Laboratory Procedure Manual

Analyte: *trans* Fatty Acids

trans-9-hexadecenoic acid (C16:1n-7t)
trans-9-octadecenoic acid (C18:1n-9t)
trans-11-octadecenoic acid (C18:1n-7t)
trans-9, _trans_-12-octadecadienoic acid (C18:2n-6t,9t)

Matrix: Plasma

Method: Analysis of total trans fatty acids in plasma and serum by GC/MS

As performed by:

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Important Information for Users

CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical

method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for testing items in the following table:

Data File Name	Variable Name	SAS Label
TFA_F	LBXHDT	*trans*-9-hexadecenoic acid (µmol/L)
	LBXOD9	*trans*-9-octadecenoic acid(µmol/L)
	LBXOD1	*trans-11*-octadecenoic acid(µmol/L)
	LBXOTT	*trans*-9,12-octadecadienoicacid(µmol/L)

1 SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

1.1 Clinical and Public Health Relevance

Trans fatty acids are unsaturated fatty acids that contain at least one double bond in the *trans* configuration. The three-dimensional structure of *trans* fatty acids is more similar to saturated fatty acids than to regular unsaturated fatty acids, which have their double bond in c*is* configuration. The *trans* configuration substantially alters the physical properties of the fatty acids and thus the properties of the oil containing these trans fatty acids 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 *trans* fatty acid intake and total as well as LDL cholesterol concentration was established, which links elevated *trans* fatty acids in blood with increased risk of coronary heart diseases. Therefore, the Institute of Medicine recommended that '*trans* fatty acid consumption be as low as possible while consuming a nutritionally adequate diet' [2]. Likewise, the conclusions in two other scientific reports, namely the Dietary Guidelines for Americans, 2000 [3] and guidelines from the National Cholesterol Education Program (NCEP) [4], were similar with recommendations to limit *trans* fat intake in the diet. In 2003, FDA amended its regulations on nutrition labeling to require that trans fatty acids be declared in the nutrition label of conventional foods and dietary supplements. This rule is intended to provide information to assist consumers in maintaining healthy dietary practices [5].

Estimating the extent of *trans* fatty acids 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 *trans* fatty acid 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. *Trans* fatty acid intake is not currently collected in U.S. national surveys [2]. 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 *trans* fatty acids.

Over 10 different trans fatty acids 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 eladidic acid (C18:1n-9t) is one major *trans* fatty acid while in dairy fats vaccenic acid (C18:1n-7t) is clearly the major isomer [6]. Another isomer reported in dairy fat and partially hydrogenated vegetable oils is palmitelaidic acid (C16:1n-7t) [7].

1.2 Test Principle

This measurement procedure determines the total (free and esterified) content of selected *trans* fatty acids in plasma and provides results in concentration units as well as percent units (*trans* fatty acids 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 pentafluorobenzylbromide (PFB-Br) as described by Stellard et al [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 ion formed in the ion source. Retention times are compared against those obtained with known standards. Quantitation is performed with standard solution using stable isotope-labeled fatty acids as internal standards.

To calculate *trans* fatty acids as percent of total fatty acids, 29 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 *trans* fatty acids: *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):

- Preparation of the samples for analysis
- Acidic and alkaline hydrolysis of the samples
- Isolation of the free fatty acids by liquid-liquid extraction
- Derivatization of fatty acids
- Analysis of derivatized fatty acids by GC/MS
- 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.

1.3 Scope

The measurement procedure described in this document is intended for quantitatively measuring the fatty acids described above 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 operation is addressed in the manufacturers' manuals and in work instructions created and maintained by the Protein Biomarker Laboratory. 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. 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 containers in the designated Base cabinet.

Organic solvents: Always wear gloves, safety glasses and a lab coat when handling organic solvents. Handle only in well-ventilated areas or as required under a chemical fume hood. Store containers in the designated flammable cabinet.

Acetonitirile: 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. Refer to the Hydrogen Safety section of the Chemical Hygiene Plan located in the trans-Fatty Acids area of the laboratory

2.3 Radioactive Hazards

There are no radioactive hazards associated with this measurement procedure.

2.4 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.

2.5 Waste Disposal

All 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 broken glass etc. must be placed into sharps containers as appropriate.

All waste disposal must be performed in compliance with CDC policies and regulations. The CDC Safety Policies and Practices Manual are located in the laboratory.
Analysts performing this measurement procedure must successfully complete

- Safety Ccourses (CDC-OHS Safety Survival Skills Parts 1 and 2, Bloodborne Pathogens courses)
- CDC-OHS Hazardous Chemical Waste Management Course
- Computer Security Awareness Course
- Records Management Training

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

Analysts must be familiar with:

- DLS Safety Manual
- Exposure Control Plan
- Chemical Hygiene Plan
- DLS Policies and Procedures Manual
- Relevant Material Safety Data Sheets
- DLS After-Hours Work Policy
- Policy on Confidentiality, Data Security and Release of Information

3 COMPUTERIZATION; 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 D.03.00.552) and Hamilton Starlet pipette (using Microlab Vector Software version 4.11.5878). 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 DLS. Assessment of bench QC results is performed using a program created with SAS software and maintained by the DLS.

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 dedicated laboratory staff.

3.2 Sample Information

All samples must be labeled as described in the latest version of the DLS Policies and Procedures Manual. No personal identifiers are used, and all samples are referenced to a blind coded sample identifier. For sample specimen handling procedure, see 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. The database is maintained by DLS staff and is routinely backed up by CDC Information Technology Services Office (ITSO). ITSO must be contacted for emergency assistance.

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 *trans* fatty acid analysis may be fresh or frozen EDTA plasma. A minimum of 150 µL plasma is needed; a 0.5-mL sample of plasma is preferable to allow for repeat analyses. A sample volume of 100 µL is required for analysis. Additional 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 in accordance to CDC and DLS policies and regulations.

Other specimen handling conditions are outlined in the Policies and Procedures Manual of the Division of Laboratory Sciences (DLS). Copies are available in the Protein Biomarker Laboratory.

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 ºC. 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.

5.1.1 Equipment, Chemicals and Consumables Used for Reagent Preparation

1.Barnstead Rotator with flat surface rotor (Lab-Line, Melrose Park, IL)
2.Vortex T-Genie 2 (Scientific Industries Inc., Bohemia, NY)
3.Kimble 5 mL disposable glass pipettes (Fisher Scientific, Suwanee, GA)
4.Various glass beakers (25 mL, 50 mL, 100 mL) (Fisher Scientific, Suwanee, GA)
5.100 mL graduated cylinders (Fisher Scientific, Suwanee, GA)
6.Capped 250 mL bottles, class A glassware (Fisher Scientific, Suwanee, GA)
7.Sodium Hydroxide, 10N solution, Certified ACS/ASTM (Fisher Scientific, Suwanee, GA)
8. Acetonitrile, HPLC Grade Reagent (Fisher Scientific, Suwanee, GA)
9.Hydrochloric Acid, 6N Solution, Certified (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.GeneVac EZ-2 Evaporation System with side bridge holders and universal rotor (GeneVac Inc., Valley Cottage, NY)
3.Hamilton Microlab STARLet Liquid Handler (using Microlab Vector Software version 4.11.5878 (Hamilton Company, Reno, NV)
4.Isotemp Mechanical Oven, 2.5 cu. ft., 120 V, temperature range from 30 ^{o}C to 275 ^{o}C, Accuracy ±3 ^{o}C (Fisher Scientific, Suwanee, GA)
5.Ultrasonic Cleaning Bath 750HT (VWR Scientific Products, Suwanee, GA)
6. Eppendorf Centrifuge 5810 R V4.2 with A-4-62 rotor (GMI, Ramsey, MN)
7.Hand Held Scanner (Symbol Technologies Inc., Bohemia, NY)
8. Analytical Balance AX 205, with printer (Mettler-Toledo, Columbus, OH)
9. Sato Label Maker CL612e and Label Making Software (Sato America, Charlotte, NC)

10. Boekel Orbitron Platform Rotator I, 115 VAC with 23 º fixed tilt angle and speed of 20 orbits/min (Boekel Scientific, Feasterville, PA)Gilson Positive Displacement Pipettes (Gilson, Inc., Middleton,WI)
    a. Gilson Microman M100 (10-100 µL)
    b. Gilson Microman M1000 (100-1000 µL)
11. Gilson Pipette Tips (Gilson Inc., Middleton, WI)
    a. Pipette tips 10 µL
    b. Pipette tips 100 µL
    c. Pipette tips 1000 µL
12. Rainin Pipettes (Rainin, Woburn, MA)
    a. Rainin SL1000 (100-1000µL)
    b. Rainin M100 (10-100 µL)
    c. Rainin AutoRep M Repeater Pipette
13. Rainin Pipette Tips (Rainin, Woburn, MA)
    a. Pipette tips, 10 µL
    b. Pipette tips, 100 µL
    c. Pipette tips, 1000 µL
14. Repeater Pipette Adapter (Fisher Scientific, Suwanee, GA)
15. Eppendorf Combitips (Eppendorf, Hauppauge, NY)
    a. Eppendorf Combitips, 5 mL
    b. Eppendorf Combitps, 50 mL
16. Conductive 300 µL Filter Tips for Hamilton (Hamilton Company, Reno, NV)
17. Conductive 1000 µL Filter Tips for Hamilton (Hamilton Company, Reno, NV)
18. Nalgene 2 mL cryovials with external thread (Fisher Scientific, Suwanee, GA)
19. Pyrex disposable glass culture tubes, (threaded, 11.5 mL, 16x100 mm (Corning Inc., Acton, MA)
20. Pyrex disposable glass culture tubes (extraction vials, rimless,11.5 mL,16x100 mm) (Corning Inc., Acton, MA)
21. Kimble black phenolic screw caps, PTFE-faced rubber liner, (Kimble Chase, Vineland, NJ)
22. Disposable glass Pyrex Pasteur pipettes, 5 3/4 inch (Corning Inc., Acton, MA)
23. Pasteur pipette bulbs (Fisher Scientific, Suwanee, GA)
24. Kimble 5 mL disposable glass pipettes (Kimble Chase, Vineland, NJ)
25. Acetonitrile, HPLC Grade Reagent (Fisher Scientific, Suwanee, GA)
26. Methanol, 99.8+% A.C.S. (Fisher Scientific, Suwanee, GA)
27. Pentafluorobenzyl Bromide (PFB-Br) (Fisher Scientific, Suwanee, GA)
28. Triethylamine (TEA) 99.7%, extra pure (Acros Organics, Morris Plains, NJ)
29. Hexane, Reagent Plus $\geq$ 99% (Sigma-Aldrich, St. Louis, MO)
30. 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 NGC 6890N Gas chromatograph and 5975B Mass selective detector for EI, PCI and NCI (Agilent Technologies, Wilmington, DE)
2.Data Processing Software: Agilent MSD ChemStation version D.03.00.552 (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 (Gerstel Inc., Linthicum, MD)
5.Hydrogen Generator Outlet, Flowrate, 500mL/min.; Purity, 99.9999%; Pressure, 10-100 psi; (Fisher Scientific, Suwanee, GA)
6.Desiccant Cartridge for Hydrogen Generator (Fisher Scientific, Suwanee, GA)
7.Chemical Ionization Gas Purifier (Agilent Technologies, Wilmington, DE)
8.Non-stick Fluorocarbon Liner Viton O-ring (Agilent Technologies, Wilmington, DE)
9.Splitless Liner, Single Taper, no glasswool deactivated (Agilent Technologies, Wilmington, DE)
10. Fixed Tapered Needle Syringe 10 µL (Agilent Technologies, Wilmington, DE)
11. Big Universal Trap 1/8" Fittings, Hydrogen (Agilent Technologies, Wilmington, DE)
12. Chemical Ionization Gas Regulator (Agilent Technologies, Wilmington, DE)
13. Advanced Green Non-stick 11mm Septa (Agilent Technologies, Wilmington, DE)
14. Varian Capillary Column CP 7421 Select FAME 200 m x 250 µm x 0.25 µm (length, inner diameter, film thickness) (Varian Inc. Palo Alto, CA)
15. Methanol, 99.8+% A.C.S. (Fisher Scientific, Suwanee, GA)
16. Hexane, Reagent Plus $\geq$ 99% (Fisher Scientific, Suwanee, GA)
17. GC vials 2-mL, Footed, Amber Glass (Fisher Scientific, Suwanee, GA)
18. Caps with septa, blue PTFE/Silicone/PTFE (Fisher Scientific, Suwanee, GA)
19. Toluene, Certified ACS/ASTM (Fisher Scientific, Suwanee, GA)
20. Deionized water with resistance to at least 18 megaOhm-cm
21. 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 1.2. Prepare 110 mL of this solution by transferring 11 mL of 6N hydrochloric acid to a 200 mL graduated cylinder and adding acetonitrile up to the 110 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. The instructions and safety information for preparing this solution can be found in the Work Instruction PBLW040003.

5.2.2 Preparation of 10% 10N NaOH in Methanol

This solution is needed for the alkaline hydrolysis step as described in section 1.2. Prepare 110 mL of this solution by transferring 11mL of 10N sodium hydroxide to a 200 mL graduated cylinder and adding methanol up to the 110 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. The instructions and safety information for preparing this solution can be found in the Work Instruction PBLW040003.

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

This solution is needed to derivatize the fatty acids for GCMS analysis as described in section 1.1. Using a positive displacement pipette add 376 µL PFB-Br to 5mL 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. The instructions and safety information for preparing this solution can be found in the Work Instruction PBLW040004.

5.3 Calibration Materials

5.3.1 Preparation of Calibrator Solutions

The standards described in Appendix 3 are used to create fatty acid stock solutions in 5 mL of toluene with the following concentrations:

Table 1: Fatty Acid Stock Solutions

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)
ALN	20	ML1	50	EPA	20	DE1	10
ARA	25	PL1	50	GLA	20	LG1	10
DHA	50	AR1	10	HGL	50	NR1	25
DP3	25	EN1	10	LNA	100	HDT	10
DP6	10	ED1	10	PM1	100	OTT	2.5
DTA	20	DA1	10	ST1	50		
VC1	25	OD1	50	OL1	100		
MR1	50	OC6	100	OD9	50		

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 5 mL volumetric flask using an analytical balance. Note the mass of fatty acid and use it to calculate exact concentration of the fatty acid stock solution.

3. Using the fatty acid stock solutions, 50 mL of a “level 40” calibrator working solution is created. The target concentration of each analyte in this working solution are as followed:

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)
ALN	400	ML1	100	EPA	800	DE1	50
ARA	2000	PL1	1200	GLA	200	LG1	200
DHA	1000	AR1	200	HGL	250	NR1	200
DP3	200	EN1	50	LNA	8000	HDT	25.0

DP6	100	ED1	50	PM1	8000	OTT	6.25
DTA	100	DA1	200	ST1	2000		
VC1	800	OD1	125	OL1	6000		
MR1	600	OC6	250	OD9	125		

4. Pipette from the individual fatty acid stock solutions the volumes listed in the table below in a 50 mL volumetric flask and fill the flask with toluene to the 50 mL mark.

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

Analyte Code	Volume of Fatty Acid Stock Solution (µL)	Analyte Code	Volume of Fatty Acid Stock Solution (µL)	Analyte Code	Volume of Fatty Acid Stock Solution (µL)	Analyte Code	Volume of Fatty Acid Stock Solution (µL)
ALN	1000	ML1	100	EPA	2000	DE1	250
ARA	4000	PL1	1200	GLA	500	LG1	1000
DHA	1000	AR1	1000	HGL	250	NR1	400
DP3	400	EN1	250	LNA	4000	HDT	125
DP6	500	ED1	250	PM1	4000	OTT	125
DTA	250	DA1	1000	ST1	2000		
VC1	1600	OD1	125	OL1	3000		
MR1	600	OC6	125	OD9	125		

5. Prepare level "30", "20", "10" calibrator working solutions in toluene using the dilution scheme described in the following table (use volumetric flasks and glass pipettes to prepare these solutions):

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

Calibrator working solution	Take volume (mL)	Dilute to volume (mL)	Name of solution created
Level 40	12.5	50	Level 30 ("CC30")
Level 30	20	50	Level 20 ("CC20")
Level 10	20	50	Level 10 ("CC10")

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^{o}C

5.3.2 Preparation of Internal Standard Solutions

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 (µL)	Desired internal Standard Fatty Acid Concentration of the Working Solution (µmol/L)
ALN_IS	5.00	2,340	200
AR1_IS	1.76	2,000	50
ARA_IS	10.00	5,000	800
DHA_IS	10.00	2,000	300
EPA_IS	9.22	2,000	300
HDT_IS	0.26	2,000	10
LNA_IS	89.48	2,000	3,000
MR1_IS	5.11	2,000	200
OD1_IS	0.86	2,000	30
OD9_IS	0.86	2,000	30
OL1_IS	45.05	2,000	1,500
OTT_IS	0.57	200	2
PL1_IS	10.00	2,684	500
PM1_IS	57.52	2,000	2,000
ST1_IS	15.99	2,000	500
VC1_IS	5.75	2,000	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 16x100 mm glass tube with screw cap using an analytical balance. Use a separate vial for each fatty acid. Note the mass of fatty acid and use it to calculate exact concentration of the fatty acid stock solution.
3. Add the amount of toluene to each vial as stated in the table.
4. For each fatty acid solution, transfer the volume needed to prepare the working solution as stated in the table in 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.

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).

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 [17] 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 or as indicated in CLIA '88 (§493.1255(b)).

With each set of samples four levels of calibration material and a low, mid, and high quality control material are analyzed. Possible shifts in calibration are assessed by comparing bench QC material data against predefined acceptance limits using a SAS software program developed and maintained by DLS (see also Section 8).

Calibration is further verified by analyzing commercial standards every 6 months and comparing the results obtained against predefined acceptance limits (+/- 10% from target value).

7 PROCEDURE OPERATION INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

All instruments are checked for correct function using the manufacturer's acceptance criteria. Specific details related to the operation instructions such as specific file names used in the execution are documented in work instructions (see specific sections in this document for references to work instructions).

7.1.1 Specimen Storage and Handling During Testing

All vials are labeled according to the DLS Policies and Procedures Manual 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 is either stored at 5 ^{o}C in the refrigerator 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 (4 levels: CC10, CC20, CC30, CC40), and 3 bench QC samples (low, medium and high). Typically 40 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 section 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 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 (for specific instrument layouts, see work instruction PBLW040017_Operation of tFA Method on Hamilton Starlet). 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 on the CDC network 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 visually inspects 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 at dedicated place in -70 ^{o}C freezer.

7.1.3 Hydrolysis of Samples

1. Add 2 mL of 10% 6N HCl in Acetonitrile solution to each vials using a graduated glass pipette.
2. Cap all vials and vortex them for 30 seconds using the T Genie 2 Vortex at highest setting.
3. Place samples in the Isotemp Mechanical 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 T Genie 2 Vortex on the highest setting.
8. Place the samples in the Isotemp Mechanical 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 (see work instruction PBLW040017_Operation of tFA Method on Hamilton Starlet for specific location).
2. Run the appropriate Hamilton Microlab STARLet Liquid Handler extraction method following work instruction PBL W040017. The robotic pipette will add 2 mL of hexane to each threaded culture tube.
3. Sonicate all vials in the sonication bath for 2 minutes (no heat, sweep sonication setting) and carefully vortex vials using the T Genie 2 Vortex at low 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 on the CDC network 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 through 7 two times (total of 3 extraction steps). The hexane layers from each vial are combined in the same vial used in step 8.
9. Scan vials each time before transferring the hexane layer. Transfer scan file in dedicated location in the Excel calculation template and assess if vials are in 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.

7.1.5 Derivatization of Fatty Acids

For the following steps please refer to the Work Instruction PBLW040017_Operation of tFA Method on Hamilton Starlet for specific samples, reagents and vials location.

13. 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
14. 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 method as described in work instruction PBL W040017.
15. Remove all vials from 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.
16. Place vials in the designated racks on Hamilton Microlab STARLet Liquid Handler instrument and keep vials at room temperature for 15 minutes for the derivatization reaction to occur.
17. Add 0.5 mL of hexane to each vial.
18. Mix all vials by vortexing for 5 seconds using the T Genie 2 Vortex on setting #7.
19. Place all vials in the designated racks on the Hamilton Microlab STARLet Liquid Handler instrument.
20. 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 on the CDC network and is imported later in the designated section of the Excel calculation template.
21. Transfer the measurement solution to the GC vials using the Hamilton instrument.

22. Cap the GC vials and visually inspect successful transfer of the measurement sample solution.
23. Transfer vials to the GC autosampler for GC/MS analysis or store as described in section 7.1.1 until GC/MS analysis.

7.1.6 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 Appendix 5 for an example of an analytical sequence).
3. The samples are loaded on the GC/MS instrument as stated in the sequence file and 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 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. Retention times and peak intensities need to be within 15% of the expected values. When instrument malfunction is indicated, the sequence is stopped, samples are stored in the refrigerator and the problem is addressed (for further specifications see Work instruction PBLW040001_Agilent GCMS Performance Verification).
6. Upon completion of the GC/MS analysis, the GC vials are recapped and stored in the designated space in the freezer at -70 ^{o}C.

The following GC/MS parameters are used (for further specific details see Appendix 6). Typical chromatograms of the retention time standard and low QC are shown in Appendix 7:

Chromatographic conditions

Injection:
Injector: CTC PAL ALS
Injection volume: 1 µL
Injection mode: Split (Split ratio: 20:1)
Split flow: 60 mL/min
Injector temperature: 240 °C
Total flow: 70.3 mL/min
Gas type: Hydrogen
Column: Varian FAME Select 200 m x 250 µm x 0.25 µm (length, inner diameter, film thickness)
Oven:
Initial temperature: 130 °C
Temperature Program:

Table 6: Temperature Program

Step	Start Temperature [°C]	Heating Rate [°C/min]	End Temperature [°C]	Temperature Hold Time (min)
1	130	5	160	0
2	160	0	160	10
3	160	1	180	0
4	180	0	180	5
5	180	1	210	0
6	210	0	210	10
7	210	35	260	0
8	260	0	260	18

Mass spectrometric conditions
Acquisition mode: Selected Ion Monitoring (SIM)
Solvent Delay: 45 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 m/z and retention times

#	Analyte Code*	m/z Ratio	Expected Retention time
1	MR1_IS	254.4	52.29
2	MR1	227.2	54.21
3	ML1	255.3	58.23
4	PM1_IS	286.5	63.79
5	PM1	225.2	65.97
6	PL1_IS	267.3	68.06
7	PL1	253.2	69
8	HDT_IS	258.4	67.78
9	HDT	253.2	67.8
10	ST1_IS	318.5	74.69
11	ST1	283.3	77.35
12	OL1_IS	299.3	80.28
13	OL1	281.3	80.39
14	OD9_IS	286.4	79.11
15	OD9	281.3	79.12
16	VC1_IS	286.4	81.20
17	VC1	281.3	81.21
18	OD1_IS	286.4	79.65
19	OD1	281.3	79.68
20	OC6	281.3	79.43
21	LNA_IS	279.3	83.19
22	LNA	279.3	83.23
			74.69

#	Analyte Code*	m/z Ratio	Expected Retention time
23	OTT_IS	284.4	82.04
24	OTT	279.3	82.05
25	ALN_IS	291.5	84.94
26	ALN	277.1	85.38
27	GLA	277.1	84.19
28	AR1_IS	350.2	83.72
29	AR1	311.3	84.89
30	HGL	305.3	89.79
31	ARA_IS	311.3	90.24
32	ARA	303.3	90.39
33	EN1	309.3	86.13
34	ED1	307.3	88.58
35	EPA_IS	306.3	92.54
36	EPA	301.1	92.72
37	DA1	339.4	90.03
38	DE1	337.3	91.34
39	DHA_IS	332.3	98.70
40	DHA	327.3	98.91
41	DTA	331.3	95.79
42	DP3	329.3	98.36
43	DP6	329.3	96.26
44	LG1	367.4	94.54
45	NR1	365.4	96.03

*For specific analyte names see Appendix 1

7.1.7 Data Processing

1. Data files generated by the GC/MS system are transferred to the dedicated place on the CDC network.
2. Using the Chemstation 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 “crd” 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.

   For further details on data processing such as specific locations on the CDC network, refer to the most recent version of work instruction PBLW040002_Data Integration Processing.
6. Integration results and calculation results are combined with relevant operator, instrument and QC sample information and transferred to a database maintained by DLS.

7.1.8 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 (μmol/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 trans fatty acid on the total fatty acids is calculated by dividing the concentration of the trans fatty acid with the sum of all fatty acid and multiplying this number by 100.

8 QUALITY ASSESSMENT AND PROFICIENCY TESTING

Quality assessment activities for this measurement procedure follow the requirements outlined in the DLS Policies and Procedures Manual.

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. [16]

The rules described in the most recent version of the DLS Policies and Procedures Manual together with the acceptance criteria are applied to measurement results obtained with the QC materials. Sample runs are rejected, if

- one bench QC result is beyond the characterization mean +/- 4SD,
- one bench QC result is outside a 3SD limit,
- current and previous bench QC results are outside the same 2SD limit
- current and previous 9 run results are on same side of the characterization mean
- the current and the previous run results differ by more than 4SD.

For further details, see the DLS Policies and Procedures Manual. Quality control evaluation is performed using a SAS program developed and maintained by DLS.

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 Laboratory Standards Institute GP29-P [13] 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 for CLIA purposes).

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

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 [14]. The reportable ranges of results are:

trans-9-hexadecenoic acid (C16:1n-7t, analyte code: HDT):	1.10 – 51.4 µmol/L
trans-9-octadecenoic acid (C18:1n-9t, analyte code: OD9):	3.60 – 146 µmol/L
trans-11-octadecenoic acid (C18:1n-7t, analyte code OD1):	4.60 – 100 µmol/L
trans-9, *trans*-12-octadecadienoic acid (C18:2n-6t, 9t, analyte code: OTT):	0.12 –6.30 µmol/L

9.2 Limit of Detection (LOD)

The limit of detection was determined using Taylor's method [15]. The limits of detection are:

trans-9-hexadecenoic acid (C16:1n-7t, analyte code: HDT):	0.50 µmol/L
trans-9-octadecenoic acid (C18:1n-9t, analyte code: OD9):	2.40 µmol/L
trans-11-octadecenoic acid (C18:1n-7t, analyte code: OD1):	2.80 µmol/L
trans-9, *trans*-12-octadecadienoic acid (C18:2n-6t, 9t, analyte code: OTT):	0.05 µmol/L

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 64 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 8 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 using the DLS SAS program for bench QC characterization.

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	9	5
trans-9-octadecenoic acid	1	7	5
trans-11-octadecenoic acid	3	4	8
trans-9, *trans*-12-octadecadienoic acid	2	11	9
Sum of fatty acids	2	5	10

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	14	16	15
trans-9-octadecenoic acid	18	18	17
trans-11-octadecenoic acid	18	18	14
trans-9, *trans*-12-octadecadienoic acid	21	23	20
Sum of fatty acids	8	9	12

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	Accuracy using GLC-603 (% of the assigned value)	Accuracy using GLC-674 (% of the assigned value)
trans-9-hexadecenoic acid	102.8	97.7
trans-9-octadecenoic acid	97.4	91.0
trans-11-octadecenoic acid	113.3	106.4
trans-9, *trans*-12-octadecadienoic acid	106.5	99.2
Sum of fatty acids	100.8	99.7

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 such as whole blood. 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 61 different substances (for specific details see section 9.3 and 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 a convenience sample size from 100 individuals was performed to obtain information on concentrations that can be expected in the general population. In this study, the following values were determined:

Table 11: Reference Range

	Values in µmol/L		Values as percent of total fatty acids	
Analyte	Mean (Range)	Median (5^{th}-95^{th} Percentile)	Mean (Range)	Median (5^{th}-95^{th} Percentile)
trans-9-hexadecenoic acid	5.75 (1.39-12.65)	5.34 (4.81 to 6.05)	0.044 (0.012-0.089)	0.043 (0.039to 0.048)
trans-9-octadecenoic acid	26.48 (5.77-77.30)	23.70 (21.32to27.85	0.204 (0.065-0.426)	0.198 (0.188to0.213)
trans-11-octadecenoic acid	30.78 (4.23-83.55)	29.02 (27.02to30.91)	0.238 (0.042-0.481)	0.233 (0.219to0.244)
trans-9, *trans*-12-octadecadienoic acid	1.48 (0.47-3.33)	1.43 (1.26to1.57)	0.0115 (0.005-0.022)	0.011 (0.009to0.012)
Sum of fatty acids	12,999 (7,588-20,399)	12,804 (12,154to13,532)	N/A	N/A

11 TEST RESULT REPORTING SYSTEM

Results are reported to 3 significant digits based on assay sensitivity calculations. Data are reported in µmol/L and % of total trans fatty acids.

The test reporting system as described in the most recent version of the DLS Policies and Procedures Manual is used when reporting test results. The system consists of review steps at multiple levels such as results verification by a DLS statistician, and DLS management.

12 ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

If the analytical system fails, we recommend that the specimens be stored at -70 °C until the analytical system is restored to functionality.

No alternate testing method exists for the measurement procedure.

13 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 DLS and CDC policy.

Standard record keeping (e.g., database, notebooks, and 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.

14 TRANSFER OR REFERRAL OF SPECIMENS

Transfer or referral of specimens will follow the procedures outlined in the most recent version of the DLS Policies and Procedures Manual.

15 CRITICAL CALL RESULTS ("PANIC VALUES"); PROTOCOL FOR REPORTING CRITICAL CALLS

There are no known critical call values for *trans* fatty acids. Therefore no protocol for critical calls is established.

16 PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTING INADEQUATELY PREPARED SLIDES

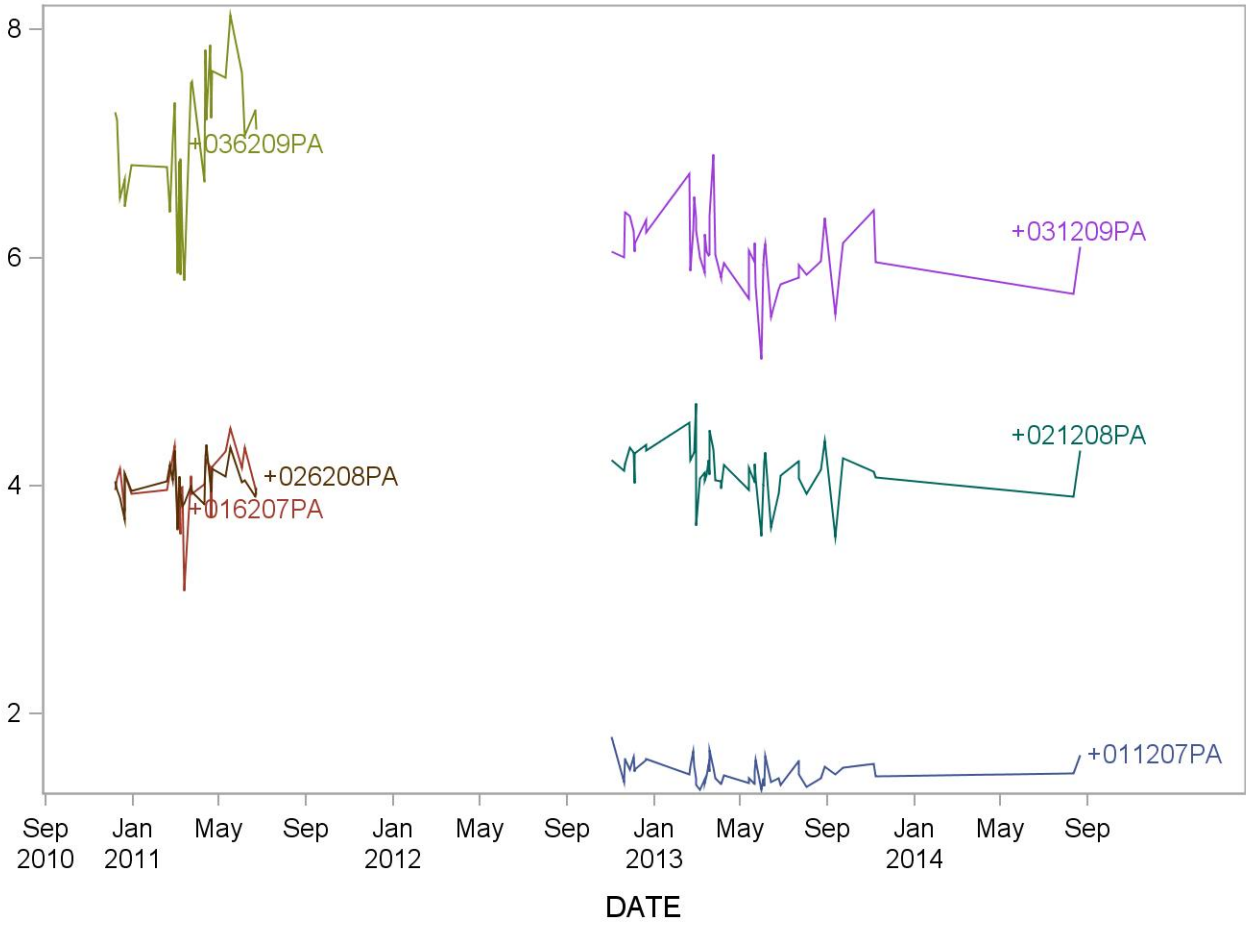
Not applicable for this procedure.

17. SUMMARY STATISTICS AND QC GRAPHS

See following pages.

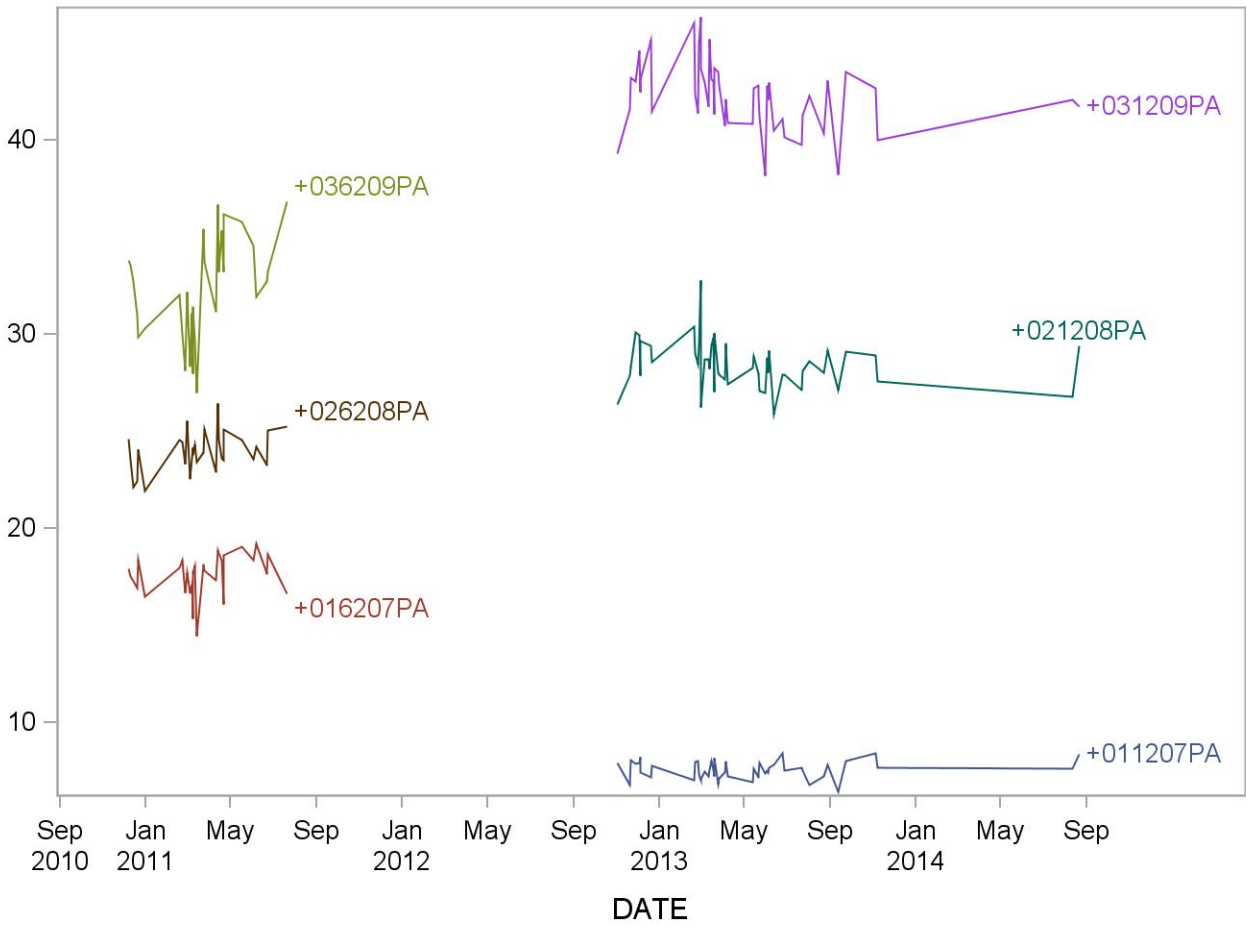
2009-2010 Summary Statistics and QC Chart for trans 9-hexadecenoic acid (µmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
+036209PA	36	07DEC10	23JUN11	7.00694	0.66828	9.5
+016207PA	36	07DEC10	23JUN11	4.01681	0.26585	6.6
+026208PA	36	07DEC10	23JUN11	3.99486	0.18990	4.8
+031209PA	55	02NOV12	21AUG14	6.04482	0.30797	5.1
+011207PA	55	02NOV12	21AUG14	1.49118	0.10960	7.3
+021208PA	55	02NOV12	21AUG14	4.13118	0.22352	5.4



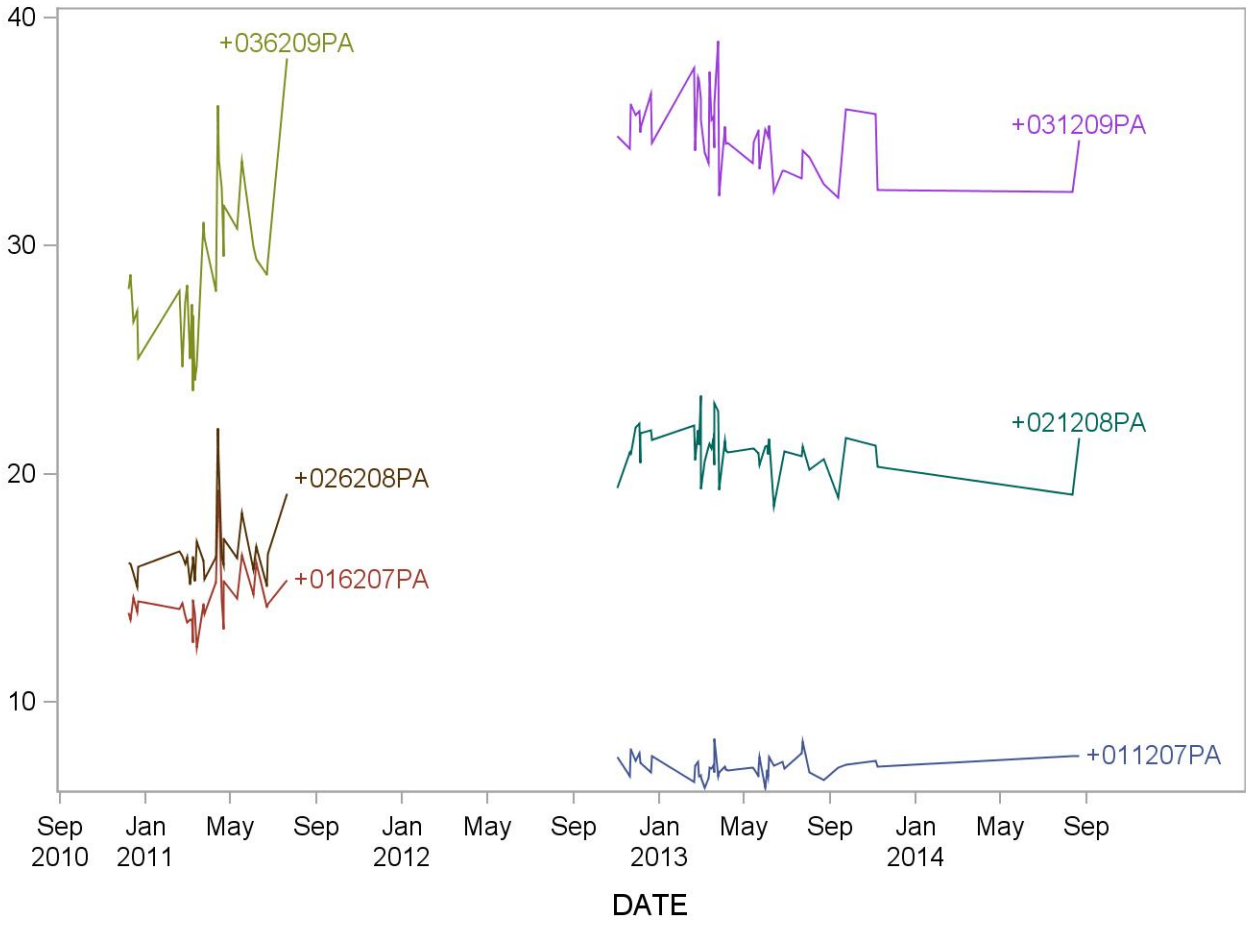
2009-2010 Summary Statistics and QC Chart for trans 11-octadecenoic acid (µmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
+036209PA	36	07DEC10	21JUL11	32.34306	2.80760	8.7
+016207PA	36	07DEC10	21JUL11	17.60694	1.05066	6.0
+026208PA	36	07DEC10	21JUL11	23.91250	1.09950	4.6
+031209PA	55	02NOV12	21AUG14	42.25455	1.77251	4.2
+011207PA	55	02NOV12	21AUG14	7.55755	0.48378	6.4
+021208PA	55	02NOV12	21AUG14	28.41909	1.27157	4.5



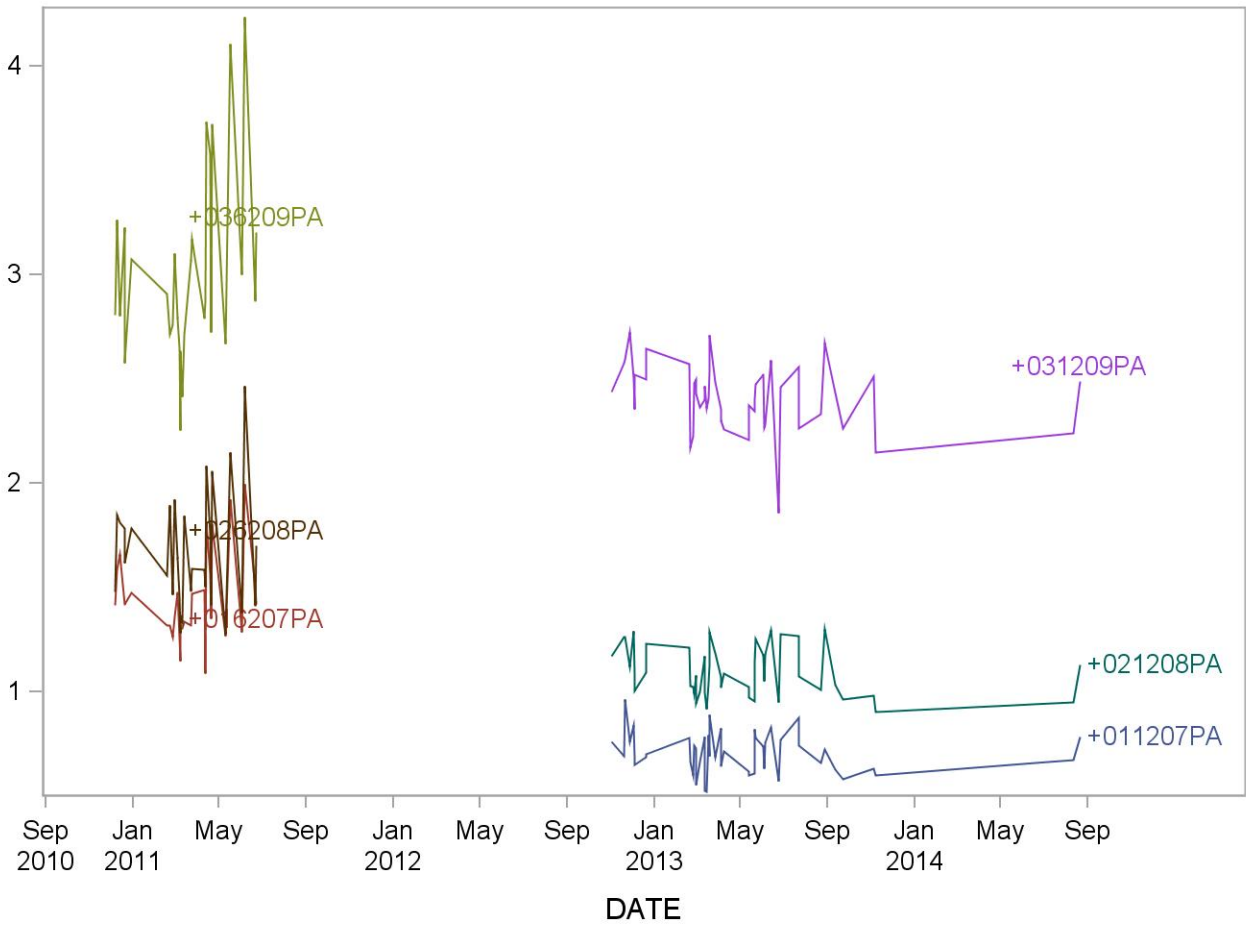
2009-2010 Summary Statistics and QC Chart for trans 9-octadecenoic acid (µmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
+036209PA	36	07DEC10	21JUL11	28.92778	3.48934	12.1
+016207PA	36	07DEC10	21JUL11	14.54750	1.42324	9.8
+026208PA	36	07DEC10	21JUL11	16.50556	1.46110	8.9
+031209PA	54	02NOV12	21AUG14	34.83241	1.55837	4.5
+011207PA	54	02NOV12	21AUG14	7.17898	0.51150	7.1
+021208PA	54	02NOV12	21AUG14	21.00833	0.98535	4.7



2009-2010 Summary Statistics and QC Chart for trans 9trans-12-octadienoic acid (µmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
+036209PA	36	07DEC10	23JUN11	3.00931	0.46795	15.6
+016207PA	36	07DEC10	23JUN11	1.45139	0.24278	16.7
+026208PA	36	07DEC10	23JUN11	1.65250	0.29282	17.7
+031209PA	49	02NOV12	21AUG14	2.41633	0.16328	6.8
+011207PA	49	02NOV12	21AUG14	0.70531	0.09940	14.1
+021208PA	49	02NOV12	21AUG14	1.09802	0.12079	11.0



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17 APPENDICES

Appendix 1. List of Fatty Acids Measured with this Measurement

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

Appendix 5. Example of Analytical Sequence

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

Appendix 7. Retention Standard And Low QC Sample Chromatogram

Appendix 8. List of Compounds Tested for Interference

Appendix 9. Related Documents

Appendix 10. Symbols, Abbreviations, Terminology

Appendix 11. Document Compliance Tables

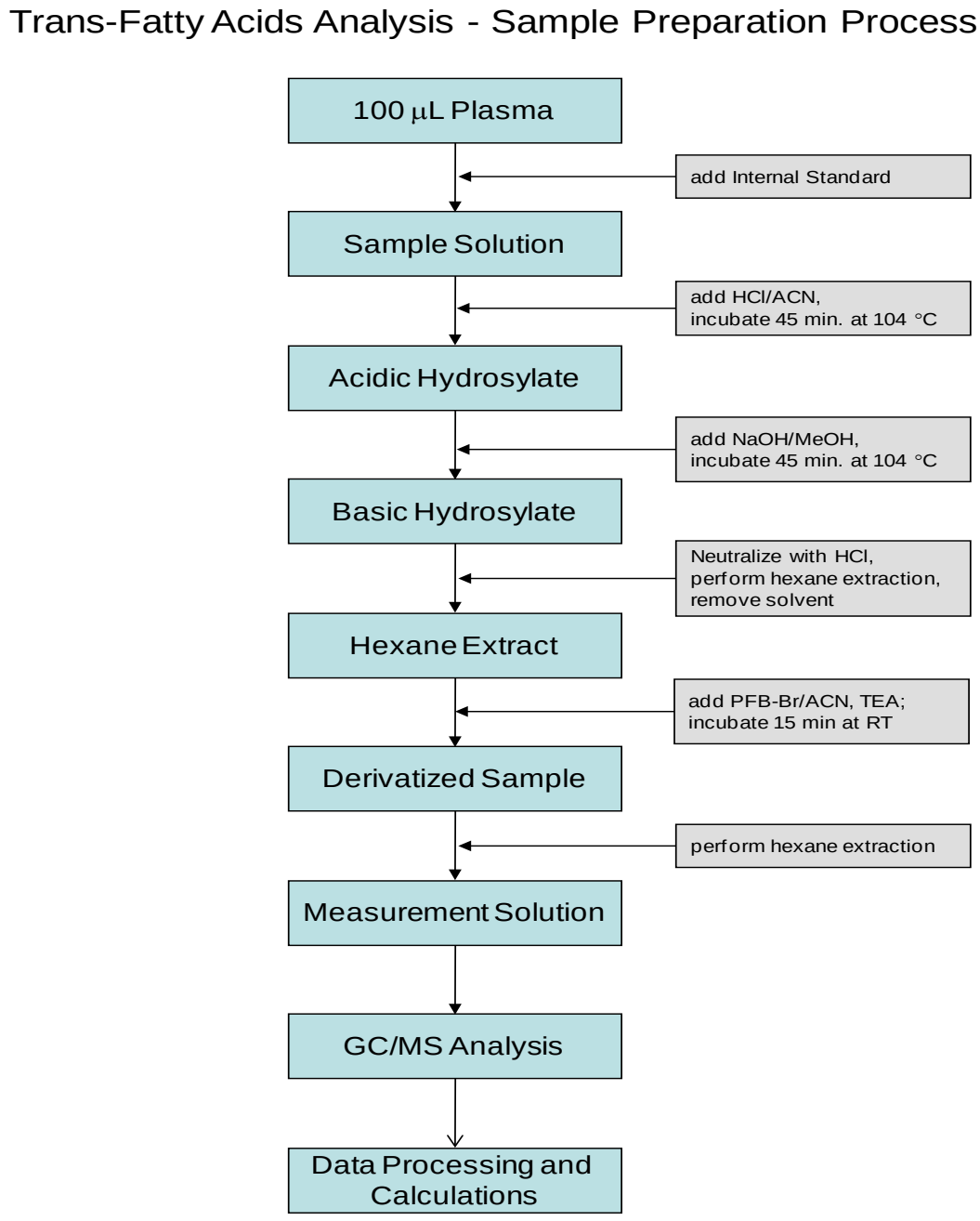
Appendix 1. List of Fatty Acids Measured with this Measurement Procedure

Nr.	IUPAC name	Common Name	Short Hand	Analyte Code
1	tetradecanoic acid	Myristic acid	C14:0	MR1*
2	cis-9-tetradecenoic acid	Myristoleic acid	C14:1n-5c	ML1
3	hexadecanoic acid	Palmitic acid	C16:0	PM1*
4	cis-9-hexadecenoic acid	Palmitoleic acid	C16:1n-7c	PL1*
5	trans 9-hexadecenoic acid	Palmitelaidic acid	C16:1n-7t	HDT*
6	octadecanoic acid	Stearic acid	C18:0	ST1*
7	cis-9-octadecenoic acid	Oleic acid	C18:1n-9c	OL1*
8	trans-9-octadecenoic acid	Elaidic acid	C18:1n-9t	OD9*
9	cis-11-octadecenoic acid	*cis*-Vaccenic acid	C18:1n-7c	VC1*
10	trans-11-octadecenoic acid	Vaccenic acid	C18:1n -7t	OD1*
11	cis-6-octadecenoic acid	Petroselinic acid	C18:1n-12c	OC6
12	cis-9, cis-12-octadecadienoic acid	Linoleic acid	C18:2n-6c,9c	LNA*
13	trans-9, trans-12-octadecadienoic acid	Linolelaidate acid	C18:2n- t6,t9	OTT*
14	cis-9, cis-12, cis-15-octadecatrienoic acid	alpha-Linolenic acid	C18:3n-3c,6c,9c	ALN*
15	cis-6, cis-9, cis-12-octadecatrienoic acid	gamma-Linolenic acid	C18:3n-6c,9c,12c	GLA
16	eicosanoic acid	Arachidic acid	C20:0	AR1*
17	cis-8, cis-11, cis-14-eicosatrienoic acid	Dihomo-gamma-Linolenic acid	C20:3n-6c,9c,12c	HGL
18	cis-5, cis-8, cis-11, cis-14-eicosatetraenoic acid	Arachidonic acid	C20:4n-6c,9c,12c,15c	ARA*
19	cis-11-eicosenoic acid	Gondoic acid	C20:1n-9c	EN1
20	cis-11, cis-14-eicosadienoic acid	Eicosadienoic acid	C20:2n-6c,9c	ED1
21	cis-5, cis-8, cis-11, cis-14, cis-17-eicosapentaenoic acid	Eicosapentaenoic acid	C20:5n-3c,6c,9c,12c,15c	EPA*
22	docosanoic acid	Behenic acid	C22:0	DA1
23	cis-13-docosenoic acid	Erucic acid	C22:1n-9c	DE1
24	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*
25	cis-7, cis-10, cis-13, cis-16-docosatetraenoic acid	Docosatetraenoic acid	C22:4n-6c,9c,12c,15c	DTA
26	cis-7, cis-10, cis-13, cis-16, cis-19-docosapentaenoic acid	Docosapentaenoic acid 3	C22:5n-3c,6c,9c,12c,15c	DP3
27	cis-4, cis-7, cis-10, cis-13, cis-16-docosapentaenoic acid	Docosapentaenoic acid 6	C22:5n-6c,9c,12c,15c,18c	DP6
28	tetracosanoic acid	Lignoceric acid	C24:0	LG1

29	cis-15-tetracosenoic acid	Nervonic acid	C24:1n-9c	NR1

*For these compounds stable isotope labeled standards 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	tetradecanoic acid	MR1	Nu-Chek-Prep, Elysian, MN	>99%	228.38
2	cis-9-tetradecenoic acid	ML1	Nu-Chek-Prep, Elysian, MN	>99%	226.38
3	hexadecanoic acid	PM1	Nu-Chek-Prep, Elysian, MN	>99%	256.43
4	cis-9-hexadecenoic acid	PL1	Nu-Chek-Prep, Elysian, MN	>99%	254.43
5	trans 9-hexadecenoate	HDT	Nu-Chek-Prep, Elysian, MN	>99%	254.40
6	octadecanoic acid	ST1	Nu-Chek-Prep, Elysian, MN	>99%	284.48
7	cis-9-octadecenoic acid	OL1	Nu-Chek-Prep, Elysian, MN	>99%	282.48
8	trans-9-octadecenoate	OD9	Nu-Chek-Prep, Elysian, MN	>99%	296.51
9	cis-11-octadecenoic acid	VC1	Nu-Chek-Prep, Elysian, MN	>99%	282.48
10	trans-11-octadecenoate	OD1	Nu-Chek-Prep, Elysian, MN	>99%	296.51
11	cis-6-octadecenoate	OC6	Sigma-Aldrich, St. Louis, MO	99+%	296.51
12	cis-9, cis-12-octadecadienoic acid	LNA	Nu-Chek-Prep, Elysian, MN	>99%	280.48
13	trans-9, trans-12-octadienoic acid	OTT	Nu-Chek-Prep, Elysian, MN	>99%	294.51
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

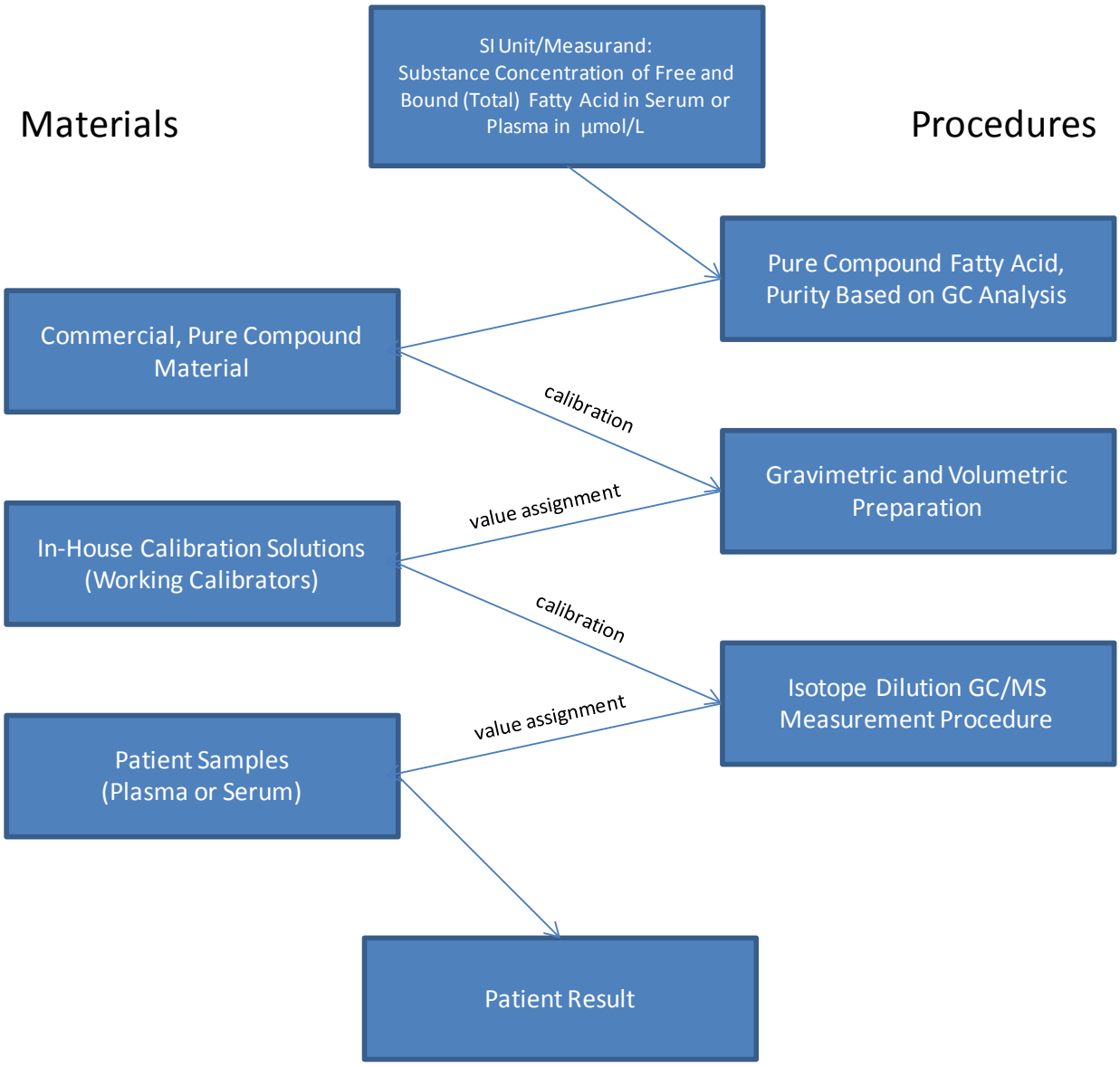
Appendix3 (continued): Stable isotope-labeled standards used for internal standards

Name	Analyte Code	Manufacturer	Purity	MW (g/mol)
Methyl ^{13}C-9-cis-hexadecenoate	PL1_IS	Spectra Stable Isotopes, Columbia, MD	>95%	284.29
Methyl ^{13}C-9-trans-hexadecenoate	HDT_IS	Spectra Stable Isotopes, Columbia, MD	≥99%	284.29
Methyl ^{13}C-9-trans-octadecenoate	OD9_IS	Spectra Stable Isotopes, Columbia, MD	>99%	314.33
Methyl ^{13}C-11-trans-octadecenoate	OD1_IS	Spectra Stable Isotopes, Columbia, MD	≥99%	314.33
Methyl ^{13}C-11-cis-octadecenoate	VC1_IS	Spectra Stable Isotopes, Columbia, MD	≥99%	314.33
Methyl ^{13}C-9-trans, 12-trans-octadecadienoate	OTT_IS	Cambridge Isotopes Laboratories, Cambridge, MA	≥98%	312.32
^{13}C-cis 9,12,15-octadecatrienoic acid	ALN_IS	Spectra Stable Isotopes, Columbia, MD	>99%	296.23
d8-cis 5,8,11,14-eicosatetraenoic acid	ARA_IS	Cayman Chemical, Ann Arbor, MI	≥99%	312.50
d5-cis 4,7,10,13,16,19-docosahexaenoic acid	DHA_IS	Cayman Chemical, Ann Arbor, MI	≥99%	333.52
d5-cis 5,8,11,14,17-eicosapentaenoic acid	EPA_IS	Cayman Chemical, Ann Arbor, MI	≥99%	307.48
^{13}C-cis 9,12-octadecadienoic acid	LNA_IS	Spectra Stable Isotopes, Columbia, MD	>99%	298.25
d31-hexadecanoic acid	PM1_IS	Cambridge Isotopes Laboratories, Cambridge, MA	≥98%	287.62
d35-octadecanoic acid	ST1_IS	Cambridge Isotopes Laboratories, Cambridge, MA	≥98%	319.69
^{13}C-cis 9-octadecenoic acid	OL1_IS	Spectra Stable Isotopes, Columbia, MD	≥99%	300.26
d27-tetradecanoic acid	MR1_IS	Cambridge Isotopes Laboratories, Cambridge, MA	≥98%	255.54
Methyl d39-eicosanoate	AR1_IS	IsoSciences, King of Prussia, PA	≥98%	265.80

Appendix3 (continued): Target concentrations of the calibrator solutions

Nr.	Name	Analyte Code	Level 40 (“CC40”) (µmol/L)	Level 30 (“CC30”) (µmol/L)	Level 20 (“CC20”) (µmol/L)	Level 10 (“CC10”) (µmol/L)
1	tetradecanoic acid	MR1	600	150	60.0	24.0
2	cis-9-tetradecenoic acid	ML1	100	25.0	10.0	4.00
3	hexadecanoic acid	PM1	8,000	2,000	800	320
4	cis-9-hexadecenoic acid	PL1	1,200	300	120	48.0
5	trans 9-hexadecenoate	HDT	25.0	6.25	2.5	1.00
6	octadecanoic acid	ST1	2,000	500	200	80
7	cis-9-octadecenoic acid	OL1	6,000	1,500	600	240
8	trans-9-octadecenoate	OD9	125	31.3	12.5	5.00
9	cis-11-octadecenoic acid	VC1	800	200	80	32
10	trans-11-octadecenoate	OD1	62.3	15.6	6.2	2.5
11	cis-6-octadecenoate	OC6	250	62.5	25.0	10.0
12	cis-9, cis-12-octadecadienoic acid	LNA	8,000	2,000	800	320
13	trans-9, trans-12-octadienoic acid	OTT	6.25	1.56	0.63	0.25
14	cis-9, cis-12, cis-15-octadecatrienoic acid	ALN	400	100	40.0	16.0
15	cis-6, cis-9, cis-12-octadecatrienoic acid	GLA	200	50.0	20.0	8.00
16	eicosanoic acid	AR1	200	50.0	20.0	8.00
17	cis-8, cis-11, cis-14-eicosatrienoic acid	HGL	250	62.5	25.0	10
18	cis-5, cis-8, cis-11, cis-14-eicosatetraenoic acid	ARA	2,000	500	200	80
19	cis-11-eicosenoic acid	EN1	50.0	12.5	5.00	2.00
20	cis-11, cis-14-eicosadienoic acid	ED1	50.0	12.5	5.00	2.00
21	cis-5, cis-8, cis-11, cis-14, cis-17-eicosapentaenoic acid	EPA	800	200	80.0	32
22	docosanoic acid	DA1	200	50.0	20.0	8.00
23	cis-13-docosenoic acid	DE1	50.0	12.5	5.00	2.00
24	cis-4, cis-7, cis-10, cis-13, cis-16, cis-19-docosahexaenoic acid	DHA	1,000	250	100	40
25	cis-7, cis-10, cis-13, cis-16-docosatetraenoic acid	DTA	100	25.0	10.0	4.00
26	cis-7, cis-10, cis-13, cis-16, cis-19-docosapentaenoic acid	DP3	200	50.0	20.0	8.00
27	cis-4, cis-7, cis-10, cis-13, cis-16-docosapentaenoic acid	DP6	100	25.0	10.0	4.00
28	tetracosanoic acid	LG1	200	50.0	20.0	8.00
29	cis-15-tetracosenoic acid	NR1	200	50.0	20.0	8.00

Appendix 4. Metrological Traceability of Trans Fatty Acids Measurements



Appendix 5. Example of Analytical Sequence

Type	Vial	Tray Name	Sample	Method / Keyword	Data File
Sample	49	Agilent ALS	RT STD	Enter method here	012610_TFP3R0183_49
Sample	1	Agilent ALS	CC_050809_10	Enter method here	012610_TFP3R0183_01
Sample	6	Agilent ALS	06+03_6209_PL01A	Enter method here	012610_TFP3R0183_06
Sample	9	Agilent ALS	0982885PA	Enter method here	012610_TFP3R0183_09
Sample	10	Agilent ALS	0982780PA	Enter method here	012610_TFP3R0183_10
Sample	11	Agilent ALS	0982781PA	Enter method here	012610_TFP3R0183_11
Sample	12	Agilent ALS	0982792PA	Enter method here	012610_TFP3R0183_12
Sample	13	Agilent ALS	0982796PA	Enter method here	012610_TFP3R0183_13
Sample	14	Agilent ALS	0982797PA	Enter method here	012610_TFP3R0183_14
Sample	15	Agilent ALS	0982808PA	Enter method here	012610_TFP3R0183_15
Sample	16	Agilent ALS	0982804PA	Enter method here	012610_TFP3R0183_16
Sample	17	Agilent ALS	0982809PA	Enter method here	012610_TFP3R0183_17
Sample	18	Agilent ALS	0982810PA	Enter method here	012610_TFP3R0183_18
Sample	19	Agilent ALS	0982811PA	Enter method here	012610_TFP3R0183_19
Sample	2	Agilent ALS	CC_050809_20	Enter method here	012610_TFP3R0183_02
Sample	7	Agilent ALS	06+02_6208_PL01A	Enter method here	012610_TFP3R0183_07
Sample	20	Agilent ALS	0982812PA	Enter method here	012610_TFP3R0183_20
Sample	21	Agilent ALS	0982813PA	Enter method here	012610_TFP3R0183_21
Sample	22	Agilent ALS	0982814PA	Enter method here	012610_TFP3R0183_22
Sample	23	Agilent ALS	0982815PA	Enter method here	012610_TFP3R0183_23
Sample	24	Agilent ALS	0982816PA	Enter method here	012610_TFP3R0183_24
Sample	25	Agilent ALS	0982817PA	Enter method here	012610_TFP3R0183_25
Sample	26	Agilent ALS	0982818PA	Enter method here	012610_TFP3R0183_26
Sample	27	Agilent ALS	0982820PA	Enter method here	012610_TFP3R0183_27
Sample	28	Agilent ALS	0982821PA	Enter method here	012610_TFP3R0183_28
Sample	29	Agilent ALS	0982822PA	Enter method here	012610_TFP3R0183_29
Sample	30	Agilent ALS	0982823PA	Enter method here	012610_TFP3R0183_30
Sample	31	Agilent ALS	0982824PA	Enter method here	012610_TFP3R0183_31
Sample	3	Agilent ALS	CC_050809_30	Enter method here	012610_TFP3R0183_03
Sample	8	Agilent ALS	06+01_6207_PL01A	Enter method here	012610_TFP3R0183_08
Sample	32	Agilent ALS	0982825PA	Enter method here	012610_TFP3R0183_32
Sample	33	Agilent ALS	0982826PA	Enter method here	012610_TFP3R0183_33
Sample	34	Agilent ALS	0982827PA	Enter method here	012610_TFP3R0183_34
Sample	35	Agilent ALS	0982828PA	Enter method here	012610_TFP3R0183_35
Sample	36	Agilent ALS	0982829PA	Enter method here	012610_TFP3R0183_36
Sample	37	Agilent ALS	0982830PA	Enter method here	012610_TFP3R0183_37
Sample	38	Agilent ALS	0982831PA	Enter method here	012610_TFP3R0183_38
Sample	39	Agilent ALS	0982832PA	Enter method here	012610_TFP3R0183_39
Sample	40	Agilent ALS	0982833PA	Enter method here	012610_TFP3R0183_40
Sample	41	Agilent ALS	0982834PA	Enter method here	012610_TFP3R0183_41
Sample	42	Agilent ALS	0982835PA	Enter method here	012610_TFP3R0183_42
Sample	43	Agilent ALS	0982836PA	Enter method here	012610_TFP3R0183_43
Sample	4	Agilent ALS	CC_050809_40	Enter method here	012610_TFP3R0183_04
Sample	5	Agilent ALS	CC_071009_00	Enter method here	012610_TFP3R0183_05
Sample	44	Agilent ALS	0982837PA	Enter method here	012610_TFP3R0183_44
Sample	45	Agilent ALS	0982838PA	Enter method here	012610_TFP3R0183_45
Sample	46	Agilent ALS	0982839PA	Enter method here	012610_TFP3R0183_46
Sample	47	Agilent ALS	0982841PA	Enter method here	012610_TFP3R0183_47
Sample	48	Agilent ALS	0982842PA	Enter method here	012610_TFP3R0183_48

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

Parameters listed as documented by Chemstation software:

Sample Inlet: GC
Injection Source: GC ALS
Mass Spectrometer: Enabled

Oven
Equilibration Time: 0.25 min
Oven Program: On
130 °C for 0 min
then 5 °C/min to 160 °C for 10 min
then 1 °C/min to 180 °C for 5 min
then 1 °C/min to 210 °C for 10 min
then 35 °C/min to 260 °C for 18 min
Run Time: 100.43 min

Front Injector

Syringe Size:	10 µL
Injection Volume:	1 µL
Injection Repetitions:	1
Injection Delay:	0 sec
Solvent A Washes (PreInj):	0
Solvent A Washes (PostInj):	8
Solvent A Volume:	8 µL
Solvent B Washes (PreInj):	2
Solvent B Washes (PostInj):	8
Solvent B Volume:	8 µL
Sample Washes:	1
Sample Wash Volume:	8 µL
Sample Pumps:	3
Dwell Time (PreInj):	0 min
Dwell Time (PostInj):	0 min
Solvent Wash Draw Speed:	300 µL/min
Solvent Wash Dispense Speed:	6000 µL/min
Sample Wash Draw Speed:	300 µL/min
Sample Wash Dispense Speed:	6000 µL/min
Injection Dispense Speed:	6000 µL/min
Viscosity Delay:	0 sec
Sample Depth:	Disabled

Sample Overlap
Sample overlap is not enabled
Front SS Inlet H2

Mode:	Split
Heater:	On set to240 °C
Pressure:	On 67.699 psi
Total Flow:	On 66 mL/min
Septum Purge Flow:	On set to 3 mL/min

Gas Saver:	On set to 20 mL/min After 2 min
Split Ratio:	20 :1
Split Flow:	60 mL/min

Thermal Aux 2 {MSD Transfer Line}

Heater:	On
Temperature Program:	On, 260 °C for 0 min
Run Time:	100.43 min

Column #1
200 m FAME Select: 1045.63196
290 °C: 200 m x 250 µm x 0.25 µm
In: Front SS Inlet H2
Out: Other

(Initial):	130 °C
Pressure:	67.699 psi
Flow:	3 mL/min
Average Velocity:	36.024 cm/sec
Holdup Time:	9.2531 min
Flow Program	On 3 mL/min for 80 min then 1 mL/min per min to 2.5 mL/min for 0 min
Run Time:	100.43 min

Signals

Test Plot	Save Off 50 Hz
Test Plot	Save Off 50 Hz
Test Plot	Save Off 50 Hz
Test Plot	Save Off 50 Hz

MS ACQUISITION PARAMETERS
General Information

Acquistion Mode	SIM

MS Information

Solvent Delay	45.00 min
EMV Mode	Gain Factor
Gain Factor	1.00
Resulting EM Voltage	1353

[Sim Parameters]
GROUP 1

Group ID	Group 1
Resolution	High
Plot 1 Ion	225.20
Ions/Dwell In Group	(Mass, Dwell) (Mass, Dwell) (Mass, Dwell) (225.20, 7) (227.20, 7) (254.40, 7)

GROUP 2

Group ID	Group 2
Resolution	High

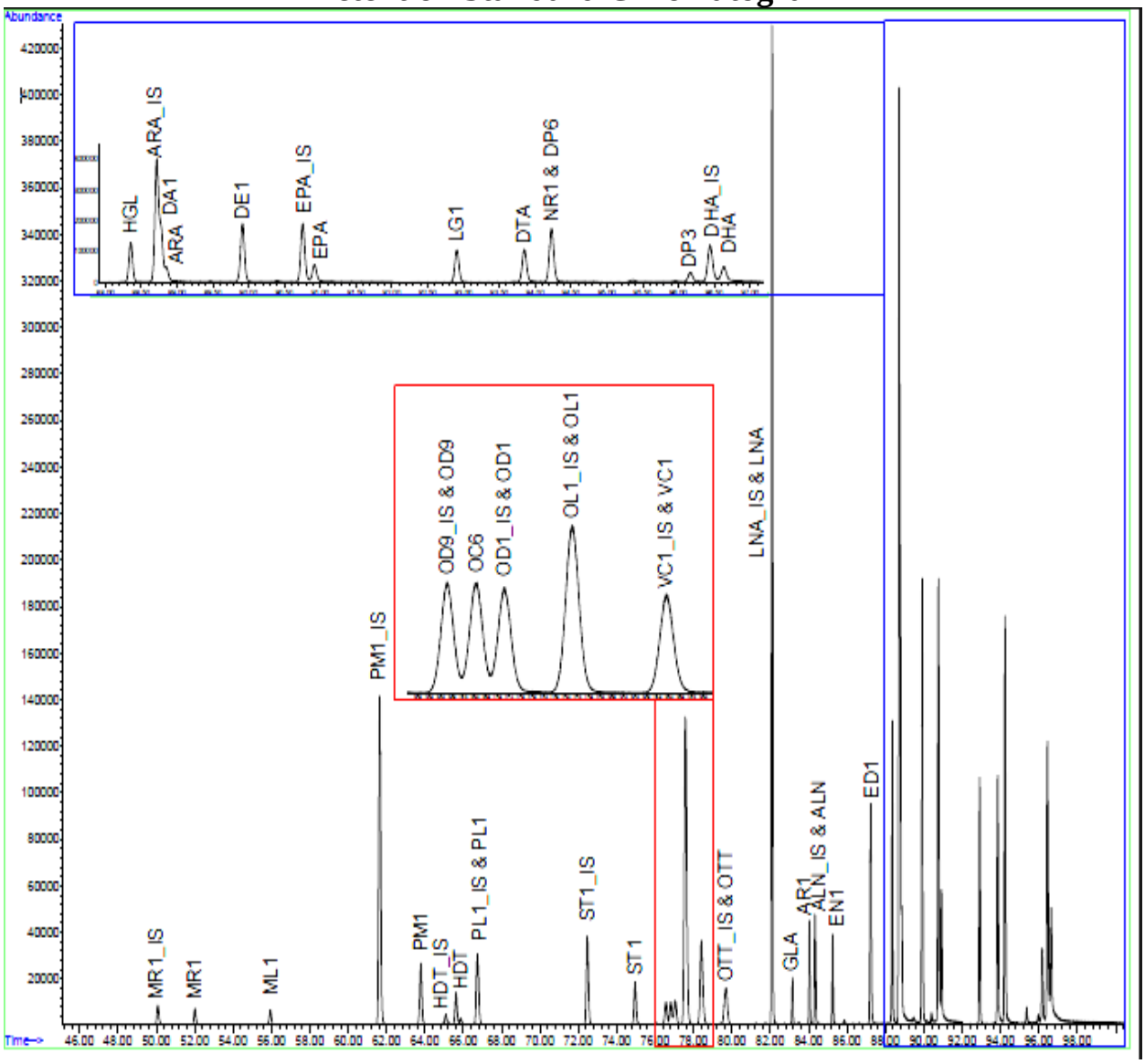
Group Start Time	57.50		
Plot 1 Ion	253.20		
Ions/Dwell In Group	(Mass, Dwell)	(Mass, Dwell)	(Mass, Dwell)
	(253.20, 3)	(255.30, 3)	(269.30, 3)
	(271.30, 3)	(286.50, 3)	
GROUP 3			
Group ID	Group 3		
Resolution	High		
Group Start Time	68.90		
Plot 1 Ion	279.30		
Ions/Dwell In Group	(Mass, Dwell)	(Mass, Dwell)	(Mass, Dwell)
	(279.30, 2)	(281.30, 2)	(283.30, 2)
	(297.30, 2)	(299.30, 2)	(318.50, 2)
GROUP 4			
Group ID	Group 4		
Resolution	High		
Group Start Time	80.60		
Plot 1 Ion	277.10		
Ions/Dwell In Group	(Mass, Dwell)	(Mass, Dwell)	(Mass, Dwell)
	(277.10, 2)	(279.30, 2)	(295.30, 2)
	(297.30, 2)	(309.30, 2)	(311.30, 2)
GROUP 5			
Group ID	Group 5		
Resolution	High		
Group Start Time	86.15		
Plot 1 Ion	301.10		
Ions/Dwell In Group	(Mass, Dwell)	(Mass, Dwell)	(Mass, Dwell)
	(301.10, 2)	(303.30, 2)	(305.30, 2)
	(306.30, 2)	(307.30, 2)	(311.30, 2)
	(337.30, 2)	(339.40, 2)	
GROUP 6			
Group ID	Group 6		
Resolution	High		
Group Start Time	91.93		
Plot 1 Ion	327.30		
Ions/Dwell In Group	(Mass, Dwell)	(Mass, Dwell)	(Mass, Dwell)
	(327.30, 2)	(329.30, 2)	(331.30, 2)
	(332.30, 2)	(365.40, 2)	(367.40, 2)

[MSZones]
MS Source : 230 C maximum 300 C
MS Quad : 150 C maximum 200 C

Trace Ion Detection is OFF.
DCPOLARITY : 1.000
CI Flow Rate: 40
CI A/B Gas : 1

Appendix 7. Retention Standard And Low QC Sample Chromatograms

Retention Standard 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

Appendix 9. Related Documents

Normative References

1. DLS Policies and Procedures Manual. http://isp-v-ehip-asp/dlsintranet/pdf/05/DLS_Policies_and_Procedures_Manual.pdf
2. CDC Safety Policies and Practices Manual. http://isp-v-ehip-asp/dlsintranet/safety_manual/
3. Clinical Laboratory Improvement Amendments of 1988 (CLIA). 42CFR493 from February 28, 1992.

Work Instructions

1. PBLW040001_Agilent GCMS Performance Verification
2. PBLW040002_Data Integration Processing
3. PBLW04003_Preparation of tFA Hydrolysis_Solutions
4. PBLW04004_PFP_Br 7% VV in Acetonitrile
5. PBLW040017_Operation of tFA Method on Hamilton Starlet
6. PBLW040023_Sample Receiving and Handling

Appendix 10. Symbols, Abbreviations, Terminology

Abbreviations

ACS.ASTM American Chemical Society. 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
CV Coefficient of Variant
DLS Division of Laboratory Sciences
EMV Electron Multiplier Voltage
EDTA Ethylenediaminetetraacetic Acid
FDA Food and Drug Administration
GC/MS Gas Chromatography/Mass Spectrometry
HCl Hydrochloric Acid
HIV Human Immunodeficiency Virus
HPLC High Performance Liquid Chromatography
ID Identification
IS Internal Standards
ISO International Organization for Standardization
ITSO Information Technology Service Office
LDL Low-density Lipoprotein
MSD Mass Selective Detector
MSDS Material Safety Data Sheets
N/A Not Applicable
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
PTEF Polytetrafluorethylene
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

Symbols

Not applicable

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)

Appendix 11. Document Compliance Tables

Table 1: Location of information required by the DLS Policies and Procures Manual

Required section	Section in this Document
requirements for specimen collection and processing, including criteria for specimen rejection	4
step-by-step performance of the procedure, including test calculations and interpretation of results	7
preparation of reagents, calibrators, controls, solutions and other materials used in testing	5
calibration and calibration verification procedures	6
the reportable range for patient test results	9.1
quality control procedures, including PT materials and programs/procedures used	8
remedial action to be taken when calibration or control results are outside acceptable limits	8.1.3
limitation in methods, including interfering substances	9.5
reference range (normal values)	10
life-threatening or "panic values"	15
pertinent literature references	16
specimen storage criteria	4.2, 7.1.1
protocol for reporting panic values	15
course of action if test system becomes inoperable	8.1.3, 12
criteria for referral of specimens (usually not needed)	14
safety considerations for performing the method	2

Table 2: Location of information as required by CLIA

Required section	Section in this Document
Requirements for patient preparation; specimen collection, labeling, storage, preservation, transportation, processing, and referral; and criteria for specimen acceptability and rejection	3.2, 4,
Microscopic examination, including the detection of inadequately prepared slides	16
Step-by-step performance of the procedure, including test calculations and interpretation of results	7
Preparation of slides, solutions, calibrators, controls, reagents, stains, and other materials used in testing	5
Calibration and calibration verification procedures	6
The reportable range for test results for the test system as established or verified	9.1
Control procedures	8
Corrective action to take when calibration or control results fail to meet the laboratory's criteria for acceptability	8.1.3
Limitations in the test methodology, including interfering Substances	9.5
Reference intervals (normal values)	10
Imminently life-threatening test results or panic or alert Values	15
Pertinent literature references	16
The laboratory's system for entering results in the patient record and reporting patient results including, when appropriate, the protocol for reporting imminent life threatening results, or panic, or alert values	3, 7.1.7, 13
Description of the course of action to take if a test system becomes inoperable	8.1.3, 12

Table 3: Location of information as required by ISO 17025

Required section	Section in this Document
appropriate identification	Title Page
Scope	1
description of the type of item to be tested or calibrated	1
parameters or quantities and ranges to be determined	1, 9.1
apparatus and equipment, including technical performance requirements	5
reference standards and reference materials required	5.3, 6.1.2
environmental conditions required and any stabilization period needed	5, 7
description of the procedure, including affixing of identification marks, handling, transporting, storing and preparation of items, checks to be made before the work is started, checks that the equipment is working properly and, where required, calibration and adjustment of the equipment before each use, the method of recording the observations and results, any safety measures to be observed	7
criteria and/or requirements for approval/rejection	4, 8
data to be recorded and method of analysis and presentation	3, 7.1.8
the uncertainty or the procedure for estimating uncertainty	9.4

Table 4: Location of information as required by ISO 15193

Required section	Section in this Document
Title page	Title Page
Contents list	List of Content
Foreword	N/A
Warning and safety precautions	2
Introduction	1
Title	Title Page
Scope	1
Normative references	Appendix 9
Definitions	Appendix 10
Symbols and abbreviations	Appendix 10
Terminology	Appendix 10
Principle and method of measurement	1
Check list	
Reagents	5
Apparatus	5
Sampling and sample	4, 7.1.1
Preparation of measuring system and analytical portion	7
Operation of measuring system	7
Data processing	3, 7.1.7
Analytical reliability	9
Special cases	N/A
Validation by inter-laboratory studies	N/A
Reporting	7.1.7
Quality assurance	8
Bibliography (Annex)	16
Dates of authorization and revision	Second page of document