store remaining sample in a dedicated place in a -70 ^{o}C freezer.

7.1.3 Hydrolysis of Samples

1. Add 2 mL of 10% 6N HCl in Acetonitrile solution to each vial using a graduated glass pipette.
2. Cap all vials and vortex them for 30 seconds using the Fisher Multi-Tube Vortexer at 2500 rpm.
3. Place samples in the Mechanical Convection oven set at 104 (+/-4) ^{o}C for 45 minutes.
4. Remove vials from the oven and place them in the chemical fume hood for 30 minutes to allow them to cool to room temperature.
5. Assess the volume in all vials by comparing it to a vial containing 2.2 mL of water. Adjust any volume lost during the hydrolysis step with acetonitrile using a positive displacement pipette and document action.
6. Add 2 mL of 10% 10 N NaOH in methanol to all vials using a graduated glass pipette.
7. Cap all vials and vortex them for 30 seconds using the Fisher Multi-Tube Vortexer at 2500 rpm.
8. Place the samples in the Mechanical Convection oven set up at 104 (+/-4) ^{o}C for 45 minutes.
9. Remove vials from the oven and let them cool for 30 minutes to room temperature in the chemical fume hood.
10. Assess the volume in all vials by comparing it to a vial containing 4.2 mL of water. Adjust any volume lost during the hydrolysis step with methanol using a positive displacement pipette and document action.
11. Add 500 µL of 6N HCl solution to each vial using a repeater pipette.
12. Cap all vials and vortex them for 5 seconds using the T Genie 2 Vortex at highest setting.

7.1.4 Extraction of Free Fatty Acids

1. Place all vials (from step 7.1.3 #12) on Hamilton Microlab STARLet Liquid Handler instrument in the designated locations.
2. Run the appropriate Hamilton Microlab STARLet Liquid Handler extraction method. The robotic pipette will add 2 mL of hexane to each threaded culture tube.
3. Shake all vials in the Eberbach Fixed Speed Reciprocal Shaker for 15 minutes (no heat) high setting until the two solvent layers disappear and the sample solution becomes opaque.
4. Transfer the threaded culture tubes to the centrifuge and centrifuge samples for 5 minutes at 21 (+/-1) ^{o}C and 3000 rpm to separate the organic layer form the aqueous layer. Before starting the centrifuge, ensure that the load is balanced.
5. Place all threaded culture tubes in the appropriate locations on the Hamilton Microlab STARLet Liquid Handler instrument.
6. Scan the barcodes of all sample vials and the empty extraction vials that will receive the hexane layer. When a barcode cannot be read, the instrument software will prompt and will allow manual entering of the barcode information. After the scanning process is successfully completed, a file is automatically created on the Hamilton instrument containing the barcode information, the location of the particular sample, calibrator and reagent information, and the current date and time. This scan file is automatically copied to a defined location according to the laboratory's specific policies and procedures and is imported later to the designated section of the Excel calculation template.
7. Transfer the upper hexane layer from each sample into the extraction vials.
8. Repeat steps 2-7 two times (total of 3 extraction steps). The hexane layers from each vial are combined in the same vial used in step 7.
9. Scan vials each time before transferring the hexane layer. Transfer scan file in dedicated location according to the laboratory's specific policies and procedures to verify that the vials are in the correct order. Make corrections if necessary before transferring hexane layer.
10. Transfer the extraction vials containing the hexane extracts into the two aluminum vial holders and place the holders in Genevac.
11. Evaporate the hexane (1.5 hours at the "Low BP", "Lamp OFF" setting).
12. Proceed to the next step immediately after drying in order to avoid sample oxidation.

Derivatization of Fatty Acids

1. Remove all the extraction vials from the GeneVac evaporator and place all vials and solutions in the designated racks on the Hamilton Microlab STARLet Liquid Handler instrument.
2. Add 100 µL of the PFB-Br solution and 10 µL of the Triethylamine (TEA) to each vial using the appropriate Hamilton Microlab STARLet Liquid Handler dilution method.

3. Remove all vials from the Hamilton Microlab STARLet Liquid Handler instrument and mix solution by vortexing all vials for 5 seconds using the T Genie 2 Vortex on setting #7.
4. Place vials in the designated racks on the Hamilton Microlab STARLet Liquid Handler instrument and keep vials at room temperature for 15 minutes for the derivatization reaction to occur.
5. Add 0.5 mL of hexane to each vial.
6. Mix all vials by vortexing for 5 seconds using the T Genie 2 Vortex on setting #7.
7. Place all vials in the designated racks on the Hamilton Microlab STARLet Liquid Handler instrument.
8. Scan the barcodes of all vials.
   When a barcode cannot be read, the instrument software will prompt and will allow manual entering of the barcode information. After the scanning process is successfully completed, a file is automatically created on the Hamilton instrument containing the barcode information, the location of the particular sample, calibrator and reagent information and the current date and time. This scan file is automatically copied to a defined location according to the laboratory's specific policies and procedures and is imported later in the designated section of the Excel calculation template.
9. Transfer the measurement solution to the GC vials using the Hamilton instrument.
10. Cap the GC vials and visually inspect successful transfer of the measurement sample solution.
11. Transfer vials to the GC autosampler for GC-MS analysis or store as described in section 7.1.1 until GC-MS analysis.

7.1.5 Analysis of Derivatized Fatty Acids by GC-MS

All samples prepared in one batch are analyzed in one batch on the same instrument. A retention time standard sample containing all analytes is added to each batch.

1. An analytical run sequence file is created by importing the file containing the sample barcode information from the Hamilton instrument (section 7.1.2, step 4) to an Excel Template. This template combines the sample ID information with additional information necessary to analyze the samples on the GC-MS system such as name, instrument location, instrument method name, and analyst ID. The sequence file creates the appropriate data file names for the individual sample data.
2. The run sequence created with the Excel template is saved as a "csv" file and the "csv" file is imported onto the Agilent GC-MS Instrument where the final run sequence file is saved as a Chemstation sequence file.
   The sequence of analyzing samples is created in a manner that at least one quality control material and one calibrator are analyzed within a 24 hour period. The first sample in a sequence is always an instrument control standard (see 0 for an example of an analytical sequence).

3. The samples are loaded on the GC-MS instrument as stated in the sequence file and the position of samples in the autosampler are verified against the information in the sequence file. Basic instrument function and settings are checked according to the GC-MS manufacturer's instructions. It is assured that the correct instrument method is loaded and all method parameters are stable.
4. The instrument run sequence is started using Chemstation/Mass Hunter software.
5. Using the retention time standard sample, the performance of the GC-MS system is assessed by inspecting retention times, peak intensities and general chromatographic parameters. When instrument malfunction is indicated, the sequence is stopped, samples are stored in the freezer and the problem is addressed.
6. Upon completion of the GC-MS analysis, the GC vials are recapped and stored in the designated space in the freezer at -70 °C.

The following GC-MS parameters are used (for further specific details see 0). Typical chromatograms of the retention time standard and low QC are shown in Appendix 7.

Chromatographic conditions

The following chromatographic conditions were found to be suitable to achieve resolution of the four trans-fatty acid isomers from neighboring peaks and to balance the response of high and low abundance peaks to ensure sufficient abundance on the low end while avoiding saturating the column on the high end. Prior to analyzing samples, it is necessary to ensure appropriate chromatographic conditions to meet the following minimum criteria: greater than 70% resolution for each trans-fatty acid from neighboring peaks, peak shapes between 0.8 and 1.2 for PM1, OL1, and LNA in the retention time standard or Level 40 calibrator, and S/N ratio better than 10 for OTT and OTT_IS in the low QC material.

Injection:	
Injector:	Gerstel MPS2
Injection volume:	1 µL
Injection mode:	Split (Split ratio: 100:1)
Injector temperature:	240 °C
Gas type:	Hydrogen
Gas flow:	2 mL/min
Column:	Agilent Select-FAME 200 m x 250 µm x 0.25 µm (length, inner diameter, film thickness)
Oven:	
Initial temperature:	50 °C
Temperature Program:	

Table 6: Temperature Program

Step	Start Temperature [°C]	Heating Rate [°C/min]	End Temperature [°C]	Temperature Hold Time (min)
1	50	40	160	10

2	160	1	175	0
3	175	0.5	210	0
4	210	35	260	25

Mass spectrometric conditions

Acquisition mode:	Selected Ion Monitoring (SIM)
Solvent Delay:	20 min
MV Mode:	Gain Factor
MS source Temperature:	230 °C
MS Quadrupole Temperature:	150 °C
CI Gas:	Methane
CI Flow Rate:	40
CI A/B Gas:	1

Table 7: Analyte and IS selected ion monitoring mass and time segments

	No.	Fatty Acid	Analyte code	SIM mass	Shorthand	Internal Standard
Group 1	1		MR1_IS	254.4	D_{27}-C14:0	
	2	Myristic acid	MR1	227.2	C14:0	MR1_IS
	3	Myristoleic acid	ML1	225.2	C14:1n-5c	MR1_IS
Group 2	4		PM1_IS	271.3	$^{13}C_{16}$-C16:0	
	5	Palmitic acid	PM1	255.3	C16:0	PM1_IS
Group 3	6		HDT_IS	258.4	$^{13}C_5$-C16:1n-7t	
	7	Palmitelaidic acid	HDT	253.2	C16:1n-7t	HDT_IS
	8		PL1_IS	269.3	$^{13}C_{16}$-C16:1n-7c	
	9	Palmitoleic acid	PL1	253.2	C16:1n-7c	PL1_IS
Group 4	10		ST1_IS	318.5	D_{35}-C18:0	
	11	Stearic acid	ST1	283.3	C18:0	ST1_IS
Group 5	12		OD9_IS	286.4	$^{13}C_5$-C18:1n-9t	
	13	Elaidic acid	OD9	281.3	C18:1n-9t	OD9_IS
	14	Petroselinic acid	OC6	281.3	C18:1n-12c	OD1_IS
	15		OD1_IS	286.4	$^{13}C_5$-C18:1n-7t	
	16	*trans*-Vaccenic acid	OD1	281.3	C18:1n-7t	OD1_IS
	17		OL1_IS	299.3	$^{13}C_{18}$-C18:1n-9c	
	18	Oleic acid	OL1	281.3	C18:1n-9c	OL1_IS
	19		VC1_IS	286.4	$^{13}C_5$-C18:1n-7c	
	20	*cis*-Vaccenic acid	VC1	281.3	C18:1n-7c	VC1_IS
Group 6	21		OTT_IS	284.4	$^{13}C_5$-C18:2n-6t,9t	
	22	Linoelaidic acid	OTT	279.3	C18:2n-6t,9t	OTT_IS
Group 7	23		LNA_IS	297.3	$^{13}C_{18}$-C18:2n-6c,9c	
	24	Linoleic acid	LNA	279.3	C18:2n-6c,9c	LNA_IS
Group 8	25		AR1_IS	350.7	D_{39}-C20:0	
	26	Arachidic acid	AR1	311.3	C20:0	AR1_IS
	27	□-Linolenic acid	GLA	277.1	C18:3n-6c, 9c,12c	ALN_IS
	28		ALN_IS	291.5	D_{14}-C18:3n-3c,6c,9c	
	29	□-Linolenic acid	ALN	277.1	C18:3n-3c,6c,9c	ALN_IS
	30	Gondoic acid	EN1	309.3	C20:1n-9c	AR1_IS
Group 9	31	Eicosadienoic acid	ED1	307.3	C20:2n-6c,9c	AR1_IS
	32	Dihomo-□-Linolenic acid	HGL	305.3	C20:3n-6c,9c,12c	ARA_IS
	33		DA1_IS	382.9	D_{43}-C22:0	
	34	Behenic acid	DA1	339.4	C22:0	DA1_IS
	35		ARA_IS	311.3	D_8-C20:4n-6c,9c,12c,15c	
	36	Arachidonic acid	ARA	303.3	C20:4n-6c,9c,12c,15c	ARA_IS
Group 10	37		EPA_IS	306.3	D_5-C20:5n-3c,6c,9c,12c,15c	
	38	Eicosapentaenoic acid	EPA	301.1	C20:5n-3c,6c,9c,12c,15c	EPA_IS

	39		LG1_IS	414.9	D_{47}-C24:0	
	40	Lignoceric acid	LG1	367.4	C24:0	LG1_IS
Group 11	41	Docosatetraenoic acid	DTA	331.3	C22:4n-6c,9c,12c,15c	DHA_IS
	42	Nervonic acid	NR1	365.4	C24:1n-9c	LG1_IS
	43	Docosapentaenoic acid 6	DP6	329.3	C22:5n-6c,9c,12c,15c,18c	DHA_IS
	44	Docosapentaenoic acid 3	DP3	329.3	C22:5n-3c,6c,9c,12c,15c	DHA_IS
	45		DHA_IS	332.3	D_5-C22:6n-3c,6c,9c,12c,15c,18c	
	46	Docosahexaenoic acid	DHA	327.3	C22:6n-3c,6c,9c,12c,15c,18c	DHA_IS

7.1.6 Data Processing

1. Data files generated by the GC-MS system are transferred to an appropriate location according to the laboratory's specific policies and procedures.
2. Using the Mass Hunter software data processing method, relevant chromatographic peaks are identified based on their retention time and m/z. The area under the curve is integrated.
3. Integrated peaks are documented as electronic files (in PDF format) and integration results are saved as Excel files.
4. The integration results are imported into an Excel template where final results are calculated.
5. Integrations and integration results are reviewed by a specially trained and dedicated individual who is not an analyst. Errors detected will be returned to the analyst for correction. Only data that passed this review process will be considered for further processing.
6. Integration results and calculation results are combined with relevant operator, instrument and QC sample information and transferred to an appropriate location according to the laboratory's specific policies and procedures.

7.1.7 Data Calculations

1. Area ratios are calculated from the analyte and internal standard area counts.
2. Calibration curves are generated with the area ratios from the calibrators and their assigned values using ordinary linear regression.
   The calibration curve is assessed for outliers and other problems resulting in non-linear behavior of data points. Analytes with invalid calibration curves are not processed further.
3. The analyte concentration in micromole per liter (□mol/L) is calculated using the area ratio calculated for a particular fatty acid and the regression parameters of the corresponding calibration curve.
   Area ratios for analytes outside the established linear range will not be used to calculate analyte concentration. These samples will be reanalyzed after appropriate dilution or concentration.

4. The sum of all fatty acids in one sample in micromole per liter is calculated. The portion of a particular TFA on the total fatty acids is calculated by dividing the concentration of the TFA with the sum of all fatty acids and multiplying this number by 100.

8 QUALITY ASSESSMENT AND PROFICIENCY TESTING

Quality assessment activities should be performed according to the laboratory's specific policies and procedures.

8.1 Quality Control Procedures

8.1.1 Quality Control Materials

Bench QC materials are used in this measurement procedure which consists of three plasma materials with levels of concentration spanning the "low-normal" to "high-normal" ranges for the analytes of interest.

The bench QC specimens are inserted in each sample batch and processed the same as the patient specimens.

8.1.2 Establishing QC Limits and Quality Control Evaluation

Acceptance criteria for values obtained with the bench QC materials ("QC limits") are established according to the procedure described by Caudill et al. [14].

8.1.3 Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria

When results of control or calibration materials fail to meet the laboratory's established criteria for acceptability, all patient test results obtained in the unacceptable test run and since the last acceptable test run must be considered adversely affected and thus cannot be reported. Specimen processing and analysis is stopped and will only resume when corrective action have been performed that ensure the reporting of accurate and reliable patient test results.

8.2 Proficiency Testing

No commercial proficiency testing/external quality assessment program exists for the analytes reported with this measurement procedure. Because of this situation, the Audit-Sample Procedure as alternative proficiency testing program as described in the guideline of the Clinical and Laboratory Standards Institute (CLSI) GP29-P [15] was selected.

In this procedure, 5 proficiency testing pools with levels spanning the full range of analyte values likely to be encountered in human specimens are prepared, characterized by measuring 30 separate vials from each pool in at least 10 different runs and performance limits are calculated. Individual vials from these pools are blinded by a different DLS laboratory. The 5 blinded vials are analyzed twice a year and results are evaluated by the DLS statistician. For the Proficiency Testing challenge to pass, at least 4 of the 5 results for each analyte need to be within the established performance limits (80% is considered passing).

If fewer than 4 of the 5 proficiency testing samples are within the limits for a given analyte, the challenge is considered as failed, no patient samples are to be analyzed and appropriate actions to correct this problem need to be initiated. Analysis of patient samples can resume after the problem was corrected and another Proficiency Testing challenge passed successfully.

9 METHOD PERFORMANCE CHARACTERISTICS

Alternative methods can be used if they achieve the analytical performance characteristics listed in this section and equivalence between the method described here and the alternate method is demonstrated. Determination of equivalence can be assessed using the protocols such as the one described in CLSI guideline EP09 [16] .

9.1 Reportable Range of Results and Linearity Limits

The reportable range of results is the range within linearity of the verified assay. The linearity for the analytes measured in this measurement procedure was determined following CLSI guideline EP6 [17]. The reportable ranges of results are:

Analyte	Linear Range µmol/L
trans 9-hexadecenoic acid	0.48 - 24.0
trans-9-octadecenoic acid	2.60 - 130
trans-11-octadecenoic acid	2.60 - 130
trans-9, trans-12-octadecadienoic acid	0.14 - 7.00
tetradecanoic acid	12.1 - 604
cis-9-tetradecenoic acid	2.12 - 106
hexadecanoic acid	161 - 8060
cis-9-hexadecenoic acid	24.6 - 1230
octadecanoic acid	40.5 - 2023
cis-9-octadecenoic acid	120 - 5990
cis-11-octadecenoic acid	14.7 - 737
cis-6-octadecenoic acid	4.86 - 243
cis-9, cis-12-octadecadienoic acid	159 - 7980
cis-9, cis-12, cis-15-octadecatrienoic acid	8.14 - 407
cis-6, cis-9, cis-12-octadecatrienoic acid	4.12 - 206
eicosanoic acid	4.06 - 203
cis-8, cis-11, cis-14-eicosatrienoic acid	5.14 - 257
cis-5, cis-8, cis-11, cis-14-eicosatetraenoic acid	36.1 - 1810
cis-11-eicosenoic acid	0.96 - 48.0
cis-11, cis-14-eicosadienoic acid	1.00 - 50.0
cis-5, cis-8, cis-11, cis-14, cis-17-eicosapentaenoic acid	14.5 - 724
docosanoic acid	3.98 - 199
cis-4, cis-7, cis-10, cis-13, cis-16, cis-19-docosahexaenoic acid	20.6 - 1030
cis-7, cis-10, cis-13, cis-16-docosatetraenoic acid	1.92 - 96.0
cis-7, cis-10, cis-13, cis-16, cis-19-docosapentaenoic acid	4.06 - 203
cis-4, cis-7, cis-10, cis-13, cis-16-docosapentaenoic acid	1.96 - 98.0
tetracosanoic acid	4.04 - 202
cis-15-tetracosenoic acid	3.92 - 196

9.2 Limit of Detection (LOD)

The limit of detection was determined using Taylor's method [18]. The limits of detection are:

Analyte	LOD µmol/L
trans 9-hexadecenoic acid	0.07
trans-9-octadecenoic acid	0.28
trans-11-octadecenoic acid	0.43
trans-9, trans-12-octadecadienoic acid	0.02
tetradecanoic acid	0.33
cis-9-tetradecenoic acid	0.29
hexadecanoic acid	17
cis-9-hexadecenoic acid	0.76
octadecanoic acid	4.02
cis-9-octadecenoic acid	14.3
cis-11-octadecenoic acid	1.06
cis-6-octadecenoic acid	0.4
cis-9, cis-12-octadecadienoic acid	4.9
cis-9, cis-12, cis-15-octadecatrienoic acid	0.82
cis-6, cis-9, cis-12-octadecatrienoic acid	0.43
eicosanoic acid	0.47
cis-8, cis-11, cis-14-eicosatrienoic acid	1
cis-5, cis-8, cis-11, cis-14-eicosatetraenoic acid	0.36
cis-11-eicosenoic acid	0.84
cis-11, cis-14-eicosadienoic acid	0.16
cis-5, cis-8, cis-11, cis-14, cis-17-eicosapentaenoic acid	1.29
docosanoic acid	1.77
cis-4, cis-7, cis-10, cis-13, cis-16, cis-19-docosahexaenoic acid	1.96
cis-7, cis-10, cis-13, cis-16-docosatetraenoic acid	0.34
cis-7, cis-10, cis-13, cis-16, cis-19-docosapentaenoic acid	0.51
cis-4, cis-7, cis-10, cis-13, cis-16-docosapentaenoic acid	0.33
tetracosanoic acid	1.59
cis-15-tetracosenoic acid	1.38

9.3 Analytical Specificity

Analytical specificity is achieved through:

- A sample preparation that isolates the analytes of interest from other components in the sample matrix
- A sample derivatization procedure that only reacts with the analytes and compounds with similar chemical characteristics
- High resolution chromatography that separates the analytes of interest and allows for compound identification based on chromatographic retention time using reference compounds and stable isotope labeled internal standards
- Mass spectrometric ionization mode that only allows for detection of the derivatives created during sample preparation
- Mass selective detection mode that only allows for detection of the mass-to-charge ratios specific to the fatty acids

Analytical specificity was tested

1. By assessing possible chromatographic coelution and MS detection using 63 different fatty acids (for the list of compounds used in this assessment see Appendix 8). None of the tested compounds showed coelution with the analytes reported in this method.
2. High, medium, and low QC pools were analyzed without addition of the internal standard to assess whether compounds in the QC samples coelute with the internal standards. No coelution was detected in this experiment.

9.4 Accuracy and Precision

Within-day imprecision was determined from 10 replicates of high, medium, and low QCs. The among day variability is assessed by measuring high, medium, and low QC pools in duplicate each over 20 days and calculating the means and standard deviations.

Table 8: Within-Day Precision

Analyte	Within-Day Precision (%CV) Low	Within-Day Precision (%CV) Medium	Within-Day Precision (%CV) High
trans 9-hexadecenoic acid	2	1	2
trans-9-octadecenoic acid	3	1	2
trans-11-octadecenoic acid	2	1	1
trans-9, trans-12-octadecadienoic acid	10	9	4
tetradecanoic acid	1	1	2
cis-9-tetradecenoic acid	2	2	2
hexadecanoic acid	1	1	1
cis-9-hexadecenoic acid	1	1	1
octadecanoic acid	1	1	1
cis-9-octadecenoic acid	1	1	1
cis-11-octadecenoic acid	1	1	1
cis-6-octadecenoic acid	1	1	2
cis-9, cis-12-octadecadienoic acid	1	1	1
cis-9, cis-12, cis-15-octadecatrienoic acid	1	2	1
cis-6, cis-9, cis-12-octadecatrienoic acid	1	2	2
eicosanoic acid	1	1	1
cis-8, cis-11, cis-14-eicosatrienoic acid	1	3	2
cis-5, cis-8, cis-11, cis-14-eicosatetraenoic acid	1	2	1
cis-11-eicosenoic acid	2	3	2
cis-11, cis-14-eicosadienoic acid	2	3	2
cis-5, cis-8, cis-11, cis-14, cis-17-eicosapentaenoic acid	1	1	1
docosanoic acid	2	5	4
cis-4, cis-7, cis-10, cis-13, cis-16, cis-19-docosahexaenoic acid	3	3	1
cis-7, cis-10, cis-13, cis-16-docosatetraenoic acid	4	5	3
cis-7, cis-10, cis-13, cis-16, cis-19-docosapentaenoic acid	4	4	3
cis-4, cis-7, cis-10, cis-13, cis-16-docosapentaenoic acid	3	6	3
tetracosanoic acid	5	7	3
cis-15-tetracosenoic acid	5	7	3
Sum of fatty acids	1	1	1

Table 9: Among-Day Precision

Analyte	Among-Day Precision (%CV) Low	Among-Day Precision (%CV) Medium	Among-Day Precision (%CV) High
trans 9-hexadecenoic acid	9	4	4
trans-9-octadecenoic acid	10	4	5
trans-11-octadecenoic acid	10	4	5
trans-9, trans-12-octadecadienoic acid	17	12	9
tetradecanoic acid	6	4	4
cis-9-tetradecenoic acid	20	9	5
hexadecanoic acid	5	4	6
cis-9-hexadecenoic acid	6	4	4
octadecanoic acid	4	4	5
cis-9-octadecenoic acid	4	3	5
cis-11-octadecenoic acid	4	3	4
cis-6-octadecenoic acid	12	4	5
cis-9, cis-12-octadecadienoic acid	4	4	6
cis-9, cis-12, cis-15-octadecatrienoic acid	5	4	4
cis-6, cis-9, cis-12-octadecatrienoic acid	6	4	4
eicosanoic acid	8	6	5
cis-8, cis-11, cis-14-eicosatrienoic acid	5	4	5
cis-5, cis-8, cis-11, cis-14-eicosatetraenoic acid	4	3	4
cis-11-eicosenoic acid	11	11	8
cis-11, cis-14-eicosadienoic acid	5	5	4
cis-5, cis-8, cis-11, cis-14, cis-17-eicosapentaenoic acid	25	13	6
docosanoic acid	7	7	7
cis-4, cis-7, cis-10, cis-13, cis-16, cis-19-docosahexaenoic acid	13	15	6
cis-7, cis-10, cis-13, cis-16-docosatetraenoic acid	5	5	4
cis-7, cis-10, cis-13, cis-16, cis-19-docosapentaenoic acid	9	6	5
cis-4, cis-7, cis-10, cis-13, cis-16-docosapentaenoic acid	6	5	5
tetracosanoic acid	8	8	8
cis-15-tetracosenoic acid	7	11	6
Sum of fatty acids	4	3	4

The accuracy was verified by analyzing commercial standards materials (GLC standard GLC-603 and GLC-674, NuCheckPrep, Elysian, MN) and comparing the assigned value to the measured values.

Table 10: Accuracy

Analyte	Abbreviation	Average Accuracy (%)	95% CI (%)
trans 9-hexadecenoic acid	HDT	95.6	94.5-96.7
trans-9-octadecenoic acid	OD9	91.7	87.8-95.5
trans-11-octadecenoic acid	OD1	89.5	86.0-93.0
trans-9, trans-12-octadecadienoic acid	OTT	89.8	88.3-91.2
tetradecanoic acid	MR1	98	96.5-99.4
cis-9-tetradecenoic acid	ML1	128.4	127.1-129.7
hexadecanoic acid	PM1	103.3	100.5-106.0
cis-9-hexadecenoic acid	PL1	99.4	97.2-101.6
octadecanoic acid	ST1	97	93.6-100.4
cis-9-octadecenoic acid	OL1	98.4	96.7-100.1
cis-11-octadecenoic acid	VC1	99	97.8-100.1
cis-6-octadecenoic acid	OC6	99.7	97.2-102.12
cis-9, cis-12-octadecadienoic acid	LNA	90.3	89.7-91.0
cis-9, cis-12, cis-15-octadecatrienoic acid	ALN	90.4	88.1-92.8
cis-6, cis-9, cis-12-octadecatrienoic acid	GLA	93	90.7-95.4
eicosanoic acid	AR1	95.6	92.4-98.7
cis-8, cis-11, cis-14-eicosatrienoic acid	HGL	92.9	89.7-96.2
cis-5, cis-8, cis-11, cis-14-eicosatetraenoic acid	ARA	95.4	91.5-99.3
cis-11-eicosenoic acid	EN1	82	80.0-83.9
cis-11, cis-14-eicosadienoic acid	ED1	95	90.0-100.1
cis-5, cis-8, cis-11, cis-14, cis-17-eicosapentaenoic acid	EPA	90.2	88.7-91.7
docosanoic acid	DA1	98.4	96.6-100.2
cis-4, cis-7, cis-10, cis-13, cis-16, cis-19-docosahexaenoic acid	DHA	92	89.9-94.0
cis-7, cis-10, cis-13, cis-16-docosatetraenoic acid	DTA	91.9	91.3-92.6
cis-7, cis-10, cis-13, cis-16, cis-19-docosapentaenoic acid	DP3	89.5	84.4-94.6
cis-4, cis-7, cis-10, cis-13, cis-16-docosapentaenoic acid	DP6	87.5	86.0-89.0
tetracosanoic acid	LG1	97.4	92.2-102.6
cis-15-tetracosenoic acid	NR1	100.6	95.2-106.1

9.5 Limitations of Method, Interfering Substances and Conditions

Interfering conditions

Analysts preparing samples and handling supplies and equipment must wear gloves at all times to minimize contamination of samples with fatty acids from the skin or skin cream products.

Limitations of the method

This method was tested for fatty acid analysis in human plasma and serum and may not be suitable for other specimens. The analytical performance parameters need to be reassessed and verified when other specimen matrices are used.

This method does not allow for analysis of fatty acids containing functional groups such as epoxy, hydroperoxy, cyclopropenyl, cyclopropyl and possibly hydroxyl and acetylenic groups. Further, it is not suitable for analysis of cis/trans and cis/cis conjugated linoleic acid isomers as they may be converted to their trans/trans isomers.

Interfering Substances

The method was tested for 63 different substances (for specific details see Appendix 8). None of these substances interfere with the analytes reported with this measurement procedure.

10 REFERENCE RANGES (NORMAL VALUES)

Population-based reference ranges have not been established yet for these trans fatty acids.

An in-house assessment using samples from 66 individuals, collected in 2013 was performed to obtain information on concentrations that can be expected in the U.S. general population. Reference ranges may be different in other countries. In this study, the following values were determined:

Table 11: Reference Range

Analyte	Values in µmol/L		Values as percent of total fatty acids	
	Mean (Range)	**Median (5^{th}-95^{th} Percentile)**	**Mean (Range)**	**Median (5^{th}-95^{th} Percentile)**
trans 9-hexadecenoic acid	2.53 (0.671-8.64)	2.13 (1.05-4.83)	0.021 (0.009-0.038)	0.02 (0.012-0.032)
trans-9-octadecenoic acid	12.3 (2.6-120)	9 (4.06-25)	0.08 (0.04-0.21)	0.07 (0.05-0.17)
trans-11-octadecenoic acid	14.7 (3.1-181.2)	10.3 (5.07-28.7)	0.11 (0.04-0.32)	0.1 (0.05-0.22)
trans-9, trans-12-octadecadienoic acid	1.66 (0.36-8)	0.9 (0.455-4.44)	0.011 (0.007-0.022)	0.01 (0.008-0.017)
tetradecanoic acid	96.3 (23.1-325.2)	72.8 (36.6-253.3)	0.86 (0.38-1.92)	0.74 (0.51-1.74)
cis-9-tetradecenoic acid	6.42 (0.69-31.38)	4.67 (1.87-16.73)	0.06 (0.01-0.19)	0.05 (0.02-0.13)
hexadecanoic acid	2272 (1086-5574)	2106 (1335-3663)	22.17 (19.08-27.45)	21.83 (19.66-25.39)
cis-9-hexadecenoic acid	205 (61-897)	162 (76.4-477)	1.92 (0.86-5.03)	1.68 (1.03-4.09)
octadecanoic acid	678 (334-1708)	648 (415-1027)	6.68 (5.4-8.71)	6.69 (5.66-7.74)
cis-9-octadecenoic acid	1785 (628-4610)	1604 (958-3268)	17.14 (12.94-25.76)	16.44 (13.63-21.91)
cis-11-octadecenoic acid	143 (53-353)	134 (79.1-249)	1.37 (0.95-1.83)	1.36 (1.06-1.71)
cis-6-octadecenoic acid	22.1 (5.5-217.5)	16.3 (9.24-41)	0.18 (0.07-0.5)	0.16 (0.1-0.34)
cis-9, cis-12-octadecadienoic acid	3230 (1447-6030)	3142 (2053-4483)	33.89 (22.19-42.47)	34.12 (26.25-40.04)
cis-9, cis-12, cis-15-octadecatrienoic acid	54.6 (19.1-199.6)	45.7 (23.5-117)	0.52 (0.24-0.93)	0.48 (0.33-0.89)
cis-6, cis-9, cis-12-octadecatrienoic acid	52.5 (16.3-127.6)	46.6 (24.9-102)	0.53 (0.18-1.25)	0.49 (0.3-0.91)
eicosanoic acid	21.5 (7.54-39.4)	20.7 (13.3-31.7)	0.22 (0.13-0.3)	0.22 (0.15-0.29)
cis-8, cis-11, cis-14-eicosatrienoic acid	127 (53-290)	118 (66.4-201)	1.26 (0.78-2.21)	1.2 (0.89-1.84)

Table 11: Reference Range (continued)

Analyte	Values in µmol/L		Values as percent of total fatty acids	
	Mean (Range)	Median (5^{th}-95^{th} Percentile)	Mean (Range)	Median (5^{th}-95^{th} Percentile)
cis-5, cis-8, cis-11, cis-14-eicosatetraenoic acid	799 (316-1382)	795 (444-1196)	8.36 (5.15-13.61)	8.45 (5.85-11.13)
cis-11-eicosenoic acid	9.79 (1.55-32.3)	8.6 (4.12-17.9)	0.09 (0.02-0.16)	0.09 (0.06-0.14)
cis-11, cis-14-eicosadienoic acid	19.6 (7.78-43.1)	19 (10.8-32.6)	0.19 (0.08-0.27)	0.19 (0.15-0.24)
cis-5, cis-8, cis-11, cis-14, cis-17-eicosapentaenoic acid	42.9 (18.3-160)	37.4 (22.6-68.6)	0.46 (0.13-1.67)	0.41 (0.28-0.77)
docosanoic acid	53.7 (2.8-102)	51.9 (26.6-90.6)	0.53 (0.03-0.86)	0.55 (0.31-0.73)
cis-4, cis-7, cis-10, cis-13, cis-16, cis-19-docosahexaenoic acid	110 (413-320)	103 (62.1-168)	1.19 (0.58-3.35)	1.14 (0.69-1.7)
cis-7, cis-10, cis-13, cis-16-docosatetraenoic acid	27.9 (7.25-59.1)	27 (14.9-46.5)	0.27 (0.07-0.41)	0.28 (0.19-0.36)
cis-7, cis-10, cis-13, cis-16, cis-19-docosapentaenoic acid	40.6 (15.6-105)	36.7 (22.6-64.9)	0.41 (0.23-1.1)	0.39 (0.3-0.53)
cis-4, cis-7, cis-10, cis-13, cis-16-docosapentaenoic acid	18.9 (5.69-39.5)	18.2 (8.53-28.1)	0.19 (0.08-0.34)	0.19 (0.13-0.29)
tetracosanoic acid	52.7 (3.32-106)	47.3 (29.6-92.7)	0.51 (0.03-0.81)	0.51 (0.33-0.67)
cis-15-tetracosenoic acid	71.8 (4.24-126)	70.2 (40-107)	0.75 (0.04-1.14)	0.77 (0.46-1)
Sum of fatty acids	9972 (4458-20865)	9618 (6071-14689)	NA	NA

11 TEST RESULT REPORTING SYSTEM

Results are reported to 3 significant digits based on assay sensitivity calculations. Data are reported in µmol/L.

12 PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Following successful completion of analysis, remaining samples will be retained until all results have been reported and sufficient time has passed for review of the results. After this time, samples are either returned to the contact person who requested the analysis or are treated according to laboratory policy.

Standard record keeping (e.g., database, notebooks, data files) is used to track specimens. Records (including related QA/QC data) are maintained for 3 years, and duplicate records are kept off-site in electronic format. Study subject confidentiality is protected by providing personal identifiers only to the medical officer if needed or remain with the contact person who requested the analyses.

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14 APPENDICES

Appendix 1. List of Fatty Acids Measured with this Measurement Procedure

Appendix 2. Flow Chart Describing Sample Processing Performed for Fatty Acids Analysis

Appendix 3. Description of Standards Used

Appendix 4. Metrological Traceability of Trans Fatty Acids Measurements

0.

Injection order	Sample Type	Vial #	Sample ID
1	Instrument Check Std	49	RT STD
2	Calibrator	4	TFAC20L06
3	Calibrator	5	TFAC10L06
4	Quality Control	8	+021208PA
5	Sample	13	QCS9019PL
6	Sample	14	QCS9029PL
7	Sample	15	QCS9030PL
8	Sample	16	QCS9031PL
9	Sample	17	QCS9033PL
10	Sample	18	QCS9034PL
11	Sample	19	QCS9035PL
12	Sample	20	QCS9036PL
13	Sample	21	QCS9063PL
14	Calibrator	1	TFAC40L06
15	Calibrator	6	TFAC00L06
16	Quality Control	7	+031209PA
17	Sample	22	QCS9064PL
18	Sample	23	QCS9065PL
19	Sample	24	QCS9068PL
20	Sample	25	QCS9069PL
21	Sample	26	QCS9073PL
22	Sample	27	QCS9074PL
23	Sample	28	QCS9075PL
24	Sample	29	QCS9079PL
25	Sample	30	QCS9080PL
26	Sample	31	QCS9081PL
27	Calibrator	3	TFAC30L06
28	Quality Control	9	+011207PA
29	Quality Control	10	+031209PA
30	Sample	32	QCS9082PL
31	Sample	33	QCS9084PL
32	Sample	34	QCS9085PL
33	Sample	35	QCS9086PL
34	Sample	36	QCS9087PL
35	Sample	37	QCS9088PL

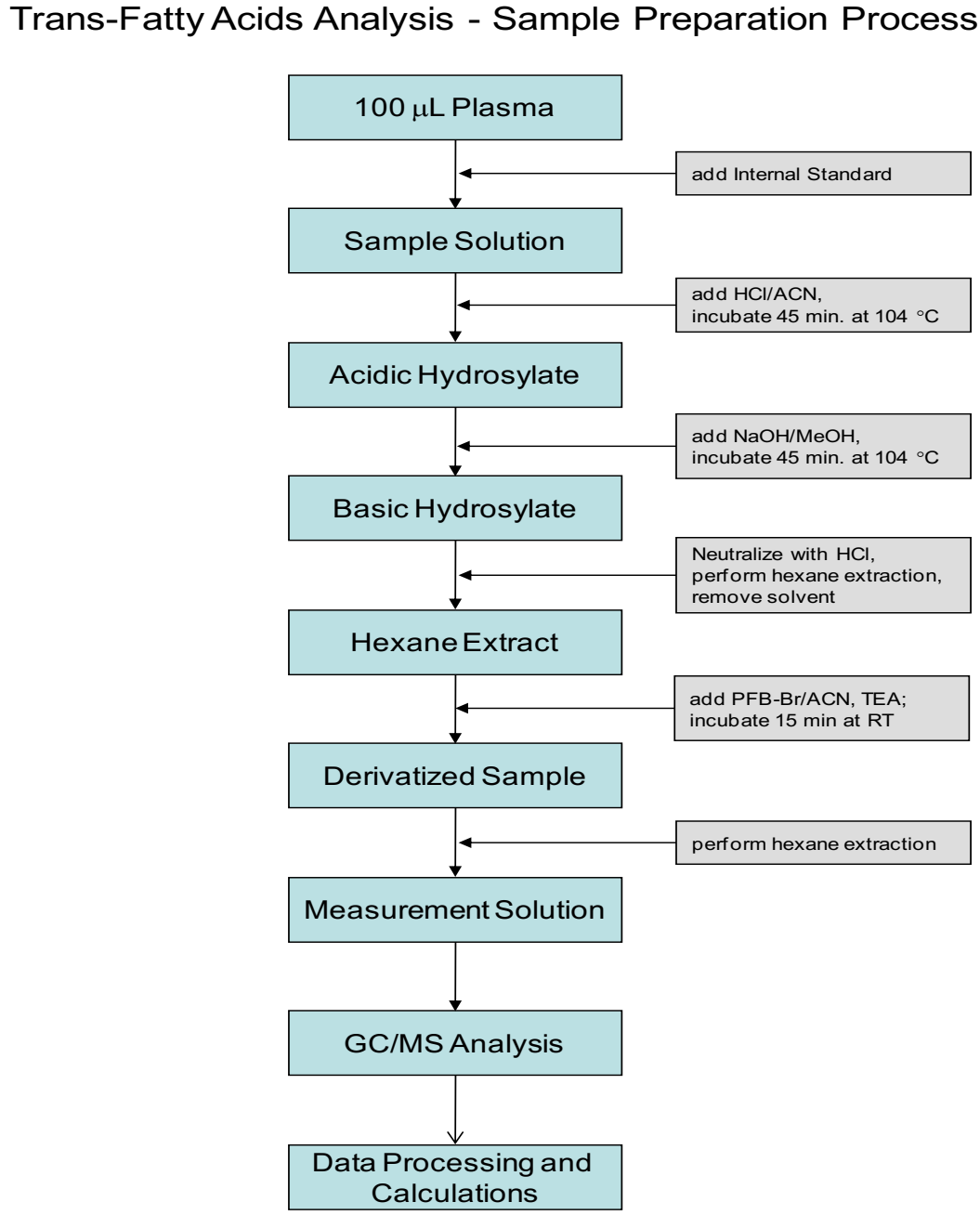
0.

Appendix 1. List of Fatty Acids Measured with this Measurement Procedure

Nr.	IUPAC name	Common Name	Short Hand	Analyte Code
1	trans 9-hexadecenoic acid	Palmitelaidic acid	C16:1n-7t	HDT*
2	trans-9-octadecenoic acid	Elaidic acid	C18:1n-9t	OD9*
3	trans-11-octadecenoic acid	Vaccenic acid	C18:1n -7t	OD1*
4	trans-9, trans-12-octadecadienoic acid	Linolelaidate acid	C18:2n- 6t, 9t	OTT*
5	tetradecanoic acid	Myristic acid	C14:0	MR1*
6	cis-9-tetradecenoic acid	Myristoleic acid	C14:1n-5c	ML1
7	hexadecanoic acid	Palmitic acid	C16:0	PM1*
8	cis-9-hexadecenoic acid	Palmitoleic acid	C16:1n-7c	PL1*
9	octadecanoic acid	Stearic acid	C18:0	ST1*
10	cis-9-octadecenoic acid	Oleic acid	C18:1n-9c	OL1*
11	cis-11-octadecenoic acid	*cis*-Vaccenic acid	C18:1n-7c	VC1*
12	cis-9, cis-12-octadecadienoic acid	Linoleic acid	C18:2n-6c,9c	LNA*
13	cis-9, cis-12, cis-15-octadecatrienoic acid	alpha-Linolenic acid	C18:3n-3c,6c,9c	ALN*
14	cis-6, cis-9, cis-12-octadecatrienoic acid	gamma-Linolenic acid	C18:3n-6c,9c,12c	GLA
15	eicosanoic acid	Arachidic acid	C20:0	AR1*
16	cis-8, cis-11, cis-14-eicosatrienoic acid	Dihomo-gamma-Linolenic acid	C20:3n-6c,9c,12c	HGL
17	cis-5, cis-8, cis-11, cis-14-eicosatetraenoic acid	Arachidonic acid	C20:4n-6c,9c,12c,15c	ARA*
18	cis-11-eicosenoic acid	Gondoic acid	C20:1n-9c	EN1
19	cis-11, cis-14-eicosadienoic acid	Eicosadienoic acid	C20:2n-6c,9c	ED1
20	cis-5, cis-8, cis-11, cis-14, cis-17-eicosapentaenoic acid	Eicosapentaenoic acid	C20:5n-3c,6c,9c,12c,15c	EPA*
21	docosanoic acid	Behenic acid	C22:0	DA1*
22	cis-4, cis-7, cis-10, cis-13, cis-16, cis-19-docosahexaenoic acid	Docosahexaenoic acid	C22:6n-3c,6c,9c,12c,15c,18c	DHA*
23	cis-7, cis-10, cis-13, cis-16-docosatetraenoic acid	Docosatetraenoic acid	C22:4n-6c,9c,12c,15c	DTA
24	cis-7, cis-10, cis-13, cis-16, cis-19-docosapentaenoic acid	Docosapentaenoic acid 3	C22:5n-3c,6c,9c,12c,15c	DP3
25	cis-4, cis-7, cis-10, cis-13, cis-16-docosapentaenoic acid	Docosapentaenoic acid 6	C22:5n-6c,9c,12c,15c,18c	DP6
26	tetracosanoic acid	Lignoceric acid	C24:0	LG1*
27	cis-15-tetracosenoic acid	Nervonic acid	C24:1n-9c	NR1

*For these compounds stable isotope labeled standards are available.

Appendix 2. Flow Chart Describing Sample Processing Performed for Fatty Acids Analysis



Appendix 3. Description of Standards Used

Standards used for creating calibrators

Nr.	Name	Analyte Code	Manufacturer	Purity	MW (g/mol)
1	trans 9-hexadecenoic acid	HDT	Nu-Chek-Prep, Elysian, MN	>99%	254.4
2	trans-9-octadecenoic acid	OD9	Nu-Chek-Prep, Elysian, MN	>99%	296.51
3	trans-11-octadecenoic acid	OD1	Nu-Chek-Prep, Elysian, MN	>99%	296.51
4	trans-9, trans-12-octadienoic acid	OTT	Nu-Chek-Prep, Elysian, MN	>99%	294.51
5	tetradecanoic acid	MR1	Nu-Chek-Prep, Elysian, MN	>99%	228.38
6	cis-9-tetradecenoic acid	ML1	Nu-Chek-Prep, Elysian, MN	>99%	226.38
7	hexadecanoic acid	PM1	Nu-Chek-Prep, Elysian, MN	>99%	256.43
8	cis-9-hexadecenoic acid	PL1	Nu-Chek-Prep, Elysian, MN	>99%	254.43
9	octadecanoic acid	ST1	Nu-Chek-Prep, Elysian, MN	>99%	284.48
10	cis-9-octadecenoic acid	OL1	Nu-Chek-Prep, Elysian, MN	>99%	282.48
11	cis-11-octadecenoic acid	VC1	Nu-Chek-Prep, Elysian, MN	>99%	282.48
12	cis-6-octadecenoic acid	OC6	Sigma-Aldrich, St. Louis, MO	99+%	296.51
13	cis-9, cis-12-octadecadienoic acid	LNA	Nu-Chek-Prep, Elysian, MN	>99%	280.48
14	cis-9, cis-12, cis-15-octadecatrienoic acid	ALN	Nu-Chek-Prep, Elysian, MN	>99%	278.48
15	cis-6, cis-9, cis-12-octadecatrienoic acid	GLA	Nu-Chek-Prep, Elysian, MN	>99%	278.48
16	eicosanoic acid	AR1	Nu-Chek-Prep, Elysian, MN	>99%	312.54
17	cis-8, cis-11, cis-14-eicosatrienoic acid	HGL	Nu-Chek-Prep, Elysian, MN	>99%	306.53
18	cis-5, cis-8, cis-11, cis-14-eicosatetraenoic acid	ARA	Nu-Chek-Prep, Elysian, MN	>99%	304.52
19	cis-11-eicosenoic acid	EN1	Nu-Chek-Prep, Elysian, MN	>99%	310.54
20	cis-11, cis-14-eicosadienoic acid	ED1	Nu-Chek-Prep, Elysian, MN	>99%	308.53
21	cis-5, cis-8, cis-11, cis-14, cis-17-eicosapentaenoic acid	EPA	Nu-Chek-Prep, Elysian, MN	>99%	302.52
22	docosanoic acid	DA1	Nu-Chek-Prep, Elysian, MN	>99%	340.59
23	cis-13-docosenoic acid	DE1	Nu-Chek-Prep, Elysian, MN	>99%	338.59
24	cis-4, cis-7, cis-10, cis-13, cis-16, cis-19-docosahexaenoic acid	DHA	Nu-Chek-Prep, Elysian, MN	>99%	328.57
25	cis-7, cis-10, cis-13, cis-16-docosatetraenoic acid	DTA	Nu-Chek-Prep, Elysian, MN	>99%	332.57
26	cis-7, cis-10, cis-13, cis-16, cis-19-docosapentaenoic acid	DP3	Nu-Chek-Prep, Elysian, MN	>99%	330.57
27	cis-4, cis-7, cis-10, cis-13, cis-16-docosapentaenoic acid	DP6	Nu-Chek-Prep, Elysian, MN	>99%	330.57
28	tetracosanoic acid	LG1	Nu-Chek-Prep, Elysian, MN	>99%	368.64
29	cis-15-tetracosenoic acid	NR1	Nu-Chek-Prep, Elysian, MN	>99%	366.63

* The chemicals described above or equivalent can be used in this measurement procedure.

Appendix 3 (continued).

Stable isotope-labeled standards used for internal standards

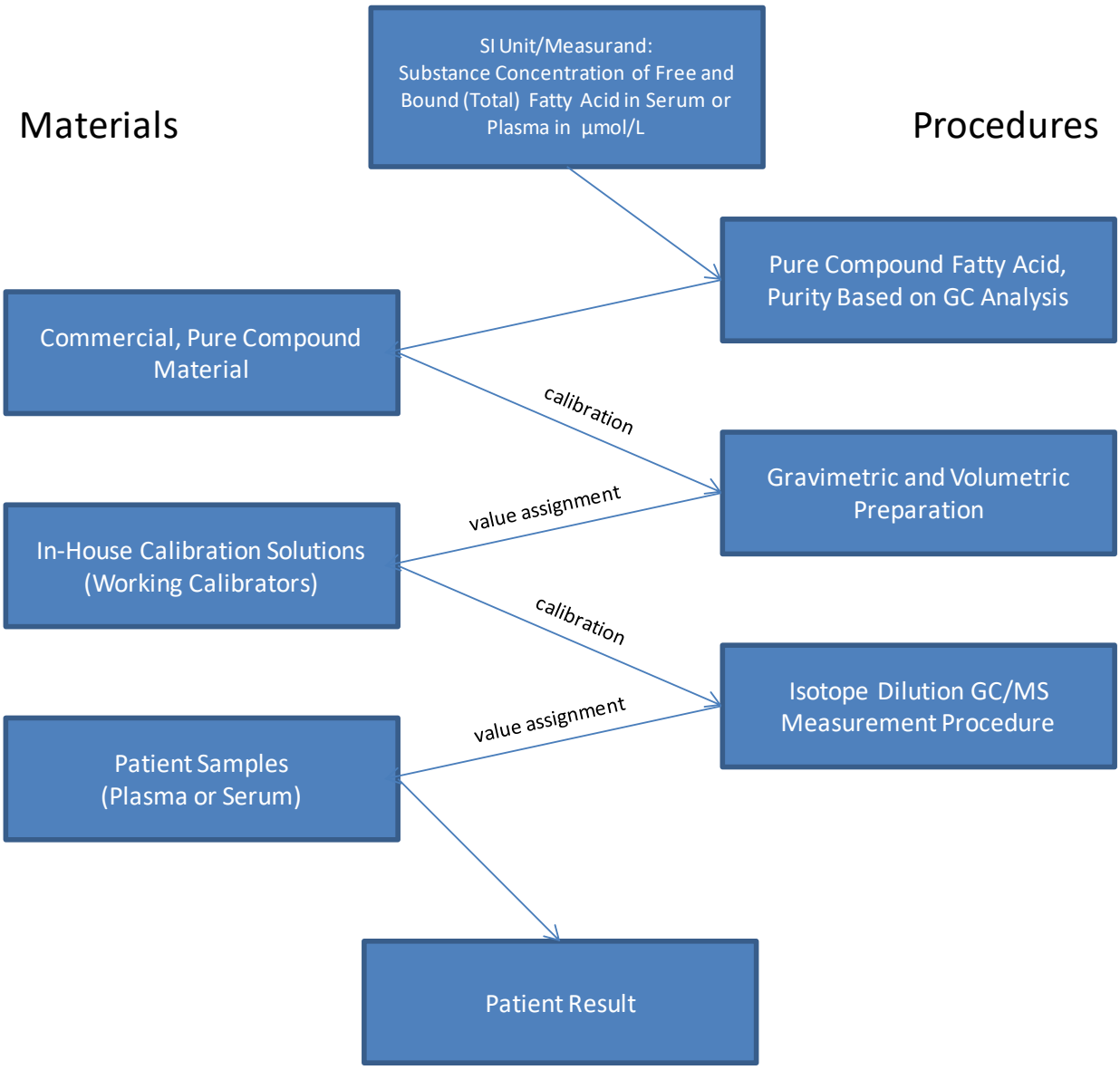
Name	Analyte Code	Manufacturer	Purity	MW (g/mol)
$^{13}C_5$-trans-9-hexadecenoic acid	HDT_IS	Sigma-Aldrich, St. Louis, MO	≥99%	259.37
$^{13}C_5$-trans-9-octadecenoic acid	OD9_IS	Sigma-Aldrich, St. Louis, MO	>99%	287.42
$^{13}C_5$-trans-11-octadecenoic acid	OD1_IS	Sigma-Aldrich, St. Louis, MO	≥99%	287.42
$^{13}C_5$-trans-9, trans-12-octadecadienoic acid	OTT_IS	Sigma-Aldrich, St. Louis, MO	≥98%	285.41
D_{27}-tetradecanoic acid	MR1_IS	Cambridge Isotopes Laboratories, Cambridge, MA	≥98%	255.54
$^{13}C_{16}$-hexadecanoic acid	PM1_IS	Sigma-Aldrich, St. Louis, MO	≥99%	272.31
$^{13}C_{16}$-cis-9-hexadecenoic acid	PL1_IS	Sigma-Aldrich, St. Louis, MO,	>99%	270.29
D_{35}-octadecanoic acid	ST1_IS	Cambridge Isotopes Laboratories, Cambridge, MA	≥98%	319.69
$^{13}C_{18}$-cis 9-octadecenoic acid	OL1_IS	IsoSciences, King of Prussia, PA	≥99%	300.27
$^{13}C_5$-cis-11-octadecenoic acid	VC1_IS	Sigma-Aldrich, St. Louis, MO	≥99%	287.42
$^{13}C_{18}$- cis-9, cis-12-octadecadienoic acid methyl ester	LNA_IS	IsoSciences, King of Prussia, PA	≥99%	298.31
D_{39}-eicosanoic acid methyl ester	AR1_IS	IsoSciences, King of Prussia, PA	≥98%	365.77
D_{14}-cis-9, cis-12, cis-15-octadecatrienoic acid	ALN_IS	Cayman Chemical, Ann Arbor, MI	>99%	292.5
D_{43}-docosanoic acid	DA1_IS	IsoSciences, King of Prussia, PA	≥99%	383.85
D_8- cis-5, cis-8, cis-11, cis-14-eicosatetraenoic acid	ARA_IS	Cayman Chemical, Ann Arbor, MI	≥96%	312.5
D_5- cis-5, cis-8, cis-11, cis-14, cis-17-eicosapentaenoic acid methyl ester	EPA_IS	IsoSciences, King of Prussia, PA	≥99%	321.52
D_{47}-tetracosanoic acid	LG1_IS	IsoSciences, King of Prussia, PA	≥99%	415.95
D_5- cis-4, cis-7, cis-10, cis-13, cis-16, cis-19-docosahexaenoic acid methyl ester	DHA_IS	IsoSciences, King of Prussia, PA	≥97%	347.56

Appendix3 (continued).

Target concentrations of the calibrator solutions

Nr.	Name	Analyte Code	Level 40 TFAC40 (µmol/L)	Level 35 TFAC35 (µmol/L)	Level 30 TFAC30 (µmol/L)	Level 20 TFAC20 (µmol/L)	Level 10 TFAC10 (µmol/L)
1	trans 9-hexadecenoic acid	HDT	25	12.5	6.25	2.5	1
2	trans-9-octadecenoic acid	OD9	125	62.5	31.3	12.5	5
3	trans-11-octadecenoic acid	OD1	125	62.5	31.3	12.5	5
4	trans-9, trans-12-octadienoic acid	OTT	8	4	2	0.8	0.32
5	tetradecanoic acid	MR1	600	300	150	60	24
6	cis-9-tetradecenoic acid	ML1	100	50	25	10	4
7	hexadecanoic acid	PM1	8,000	4,000	2,000	800	320
8	cis-9-hexadecenoic acid	PL1	1,200	600	300	120	48
9	octadecanoic acid	ST1	2,000	1,000	500	200	80
10	cis-9-octadecenoic acid	OL1	6,000	3,000	1,500	600	240
11	cis-11-octadecenoic acid	VC1	800	400	200	80	32
12	cis-6-octadecenoic acid	OC6	250	125	62.5	25	10
13	cis-9, cis-12-octadecadienoic acid	LNA	8,000	4,000	2,000	800	320
14	cis-9, cis-12, cis-15-octadecatrienoic acid	ALN	400	200	100	40	16
15	cis-6, cis-9, cis-12-octadecatrienoic acid	GLA	200	100	50	20	8
16	eicosanoic acid	AR1	200	100	50	20	8
17	cis-8, cis-11, cis-14-eicosatrienoic acid	HGL	250	125	62.5	25	10
18	cis-5, cis-8, cis-11, cis-14-eicosatetraenoic acid	ARA	2,000	1,000	500	200	80
19	cis-11-eicosenoic acid	EN1	50	25	12.5	5	2
20	cis-11, cis-14-eicosadienoic acid	ED1	50	25	12.5	5	2
21	cis-5, cis-8, cis-11, cis-14, cis-17-eicosapentaenoic acid	EPA	800	400	200	80	32
22	docosanoic acid	DA1	200	100	50	20	8
23	cis-13-docosenoic acid	DE1	50	25	12.5	5	2
24	cis-4, cis-7, cis-10, cis-13, cis-16, cis-19-docosahexaenoic acid	DHA	1,000	500	250	100	40
25	cis-7, cis-10, cis-13, cis-16-docosatetraenoic acid	DTA	100	50	25	10	4
26	cis-7, cis-10, cis-13, cis-16, cis-19-docosapentaenoic acid	DP3	200	100	50	20	8
27	cis-4, cis-7, cis-10, cis-13, cis-16-docosapentaenoic acid	DP6	100	50	25	10	4
28	tetracosanoic acid	LG1	200	100	50	20	8
29	cis-15-tetracosenoic acid	NR1	200	100	50	20	8

Appendix 4. Metrological Traceability of Trans Fatty Acids Measurements



Appendix 5. Example of Analytical Sequence

Injection order	Sample Type	Vial #	Sample ID
1	Instrument Check Std	49	RT STD
2	Calibrator	4	TFAC20L06
3	Calibrator	5	TFAC10L06
4	Quality Control	8	+021208PA
5	Sample	13	QCS9019PL
6	Sample	14	QCS9029PL
7	Sample	15	QCS9030PL
8	Sample	16	QCS9031PL
9	Sample	17	QCS9033PL
10	Sample	18	QCS9034PL
11	Sample	19	QCS9035PL
12	Sample	20	QCS9036PL
13	Sample	21	QCS9063PL
14	Calibrator	1	TFAC40L06
15	Calibrator	6	TFAC00L06
16	Quality Control	7	+031209PA
17	Sample	22	QCS9064PL
18	Sample	23	QCS9065PL
19	Sample	24	QCS9068PL
20	Sample	25	QCS9069PL
21	Sample	26	QCS9073PL
22	Sample	27	QCS9074PL
23	Sample	28	QCS9075PL
24	Sample	29	QCS9079PL
25	Sample	30	QCS9080PL
26	Sample	31	QCS9081PL
27	Calibrator	3	TFAC30L06
28	Quality Control	9	+011207PA
29	Quality Control	10	+031209PA
30	Sample	32	QCS9082PL
31	Sample	33	QCS9084PL
32	Sample	34	QCS9085PL
33	Sample	35	QCS9086PL
34	Sample	36	QCS9087PL
35	Sample	37	QCS9088PL

36	Sample	38	QCS9089PL
37	Sample	39	QCS9090PL
38	Sample	40	QCS9091PL
39	Sample	41	QCS9092PL
40	Calibrator	2	TFAC35L06
41	Quality Control	11	+021208PA
42	Quality Control	12	+011207PA
43	Sample	42	QCS9094PL
44	Sample	43	QCS9095PL
45	Sample	44	QCS9096PL
46	Sample	45	QCS9097PL
47	Sample	46	QCS9098PL
48	Sample	47	QCS9100PL
49	Sample	48	QCS9101PL

Appendix 6. GC-MS Settings (Instrument Control Parameters)

Parameters listed as documented by Mass Hunter software:

Sample Inlet:	GC
Mass Spectrometer:	Enabled
Injection Location:	Front
GC Oven Temperature	
Setpoint	On
(Initial)	50 °C
Hold Time	0 min
Post Run	130 °C
Program	
#1 Rate	40 °C/min
#1 Value	160 °C
#1 Hold Time	10 min
#2 Rate	1 °C/min
#2 Value	175 °C
#2 Hold Time	0 min
#3 Rate	0.5 °C/min
#3 Value	210 °C
#3 Hold Time	0 min
#4 Rate	35 °C/min
#4 Value	260 °C
#4 Hold Time	20 min
Equilibration Time	0.25 min
Max Temperature	290 °C
Maximum Temperature Override	Disabled
Slow Fan	Disabled
Cryo	Off
Front SS Inlet H2	
Mode	Split
Heater	On 240 °C
Pressure	On 40.264 psi
Total Flow	On 245 mL/min
Septum Purge Flow	On 3 mL/min
Gas Saver	On 20 mL/min after 2 min
Split Ratio	100 :1
Split Flow	240 mL/min

Thermal Aux 2 (MSD Transfer Line) Temperature	
Setpoint	On
(Initial)	260 °C
Post Run	0 °C
Column	
Column #1	
Flow	
Setpoint	Off
(Initial)	2 mL/min
Post Run	0.57353 mL/min
Agilent Varian CP7421	
FAME SELECT 200 m x 250 µm x 0.25 µm	
In	
Front SS Inlet H2	
Out	MSD
(Initial)	50 °C
Pressure	40.264 psi
Flow	2 mL/min
Average Velocity	29.639 cm/sec
Holdup Time	11.247 min
Column Outlet Pressure	0 psi
GERSTEL MAESTRO SYSTEM SETTINGS	
Maestro Runtime	119.18 min
GC Cool Down Time	10.00 min
GERSTEL MPS Liquid Injection	
Syringe	10ul
SAMPLE PARAMETERS	
Sandwich	not used
Inj. Volume	1.0 uL
Air Volume below	0.0 uL
Inj. Speed	50.00 uL/s
Fill Volume	1.0 uL
Fill Strokes	3
Fill Speed	1.00 uL/s
Viscositiy Delay	0 s
Eject Speed	50.00 uL/s

Pre Inj. Delay	0 s
Post Inj. Delay	0 s
Inj. Penetration mm	40.00
Sample Tray Type	VT54
Vial Penetration mm	35.00

CLEANING PARAMETERS
Preclean Sample : 1

Wash Station 1	Wash1
Preclean Solv.1	0
Postclean Solv.1	8
Fill Speed Solv.1	5.00 uL/s
Viscosity Delay Solv.1	0 s
Eject Speed Solv.1	50.00 uL/s
Information Solv.1	acetone
Wash Station 2	Wash2
Preclean Solv.2	2
Postclean Solv.2	8
Fill Speed Solv.2	5.00 uL/s
Viscosity Delay Solv.2	0 s
Eject Speed Solv.2	50.00 uL/s
Information Solv.2	hexane

MS Information

Acquisition Mode	SIM
Solvent Delay (minutes)	20
EM Setting mode Delta	200
Number of SIM Groups	12

[SIM Parameters]

Group 1	Group ID	1
Resolution		1
Group Start Time		20
Number of Ions		2
Ions		
Dwell In Group (Mass, Dwell):		(171.30, 200)
		(199.30, 200)
Group 2	Group ID	2
Resolution		1
Group Start Time		51
Number of Ions		3
Ions		
Dwell In Group (Mass, Dwell):		(225.20, 125)
		(227.20, 125)
		(254.40, 125)
Group 3	Group ID	3
Resolution		1
Group Start Time		70
Number of Ions		2
Ions		
Dwell In Group (Mass, Dwell):		(255.30, 200)
		(271.30, 200)
Group 4	Group ID	4
Resolution		1
Group Start Time		77.5
Number of Ions		3
Ions		
Dwell In Group (Mass, Dwell):		(253.20, 125)
		(258.40, 125)
		(269.30, 125)
Group 5	Group ID	5
Resolution		1
Group Start Time		84
Number of Ions		2
Ions		
Dwell In Group (Mass, Dwell):		(283.30, 200)
		(318.50, 200)
Group 6	Group ID	6
Resolution		1
Group Start Time		94.5
Number of Ions		3
Ions		
Dwell In Group (Mass, Dwell):		(281.30, 125)
		(286.40, 125)
		(299.30, 125)
Group 7	Group ID	7
Resolution		1
Group Start Time		98.7
Number of Ions		2
Ions		
Dwell In Group (Mass, Dwell):		(279.30, 200)
		(284.40, 200)
Group 8	Group ID	8
Resolution		1
Group Start Time		99.5

Number of Ions 2
Ions
Dwell In Group (Mass, Dwell): (279.30, 200)
(297.30, 200)
Dwell In Group (Mass, Dwell): (327.30, 80)
(329.30, 80)
(331.30, 80)
(332.30, 80)
(365.40, 80)

MS Source : 230 C maximum 300 C
MS Quad : 150 C maximum 200 C

Group 9 Group ID 9
Resolution 1
Group Start Time 100.8
Number of Ions 5
Ions
Dwell In Group (Mass, Dwell): (277.10, 80)
(291.50, 80)
(309.30, 80)
(311.30, 80)
(350.70, 80)

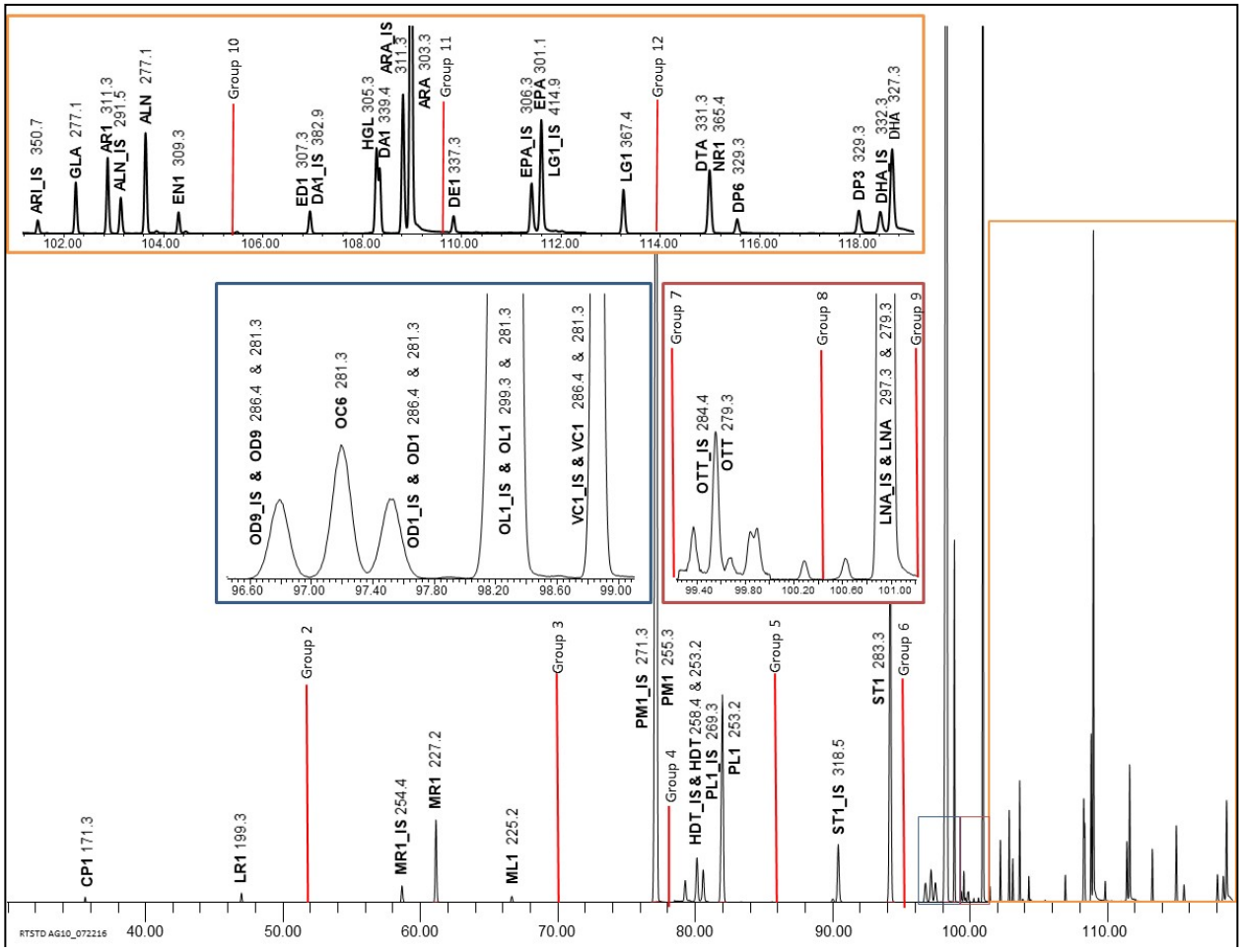
Group 10 Group ID 10
Resolution 1
Group Start Time 104.5
Number of Ions 6
Ions
Dwell In Group (Mass, Dwell): (303.30, 65)
(305.30, 65)
(307.30, 65)
(311.30, 65)
(339.40, 65)
(382.85, 65)

Group 11 Group ID 11
Resolution 1
Group Start Time 109.1
Number of Ions 5
Ions
Dwell In Group (Mass, Dwell): (301.10, 80)
(306.30, 80)
(337.30, 80)
(367.40, 80)
(414.95, 80)

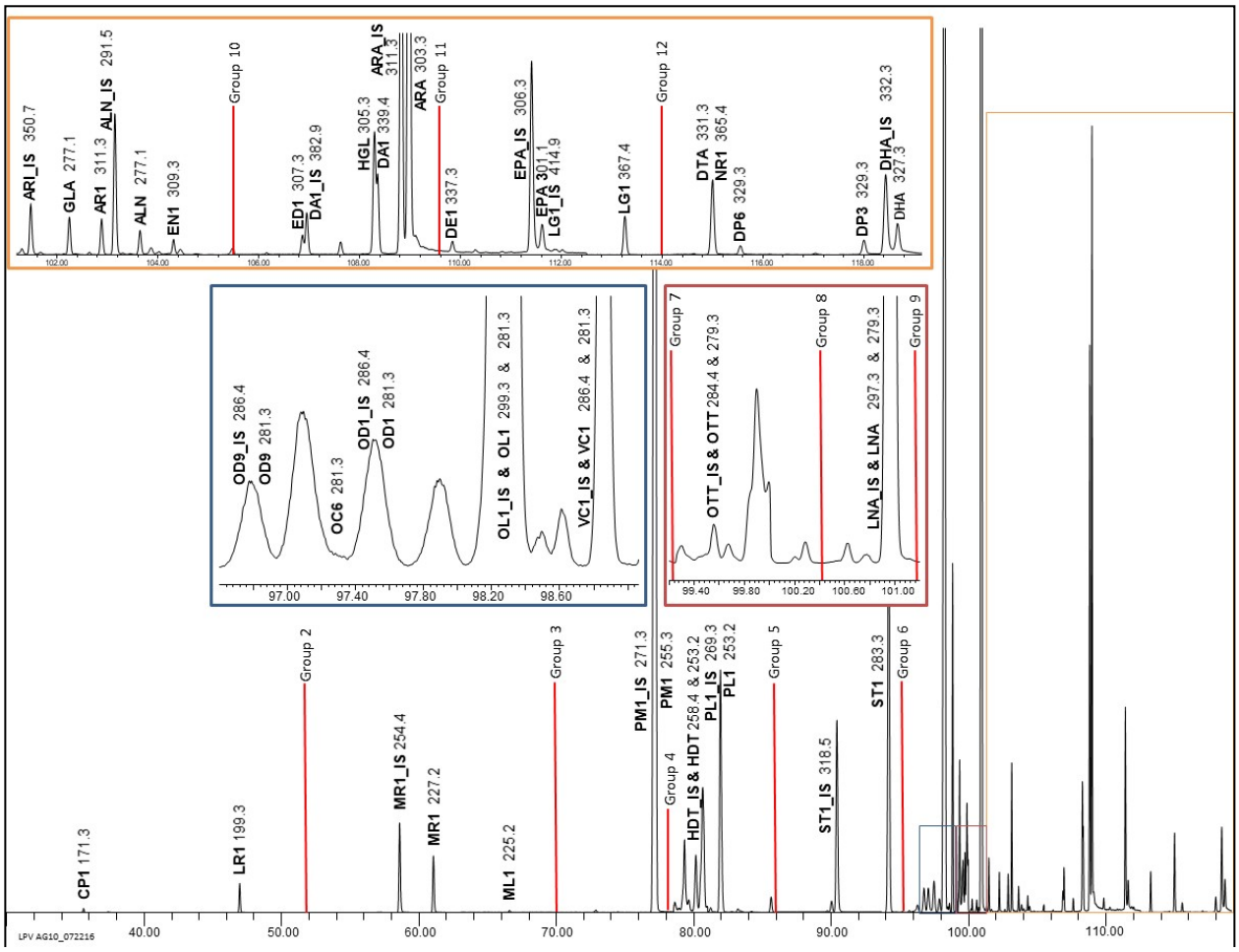
Group 12 Group ID 12
Resolution 1
Group Start Time 113.5
Number of Ions 5
Ions

Appendix 7. Retention Standard And Low QC Sample Chromatograms

Retention Standard Chromatogram



Low QC Chromatogram



Appendix 8. List of Compounds Tested for Interference

Number	Analyte	Short Hand	m/z
1	2-hydroxy-decanoic acid		187.3
2	undecanoic acid	C11:0	185.9
3	dodecanoic acid	C12:0	199.3
4	tridecanoic acid	C13:0	213.4
5	12-tridecenoic acid	C13:1n-1	211.4
6	12-methyl-tetradecanoic acid		241.4
7	13-methyl-tetradecanoic acid		241.4
8	pentadecanoic acid	C15:0	241.4
9	trans-10-pentadecenoic acid	C15:1n-5t	239.4
10	14-methyl-pentadecanoic acid		255.4
11	cis-10-pentadecenoic acid	C15:1n-5c	239.4
12	14-pentadecenoic acid	C15:1n-1	239.4
13	9R,10S-methylene-hexadecanoic acid		267.4
14	15-methyl-hexadecanoic acid		269.5
15	2-hydroxy-hexadecanoic acid		271.4
16	heptadecanoic acid	C17:0	269.5
17	trans-10-heptadecenoic acid	C17:1n-7t	267.5
18	cis-9, cis-12-hexadecadienoic acid	C16:2n-4c,7c	251.4
19	cis-10-heptadecenoic acid	C17:1n-7c	267.5
20	9S,10R-methylene-octadecanoic acid		295.5
21	trans-6-octadecenoic acid	C18:1n-12t	281.5
22	2-hydroxy-dodecanoic acid		215.3
23	3-hydroxy-dodecanoic acid		215.3
24	cis-6, cis-9, cis-12, 15-hexadecatetraenoic acid	C16:4n-1,4c,7c,10c	247.4
25	nonadecanoic acid	C19:0	297.5
26	cis-9, trans-12-octadecadienoic acid	C18:2n-6t,9c	279.5
27	cis-11, cis-14-octadecadienoic acid	C18:2n-4c,7c	279.5
28	trans-9, cis-12-octadecadienoic acid	C18:2n-6c,9t	279.5
29	trans-7-nonadecenoic acid	C19:1n-12t	295.5
30	trans-10-nonadecenoic acid	C19:1n-9t	295.5
31	2-hydroxy-tetradecanoic acid		243.4
32	cis-7-nonadecenoic acid	C19:1n-12c	295.5
33	trans-9, trans-12, trans-15-octadecatrienoic acid	C18:3n-3t,6t,9t	277.5
34	cis-10-nonadecenoic acid	C19:1n-9c	295.5
35	cis-9, trans-12, trans-15-octadecatrienoic acid	C18:3n-3t,6t,9c	277.5
36	trans-9, cis-12, trans-15-octadecatrienoic acid	C18:3n-3t,6c,9t	277.5
37	trans-9, trans-12, cis-15-octadecatrienoic acid	C18:3n-3c,6t,9t	277.5
38	cis-9, cis-12, trans-15-octadecatrienoic acid	C18:3n-3t,6c,9c	277.5
39	cis-9, trans-12, cis-15-octadecatrienoic acid	C18:3n-3c,6t,9c	277.5
40	cis-9, cis-11, cis-14-octadecatrienoic acid	C18:3n-4c,7c,9c	277.5
41	trans-9, cis-12, cis-15-octadecatrienoic acid	C18:3n-3c,6c,9t	277.5
42	cis-5-eicosenoic acid	C20:1n-15c	309.5
43	cis-10, cis-13-nonadecadienoic acid	C19:2n-6c,9c	293.5
44	trans-11-eicosenoic acid	C20:1n-9t	309.5
45	cis-8-eicosenoic acid	C20:1n-12c	309.5
46	cis-6, cis-9, cis-12, cis-15-octadecatetraenoic acid	C18:4n-3c,6c,9c,12c	275.5
47	3-hydroxy-tetradecanoic acid		243.4
48	12-hydroxy-cis-9-octadecenoic acid		297.5
49	12-hydroxy-trans-9-octadecenoic acid		297.5
50	cis-12-heneicosenoic acid	C21:1n-9c	323.6
51	cis-11, cis-14, cis-17-eicosatrienoic acid	C20:3n-3c,6c,9c	305.5
52	trans-13-docosenoic acid	C22:1n-9t	337.6
53	cis-12, cis-15-heneicosadienoic acid	C21:2n-6c,9c	321.6
54	cis-11-docosenoic acid	C22:1n-11	337.6
55	cis-13-docosenoic acid	C22:1n-9c	337.4
56	tricosanoic acid	C23:0	353.6
57	cis-13, cis-16-docosadienoic acid	C22:2n-6c,9c	335.6
58	cis-14-tricosenoic acid	C23:1n-9c	351.6
59	cis-13, cis-16, cis-19-docosatrienoic acid	C22:3n-3c,6c,9c	333.6
60	pentacosanoic acid	C25:0	381.7
61	hexacosanoic acid	C26:0	395.7
62	octacosanoic acid	C28:0	423.7
63	nonacosanoic acid	C29:0	437.8

Appendix 9. Symbols, Abbreviations, Terminology

Abbreviations

ACS	American Chemical Society
ASTM	American Society for Testing and Material
BP	Boiling Point
CDC	Centers for Disease Control and Prevention
CC	Calibrators
CI	Chemical Ionization
CLIA	Clinical Laboratory Improvement Act/Amendment
CLSI	Clinical and Laboratory Standards Institute
CV	Coefficient of Variant
EMV	Electron Multiplier Voltage
EDTA	Ethylenediaminetetraacetic Acid
FDA	Food and Drug Administration
GC-NCI-MS	Gas Chromatography-Negative Chemical Ionization-Mass Spectrometry
HCl	Hydrochloric Acid
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
ID	Identification
IS	Internal Standards
ISO	International Organization for Standardization
LDL	Low-density Lipoprotein
MSD	Mass Selective Detector
MSDS	Material Safety Data Sheets
NaOH	Sodium Hydroxide
NCEH	National Center of Environmental Health
NCEP	National Cholesterol Education Program
OHS	Occupational Health and Safety
PFB-Br	Pentafluorobenzyl Bromide
PT	Proficiency Testing/External Quality Assurance Testing
QA	Quality Assurance
QC	Quality Control
SAS	Statistical Analysis Software
SD	Standard Deviation
SIM	Single Ion Monitoring
SAS	Statistical Analysis System
tFA	Trans Fatty Acid

Terminology

The terminology defined in CLIA ’88 (57 FR 7139 Subpart A Sec Sec. 493.2) is used in this document. Otherwise the terminology described in the Clinical and Laboratory Standards Institute’s terminology database was used. The database can be accessed at: (http://www.clsi.org/Content/NavigationMenu/Resources/HarmonizedTerminologyDatabase/Harmonized_Terminolo.htm)