Laboratory Procedure Manual

Analyte: ***Profile of 21 fatty acids - alpha-Linolenic, Arachidic, Arachidonic, Docosanoic, Docosahexaenoic, Docosapentaenoic (n3), Docosapentaenoic (n6), Docosatetraenoic, Eicosadienoic, Eicosenoic, Eicosapentaenoic, gamma-Linolenic, homo-gamma-Linolenic, Lignoceric, Linoleic, Myristic, Nervonic, Oleic, Palmitic, Palmitoleic, and Stearic***

Matrix: **Red blood cell**

Method: **Gas Chromatography – Mass Spectrometry**

Method No: 4030.03

Revised: Dec 2022

as performed by: Nutritional Biomarkers Branch (NBB)
Division of Laboratory Sciences (DLS)
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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.

Images are included in this document as visual aids for certain topics. They are intended to be representative images only and should not be construed as absolute references. Discrepancies between the images in this document and the actual application design are not a cause for revisions to this document.

Data Set (FAR_K_R) Information

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

File Name	Variable Name	SAS Label
FAR_K_R	LBXPAN	alpha-Linolenic acid (C18:3n3) (%)
	LBXP1A	Arachidic acid (C20:0) (%)
	LBXPRA	Arachidonic acid (C20:4n6) (%)
	LBXPDA	Docosanoic acid (C22:0) (%)
	LBXPHA	Docosahexaenoic acid (C22:6n3) (%)
	LBXPD3	Docosapentaenoic acid 3 (C22:5n3) (%)
	LBXPD6	Docosapentaenoic acid 6 (C22:5n6) (%)
	LBXPTA	Docosatetraenoic acid (C22:4n6) (%)
	LBXPED	11,14-Eicosadienoic acid (C20:2n6) (%)
	LBXP1E	11-Eicosenoic acid (C20:1n9) (%)
	LBXPPE	Eicosapentaenoic acid (C20:5n3) (%)
	LBXPLG	gamma-Linolenic acid (C18:3n6) (%)
	LBXPGH	homo-gamma-Linolenic acid (C20:3n6) (%)
	LBXP1G	Tetracosanoic acid (C24:0) (%)
	LBXPNL	Linoleic acid (C18:2n6) (%)
	LBXPMR	Myristic acid (C14:0) (%)
	LBXPNR	15-Tetracosenoic acid (C24:1n9) (%)
	LBXPOL	Oleic acid (C18:1n9) (%)
	LBXPPL	Palmitoleic acid (C16:1n7) (%)
	LBXPPM	Palmitic acid (C16:0) (%)
	LBXPST	Stearic acid (C18:0) (%)

1. Summary of Clinical Relevance and Principle

A. Clinical Relevance

The analysis of red blood cell (RBC) fatty acids (FA) is an indicator of long term (4 months) FA status. American Heart Association recommendation for those with no history of coronary heart disease (CHD) is to eat two fatty fish servings per week and for those with a history of CHD to consume 1 g/day of two omega-3 fatty acids, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid **[1]**. The Omega-3 Index (sum of EPA and DHA as a percent of 21 fatty acids) has been suggested as a risk factor for death from CHD **[2]**. A few studies have shown that the Omega-3 Index is inversely correlated with cardiac death and acute coronary syndrome **[3,4]**. The goal of this method is to obtain US reference ranges for 21 *cis*-fatty acids and to assess the Omega-3 Index.

B. Test Principle

Esterified FA are hydrolyzed from washed RBC membranes using sequential treatment with mineral acid and base in the presence of heat. Using a modification of Lagerstedt et al. **[5]**, total FA are hexane-extracted from the matrix (100 µL 50:50 saline diluted RBC) along with an internal standard solution containing thirteen stable isotopically-labeled FA to account for recovery. The extract is derivatized with pentafluorobenzyl bromide (PFBBr) in the presence of triethylamine to form pentafluorobenzyl esters. The reaction mixture is injected onto a capillary gas chromatograph column to resolve individual FA of interest from other matrix constituents. FA are detected using electron capture negative-ion mass spectrometry within 34 minutes. Six saturated FA (SFA), four monounsaturated FA (MUFA), and eleven polyunsaturated FA (PUFA) (21 FA in total) are measured using selected ion monitoring. Quantitation is accomplished by comparing the peak area ratio of the analyte to internal standard in the unknown sample with the peak area ratio of a known amount of analyte to internal standard in a calibrator solution.

Saturated	**Fatty acids**	**Database Analyte Code (weight percent)**	**Carbon: Double bonds**
1	Myristic acid	MR1P	C14:0
2	Palmitic acid	PM1P	C16:0
3	Stearic acid	ST1P	C18:0
4	Arachidic acid	AR1P	C20:0
5	Docosanoic acid	DA1P	C22:0
6	Lignoceric acid	LG1P	C24:0
Monounsaturated			
1	Palmitoleic acid	PL1P	C16:1n-7
2	Oleic acid	OL1P	C18:1n-9
3	Eicosenoic acid	EN1P	C20:1n-9
4	Nervonic acid	NR1P	C24:1n-9
Polyunsaturated			
1	Linoleic acid	LNAP	C18:2n-6
2	*alpha*-Linolenic acid	ALNP	C18:3n-3
3	*gamma*-Linolenic acid	GLAP	C18:3n-6
4	Eicosadienoic acid	ED1P	C20:2n-6
5	*homo-gamma*-Linolenic acid	HGLP	C20:3n-6
6	Arachidonic acid	ARAP	C20:4n-6
7	Eicosapentaenoic acid	EPAP	C20:5n-3
8	Docosatetraenoic acid	DTAP	C22:4n-6

9	Docosapentaenoic acid n-3	DP3P	C22:5n-3
10	Docosapentaenoic acid n-6	DP6P	C22:5n-6
11	Docosahexaenoic acid	DHAP	C22:6n-3

2. Safety Precautions

Consider all RBC specimens potentially positive for infectious agents including HIV and the hepatitis B virus. We recommend the hepatitis B vaccination series for all analysts working with whole blood, RBCs and/or serum/plasma. Observe universal precautions; wear protective gloves, laboratory coats, and safety glasses during all steps of this method. Place disposable plastic, glass, and paper (pipette tips, autosampler vials, gloves, etc.) that contact RBC in a biohazard autoclave bag and keep these bags in appropriate containers until sealed and autoclaved. Wipe down all work surfaces with 10% bleach solution prepared fresh daily or All Safe™ when work is finished.

Handle acids and bases with extreme care; they are corrosive or caustic. Handle organic solvents only in a well-ventilated area or, as required, under a chemical fume hood. The derivatizing agent, pentafluorobenzyl bromide (PFBBr), is a combustible liquid and vapor. PFBBr can cause respiratory tract, eye, and skin burns, use extreme care when handling and change gloves after handling.

Organic solvents, such as methanol and acetonitrile, containing either acid or base are heated to ~104°C for complete hydrolysis. These steps should be done in an oven designed for volatile organic solvents.

Reagents and solvents used in this study include those listed in Section 6. Safety data sheets (SDS) for these chemicals are readily accessible as hard copies in the lab. If needed, SDS for other chemicals can be viewed at http://intranet.cdc.gov/ossam/workplace-safety/safety-practices/chemical-safety/index.html. Additional information on hazard identification, risk evaluation, and risk mitigation for this method can be found in the method risk assessment form.

3. Computerization; Data System Management

(A) During sample preparation and analysis, samples are identified by their sample ID. The sample ID is a number that is unique to each sample that links the laboratory information to demographic data recorded by those who collected the sample.

(B) The raw data files from the mass spectrometer are collected using the instrument software and stored on the instrument workstation. The raw data files are reviewed on the instrument workstation and results files are created. Results are typically generated by auto-integration but may require in some cases manual integration. The data file folders containing the results files are transferred to the CDC network. The results file (including analyte and internal standard names, peak areas, retention times, sample dilution factor, data file name, acquisition time, etc.) is imported into the STARLIMS database for review of the patient data, statistical evaluation of the QC data, and approval of the results. See Job Aid **"JA-4030-DR-01-Computerization & Data System Management" found in Appendix B section D** for a step-by-step description of data transfer, review, and approval.

(C) Files stored on the CDC network are automatically backed up by ITSO support staff.

4. Specimen Collection, Storage, and Handling Procedures; Criteria for Specimen Rejection

Specimens for FA analysis may be fresh or frozen washed RBC. This method is not approved for use with capillary RBC samples.

A 0.5-mL RBC sample is required to allow for repeat analyses; a volume of 100 µL is required per analysis.

The appropriate amount of RBC is dispensed into a Nalge 2.0-mL cryovial or other plastic screw-capped vial labeled with the specimen ID.

Specimens collected in the field are typically frozen at ≤-30°C for about 1 week and then shipped on dry ice by overnight carrier. Once received, they should be kept frozen during 'in-processing', which is typically completed within less than 4 hours and then stored frozen at ≤-50°C for up to 15 business days until they are transferred to the testing laboratory for long-term storage at deep frozen condition (-50°C to -90°C), typically at temperatures ≤-60°C. Up to three short (2 h) freeze-thaw cycles cause relatively minor (<16%) analyte degradation.

The criteria for unacceptable specimens are suspected contamination such as leaking, or a damaged sample container. Record a description of reasons for each rejected sample, such as leaking or damaged container. Specimens should arrive frozen. Specimens stored at ≥-20°C for >4 weeks are rejected because PUFA in RBCs start to degrade >4 weeks **[6]**.

Specimen handling conditions are outlined in the DLS Policies and Procedures Manual. The protocol discusses in general collection and transport of specimens and the special equipment required. If there is more than one test of interest in the specimen and it needs to be divided, the appropriate amount of RBC should be transferred into a Nalge cryovial labeled with a new specimen ID linked to the participant's ID; avoid cross-contamination. A series of standard comment codes are available in the LIMS database to identify any issues related to sample quality. These codes can be used, along with text descriptions, to document why a result was not reported (specimen rejection) or that a result should be interpreted with caution based on the sample quality.

5. Procedures for Microscopic Examinations; Criteria for Rejection of Inadequately Prepared Slides

Not applicable for this procedure.

6. Preparation of Reagents, Calibration (Standards), Controls, and All Other Materials; Equipment and Instrumentation

Chemicals and other materials used in the preparation of reagents, calibrators, and quality controls materials are tracked as indicated in Job Aid "**JA-4030-R&S-01-Reagent Tracking" found in Appendix B section B.** To facilitate tracking of solvents and other chemicals transferred from the original manufacturer containment into a secondary container (e.g., solvent bottle), include the expiration date provided by the manufacturer or the lot number on the secondary container.

A. Reagent Preparation

Prepare all solutions, samples and standards with HPLC-grade solvents and reagents. Use Class A volumetric glassware in all cases. Perform all steps involving concentrated acids, bases, and organic solvents in a chemical fume hood. Though each reagent preparation specifies a total volume of reagent prepared, these directions may be scaled up or down to prepare slightly larger or smaller quantities if desired.

1) Acetonitrile: 6 N Hydrochloric acid, (90:10, v:v)

Add 100 mL of 6 N HCl to 900 mL of acetonitrile and mix. Store at room temperature. Reagent expires 3 weeks after preparation or when crystals appear.

CAUTION!!! HCl is corrosive. Wear acid-resistant gloves, safety glasses (face shields are available if desired), lab coat and\or apron.

2) Methanol: 10 N Sodium Hydroxide, (90:10, v:v)

Add 100 mL of 10 N sodium hydroxide to 900 mL with methanol and mix. Store at room temperature. Reagent expires 3 weeks after preparation or when crystals appear.

CAUTION!!! NaOH is caustic. Wear base-resistant gloves, safety glasses (face shields are available if desired), lab coat and\or apron.

3) Derivatizing Solution

Prepare the 7% pentafluorobenzyl bromide and 10% triethylamine in acetonitrile solution as shown in the table below. Derivatizing solution should be prepared within 15 minutes of use.

# of samples	Pentafluorobenzyl Bromide (PFBBr)	Acetonitrile (ACN)	Triethylamine (TEA)
< 20	140 µL	1.86 mL	200 µL
< 25	175 µL	2.33 mL	250 µL
< 30	210 µL	2.79 mL	300 µL
< 35	245 µL	3.26 mL	350 µL
< 40	280 µL	3.72 mL	400 µL
< 45	315 µL	4.19 mL	450 µL
< 50	350 µL	4.65 mL	500 µL
< 55	385 µL	5.12 mL	550 µL
< 60	420 µL	5.58 mL	600 µL
< 65	455 µL	6.05 mL	650 µL
< 70	490 µL	6.51 mL	700 µL
< 75	525 µL	6.98 mL	750 µL
< 80	560 µL	7.44 mL	800 µL
< 85	595 µL	7.90 mL	850 µL
< 90	630 µL	8.37 mL	900 µL
< 95	665 µL	8.84 mL	950 µL
< 100	700 µL	9.30 mL	1 mL

CAUTION!!! PFBBr is a combustible liquid. Wear chemical-resistant gloves, safety glasses (face shields are available if desired), lab coat and/or apron. Remove gloves, wash hands, and replace with new gloves after handling/pipetting PFBBr.

B. Standards Preparation

1) Individual stock standard solutions are prepared as follows:

	Fatty Acid Name	Analyte Code	MW	Target Stock Concentration (mM)	Target Amount in 5.0 mL Toluene (mg)
1	*alpha*-Linolenic	ALN	278.48	50	70
2	Arachidic	AR1	312.54	25	39
3	Arachidonic	ARA	304.52	250	381
4	Docosanoic	DA1	340.59	25	43
5	Docosahexaenoic	DHA	328.57	75	123
6	Docosapentaenoic n-3	DP3	330.57	25	41
7	Docosapentaenoic n-6	DP6	330.57	25	41
8	Docosatetraenoic	DTA	332.57	75	125
9	Eicosadienoic	ED1	308.53	25	39
10	Eicosenoic	EN1	310.54	25	39
11	Eicosapentaenoic	EPA	302.52	50	76
12	*gamma*-Linolenic	GLA	278.48	75	104
13	*homo-gamma*-Linolenic	HGL	306.53	125	192
14	Lignoceric	LG1	368.64	15	28
15	Linoleic	LNA	280.48	500	701
16	Myristic	MR1	228.38	100	114
17	Nervonic	NR1	366.63	50	92
18	Oleic	OL1	282.48	500	706
19	Palmitoleic	PL1	254.43	125	159
20	Palmitic	PM1	256.43	500	641
21	Stearic	ST1	300.48	150	225

a) Weigh all materials into labeled 16-mm x 100-mm screw-top culture tubes.

b) After weighing materials, calculate volume of toluene to be added based on actual amount weighed: actual weight/target weight * 5 mL. Cap and mix by gentle inversion. If necessary, sonicate stock solutions until analyte is in solution.

2) Purity Check for individual stock standard solutions – in addition to obtaining manufacturer purity information

c) Aliquot 50 µL of each individual stock standard solution to a 13-mm x 100-mm culture tube. If analyte is in methyl ester form, then you must take the 50 µL sample through the sample preparation steps listed in Section 8 subsections B-D.

d) Add 100 µL of derivatizing solution (7% pentafluorobenzyl bromide and 10% triethylamine in acetonitrile solution; preparation shown in section 6.a.3 Reagent preparation)

e) Wait 15 minutes for derivatization to occur

f) Add 9.85 mL hexane to each tube and mix

g) Transfer 1 mL hexane to labeled GCMS vial and run in SIM and Scan modes on GCMS

h) After analysis and data review, for each stock standard solution, sum peak areas of all known peaks. Divide peak area for analyte of interest by the sum peak areas of all known peaks to obtain percent purity. Percent purity should be taken into account for all analytes when assigning calibration values.

3) Composition of mixed intermediate stock 1 (prepared in 250-mL volumetric flask).

	Fatty Acid Name	**Analyte Code**	**Target stock concentration (mM)**	**mL stock to prepare intermediate stock 1**	**Target intermediate stock 1 Concentration (uM)**
1	*alpha*-Linolenic	ALN	50	7.5	1500
2	Arachidic	AR1	25	2.5	250
3	Arachidonic	ARA	250	6.25	6250
4	Docosanoic	DA1	25	5	500
5	Docosahexaenoic	DHA	75	6.25	1875
6	Docosapentaenoic n-3	DP3	25	6.25	625
7	Docosapentaenoic n-6	DP6	25	3.75	375
8	Docosatetraenoic	DTA	75	1.25	375
9	Eicosadienoic	ED1	25	2.5	250
10	Eicosenoic	EN1	25	2.5	250
11	Eicosapentaenoic	EPA	50	9	1800
12	*gamma*-Linolenic	GLA	75	2.5	750
13	*homo-gamma*-Linolenic	HGL	125	2.5	1250
14	Lignoceric	LG1	15	6.25	375
15	Linoleic	LNA	500	12.5	25000
16	Myristic	MR1	100	6.25	2500
17	Nervonic	NR1	50	2.5	500
18	Oleic	OL1	500	12.5	25000
19	Palmitoleic	PL1	125	12.5	6250
20	Palmitic	PM1	500	12.5	25000
21	Stearic	ST1	150	12.5	7500

4) Intermediate Stock 2 solution: Add 20 mL of Intermediate Stock 1 to a 250-mL volumetric flask, then fill to the mark with Toluene. Mix by Inversion.

5) Standards 1-6 are prepared in 250-mL volumetric flasks as follows:

Standard	Intermediate stock used	mL Intermediate stock used
Std6	1	100
Std5	1	60
Std4	1	30
Std3	2	125
Std2	2	12.5
Std1*	2	5

*Std1 was prepared but not used for this method.

a) For above standards, add volume specified of Intermediate Stock 1 or 2 to a 250-mL volumetric flask, then fill to the mark with Toluene. Mix by Inversion.

b) A dilution of Standards 3 and 2 are done with each run to obtain lower concentrations for some analytes. The dilution is done by using smaller volumes of the standards as follows:

Standard	Standard used	µL
Std3_60	Std3	60
Std3_30	Std3	30
Std2_50	Std2	50

6) The blank (Std0) is prepared by adding Toluene directly to labeled amber GC vials containing fused glass inserts.

7) Aliquot 225 µL final standard solutions into appropriately labeled amber calibrator vials.

8) Calibrators are stable for at least 9 years when stored deep frozen (-50°C to -90°C).

Table of target concentrations for calibrators (5-6 levels per analyte): Historically, the CDC laboratory used molar concentrations for preparing fatty acid standards. A Starlims macro converts the molar concentrations (μM) to weight concentrations (mg/L) and finally weight percentages (wt %).

	Fatty Acid	Analyte Code	Std6 (μM)	Std5 (μM)	Std4 (μM)	Std3 (μM)	Std3_60 (μM)	Std3_30 (μM)	Std2 (μM)	Std2_50 (μM)
1	*alpha*-Linolenic	ALN			200	60	36	18	6	3
2	Arachidic	AR1			30	10	6	3	1	
3	Arachidonic	ARA		1500	750	250	150	75		
4	Docosanoic	DA1		120	60	20	12	6		
5	Docosahexaenoic	DHA			225	75	45	23	8	
6	Docosapentaenoic n-3	DP3		150	75	25	15	8		
7	Docosapentaenoic n-6	DP6			45	15	9	5	2	
8	Docosatetraenoic	DTA	150	90	45	15	9			
9	11,14-Eicosadienoic	ED1			30	10	6	3	1	
10	11-Eicosenoic	EN1			30	10	6	3	1	
11	Eicosapentaenoic	EPA				72	43	22	7	4
12	*gamma*-Linolenic	GLA			90	30	18	9	3	2
13	*homo-gamma*-Linolenic	HGL			150	50	30	15	5	2
14	Lignoceric	LG1	150	90	45	15	9			
15	Linoleic	LNA				1000	600	300	100	50
16	Myristic	MR1				100	60	30	10	5
17	Nervonic	NR1	200	120	60	20	12			
18	Oleic	OL1				1000	600	300	100	50
19	Palmitoleic	PL1				250	150	75	25	13
20	Palmitic	PM1			3000	1000	600	300	100	
21	Stearic	ST1			900	300	180	90	30	

9) Internal Standard Solutions

Individual internal standard stock solutions are prepared so that each will yield the following final concentration in the final solution. If the deuterated form cannot be obtained, then the 13C form will also work, just be sure to use the correct MW when calculating the amount needed to yield the target concentration and for the instrument method. The final concentration of the mixed internal standard solution should be calculated (including correction for purity). Purity is confirmed using the same methodology as shown in section B (2) Purity Check. Mixed internal standard solution is aliquoted into glass vials with PTFE caps (18mL/vial) and stored deep frozen (-50°C to -90°C). Mixed internal standard solution is stable for at least 10 years when stored deep frozen (-50°C to -90°C).

	Internal Standard Acid Name	**Label**	**Analyte Code**	**Target (uM)**
1	*alpha*-Linolenic	d-14	ALN _IS_N	65
2	Arachidic	d-39	AR1_IS_N	25
3	Arachidonic	d-8	ARA_IS_N	800
4	Docosahexaenoic	d-5	DHA_IS_N	125
5	Docosanoic	d-4	DA1_IS_N	60
6	Eicosapentaenoic	d-5	EPA_IS_N	40
7	Lignoceric	d-4	LG1_IS_N	50
8	Linoleic	13C	LNA_IS_N	3500
9	Myristic	d-27	MR1_IS_N	100
10	Oleic	13C	OL1_IS_N	2100
11	Palmitic	d-31	PM1_IS_N	2700
12	Palmitoleic	d-14	PL1_IS_N	200
13	Stearic	d-35	ST1_IS_N	700

C. Preparation of Quality Control Materials

Normal saline diluted RBC (50:50) containing 10% 0.014g BHT/mL methanol solution is aliquoted into 2.0-mL Nalgene cryovials, capped, and frozen. If possible, each level should fall within lower 1/3, middle 1/3, and upper 1/3 of the US population reference range values for key FA (Palmitic, Oleic, Linoleic, Stearic and Arachidonic). The QC pools should be stored deep frozen (-50°C to -90°C), typically at temperatures ≤-60°C.

Characterization limits for all pools are established by analyzing duplicates for at least 20 runs.

For more detailed information on the preparation of QC materials, homogeneity testing, and characterization refer to **SOP "NBB-OC-LABOP.01.01 QC Materials"**.

D. Other Materials

With some exceptions, a material listed herein may be substituted with equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed. In the case of standards, internal standards, chemicals and reagents, the chemical and/or isotopic purity of the substitute must meet or exceed that of the listed product. In the case of the GC column, equivalent performance must be demonstrated experimentally in accordance with DLS policies and procedures.

a) General Supplies

   a) Pyrex Screw caps with teflon liners 415\15 (Corning, Inc., Corning, NY)
   b) 16- x 100-mm Pyrex Disposable screw caps culture tubes (Corning, Inc.)
   c) 13- x 100-mm Pyrex culture tubes (Corning, Inc.)
   d) 13- x 100-mm Pyrex Disposable screw cap culture tubes (Corning, Inc.)
   e) Pyrex Screw caps with teflon liners 415\13 (Corning, Inc.)
   f) 2.0-mL Polypropylene cryovials (Nalgene Company, Rochester, NY)
   g) 6” Disposable glass Pasteur pipettes (Kimble Glass, Vineland, NJ)
   h) Blue tips (50-1000 µL) for Eppendorf pipette (Brinkmann Instruments Inc., Westbury, NY)
   i) Yellow tips (2-200 µL) for Eppendorf pipette (Brinkmann Instruments Inc.)
   j) Combitip Plus (0.5 mL) for Eppendorf repeater pipette (Brinkmann Instruments Inc.)
   k) Combitip Plus (5.0 mL) for Eppendorf repeater pipette (Brinkmann Instruments Inc.)
   l) Combitip Plus (50 mL) for Eppendorf repeater pipette (Brinkmann Instruments Inc.)
   m) 10mL glass serological disposable pipettes, sterile (7077-10N; Fisher Scientific, Fair Lawn, NJ)
   n) 5mL glass serological disposable pipettes, sterile (7077-5N; Fisher Scientific, Fair Lawn, NJ)
   o) Rainin Positive Displace tips (C-1000, C-250, C-50 and C-25, Mettler-Toledo Rainin, LLC, Oakland, CA)
   p) Gilson positive displacement pipette tips (CP100ST, Gilson, Middleton, WI)
   q) Hamilton high volume (1mL) tips without filter (235904, Hamilton Company, Reno, NV)
   r) Hamilton standard volume (300µL) tips without filter (235902, Hamilton Company)
   s) 20mL Disposable glass scintillation vials with caps
   t) Amber GC vials with fused glass inserts (C4000LV2; Fisher Scientific)
   u) Amber GC vials (C4000-2W; Fisher Scientific)
   v) GC glass inserts (; Fisher Scientific)
   w) GC vial caps (C4000-53B; Fisher Scientific)
   x) Various glass beakers, graduated cylinders and glass bottles, class A glassware

10) GCMS Supplies

   a) Helium, ultrapure (>99.99% purity) (Air Products, Inc., Atlanta, GA)
   b) Methane, ultrapure (>99.99% purity) (Air Products, Inc.)

c) Thermo Trace Gold TG-POLAR GC Column, 60.0m x 0.25mm x 0.25 µm (Thermo Fisher Scientific, Waltham, MA)

d) Split/splitless liner (19251-60540, Agilent)

e) Liner o-ring (5188-5365, Agilent)

f) Split gold seal disk (5182-9652, Agilent)

g) Silver washer (5061-5869, Agilent)

h) Inlet Ferrule, 0.4mm 15%/85% graphite/vespel (5181-3323, Agilent)

i) Column nut for GC capillaries (5181-8830, Agilent)

j) Ferrule, 0.4mm 15%/85% graphite/vespel (5062-3508, Agilent)

k) Column nut for MS interface (05988-20066, Agilent)

l) Lens insulator, 597X MSD (G1370-20530, Agilent)

m) 73CI Repeller (G1999-20432, Agilent)

n) CI Filament for the 5973 and 5975 MSD (G1099-80053, Agilent)

o) ALS Syringe, 10 µl tapered, fixed needle (5181-3360, Agilent)

p) Advanced Green 11 mm septa (5183-4759, Agilent)

q) 4mL wash vial with fill markings and cap (5182-0551, Agilent)

r) Diffusion caps for 4 mL wash vials (07673-40180, Agilent)

s) Various Swagelok fittings (Agilent or equivalent)

t) Copper tubing cutter (Agilent or equivalent)

11) Chemicals

a) Methanol, HPLC grade (Honeywell Burdick & Jackson AH230-4, Fisher Scientific)

b) Acetonitrile, HPLC grade (Acros UN1648, Fisher Scientific)

c) Hexanes, HPLC grade (Tedia Company Inc UN1208, Fairfield, OH)

d) Toluene (T323-4, Fisher Scientific)

e) 10N Sodium Hydroxide (SS255-1, Fisher Scientific)

f) Pentafluorobenzyl bromide (PI-58220, Fisher Scientific)

g) 6N Hydrochloric acid (SA56-1, Fisher Scientific)

h) Triethylamine, HPLC grade (O4884-100, Fisher Scientific)

12) Standards (acid form)

a) *alpha*-Linolenic (U-62A, Nu-Chek Prep, Elysian, MN)

b) Arachidic (N-20A, Nu-Chek Prep)

c) Arachidonic (U-71A, Nu-Chek Prep)

d) Docosanoic (N-22A, Nu-Chek Prep)

e) Docosahexaenoic (U-84A), Nu-Chek Prep

f) Docosapentaenoic n-3 (U-101, Nu-Chek Prep)

g) Docosapentaenoic n-6 (U-102, Nu-Chek Prep)

h) Docosatetraenoic (U-83A, Nu-Chek Prep)

i) 11,14-Eicosadienoic (U-68A, Nu-Chek Prep)

j) 11-Eicosenoic (U-66A, Nu-Chek Prep)

k) Eicosapentaenoic (U-99A, Nu-Chek Prep)

l) *gamma*-Linolenic (U-63A, Nu-Chek Prep)

m) *homo-gamma*-Linolenic (U-69A, Nu-Chek Prep)

n) Lignoceric (Tetracosanoic) (N-24A, Nu-Chek Prep)

o) Linoleic (U-59A, Nu-Chek Prep)

p) Myristic (N-14A, Nu-Chek Prep)

q) Nervonic (U-88A, Nu-Chek Prep)

r) Oleic (U-46A, Nu-Chek Prep)

s) Palmitoleic (U-40A, Nu-Chek Prep)

t) Palmitic (N-16A, Nu-Chek Prep)

u) Stearic (N-18A, Nu-Chek Prep)

13) Isotopically Labeled Standards (acid form)

    a) Arachidic, d-39 (D-1617, C/D/N Isotopes, Pointe-Claire, Quebec, Canada)

    b) Arachidonic, d-8 (custom synthesis, Cayman Chemical or IsoSciences)

    c) Docosanoic, d-4 (custom synthesis, IsoSciences, Ambler, PA)

    d) Docosahexaenoic, d-5 (custom synthesis, IsoSciences)

    e) Eicosapentaenoic, d-5 (custom synthesis, IsoSciences)

    f) Lignoceric, d-4 (custom synthesis, IsoSciences)

    g) Linoleic, 13C (custom synthesis, IsoSciences)

    h) *alpha*-Linolenic, d-14 (custom synthesis, Cayman Chemical)

    i) Myristic, d-27 (I1-7220D, Cambridge Isotopes, Tewksbury, MA)

    j) Oleic, 13C (custom synthesis, IsoSciences)

    k) Palmitic, d-31 (I1-10006B, Cambridge Isotopes)

    l) Palmitoleic, d-14 (custom synthesis, Cayman Chemical)

    m) Stearic, d-35 (I1-7911A, Cambridge Isotopes)

E. Instrumentation

In the case of simple laboratory instrumentation (e.g., pipettes, vortex mixer, analytical balance, etc.) a product listed herein may be substituted with equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed. In the case of analysis instrumentation (e.g., GC components, mass spectrometer) equivalent performance must be demonstrated experimentally in accordance with DLS Policies and Procedures Manual if a product substitution is made. Equivalent performance must also be demonstrated in accordance with DLS policies and procedures when multiple analysis systems are used in parallel, even if they are of the exact same type. For details, see Job Aid **"JA-4030-I-01-GCMS Instrument Comparison and System Verification" found in Appendix B section C**.

1) Agilent GCMS (Agilent)

    a) GC model: 7890A

    b) Autosampler tray and tower model:7683 and 7683B

    c) Mass Spectrometer model: 5975C

    d) Software model: Mass Hunter GCMS 10.1.49

2) Thermo Scientific FREAS Mechanical Convention Oven model 625S (Thermo Fisher Scientific)

3) Savant Speedvac Plus SPD210-115 (Thermo Fisher Scientific)

4) Mixer Type 16700 Model # M16715 (Barnstead International, Dubuque, IA)

5) Magnetic stirrer (Baxter Scientific Products, Stone Mountain, GA)

6) Eppendorf micropipette (Eppendorf North America, Hauppauge, NY)

7) Eppendorf repeater pipette (Eppendorf North America)

8) Rainin Pos-D positive displacement pipettes various sizes (Mettler-Toledo Rainin LLC)

9) Gilson Microman M100 positive displacement pipette (Gilson)

10) Mettler Toledo AG 104 balance (Mettler-Toledo, LLC, Columbus, OH)

11) Hamilton Starlet 8-channel with auto-load arm (Hamilton Company)

    a) Various carriers (sample, reagent, and tip)

7. Calibration and Calibration Verification Procedures

A. Method Calibration

At the beginning of each run, an equilibration sample (typically the highest calibrator from current run) is run to determine that the retention times and analyte responses are within expected limits.

Eight calibration standards plus a reagent blank are prepared and extracted along with the samples. The calibration standards are injected at the beginning of the analytical run. Each analyte uses a five-point calibration curve (except ALNP, GLAP, and HGLP, which use six-point calibration curves). The following table lists calibration curve type and weighting associated with each analyte:

	Fatty Acid	**Analyte Code (µM)**	**Curve Type**	**Weighting**
1	*alpha*-Linolenic	ALN	linear	$1/x^2$
2	Arachidic	AR1	linear	$1/x^2$
3	Arachidonic	ARA	linear	$1/x^2$
4	Docosanoic	DA1	linear	$1/x^2$
5	Docosahexaenoic	DHA	linear	$1/x^2$
6	Docosapentaenoic n-3	DP3	linear	$1/x^2$
7	Docosapentaenoic n-6	DP6	linear	$1/x^2$
8	Docosatetraenoic	DTA	quadratic	$1/x^2$
9	11-14, Eicosadienoic	ED1	quadratic	$1/x^2$
10	11-Eicosenoic	EN1	quadratic	$1/x^2$
11	Eicosapentaenoic	EPA	quadratic	$1/x^2$
12	*gamma*-Linolenic	GLA	linear	$1/x^2$
13	*homo-gamma*-Linolenic	HGL	quadratic	$1/x^2$
14	Lignoceric	LG1	linear	$1/x^2$
15	Linoleic	LNA	linear	$1/x^2$
16	Myristic	MR1	quadratic	$1/x^2$
17	Nervonic	NR1	quadratic	$1/x^2$
18	Oleic	OL1	linear	$1/x^2$
19	Palmitoleic	PL1	quadratic	$1/x^2$
20	Palmitic	PM1	quadratic	$1/x^2$
21	Stearic	ST1	quadratic	$1/x^2$

Following the completion of the analytical run, calibration data are calculated using MassHunter software (see calculation section at the end of Section 8 for analyte specific calibration curve information). Each chromatographic peak is reviewed for correct delineation of the baseline and proper

retention. Fatty acid concentrations in unknown samples are calculated from their respective calibration curves. The calculated concentrations for the calibration solutions should fall within 15% of the target values, with the exception of low concentration calibrators that are approaching the LOD.

After verifying integration is correct, if the calibration curve R^2 is < 0.985 and any point deviates more than 15% from its nominal value, then one calibration point may be removed to assess whether the calibration curve fit is improved.

This method uses toluene as the calibration matrix. RBC matrix-based calibration was evaluated by comparing the average slopes for each of the 21 FA from six 10-point toluene-based calibration curves and six 10-point toluene-based calibration curves spiked with an RBC pool. A ≤ 5% slope difference on average was observed between the six RBC- and six toluene-based calibrations for 11/21 FA and > 5% difference was observed for 10/21 FA (8% to 11% for 6/10 FA and 26% to 42% for 4/10 FA). Unfortunately, the RBC matrix adds variability to the calibration curves, which is not desirable. Across all 21 FA the mean CV of the six slopes was 8% for RBC-based, but only 4% for toluene-based, which was similar to the historical mean CV of 5% for 25 toluene-based calibration curves. For the 11/21 FA with slope differences ≤ 5%, the mean CV of the six slopes was 4% (RBC-based) and 3% (toluene-based). For the 10 FA with slope differences > 5%, the mean CV of the six slopes was 13% (RBC-based) and 5% (toluene-based). For the 4/10 FA with slope differences > 20%, the mean CV of the six slopes was 22% (RBC-based) and 7% (toluene-based). Additionally, the R^2 indicates more variability with the RBC-based calibration curves. The mean R^2 across the 21 FA from this comparison was 0.944 (RBC-based) and 0.994 (toluene-based), which was similar to the historical mean R^2 of 0.995 for 25 toluene-based calibration curves. For the 11/21 FA with slope differences ≤ 5%, the mean R^2 was 0.988 (RBC-based) and 0.997 (toluene-based). For the 10 FA with slope differences >5%, the mean R^2 was 0.895 (RBC-based) and 0.991 (toluene-based). For the 4/10 FA with slope differences > 20%, the mean R^2 was 0.832 (RBC-based) and 0.989 (toluene-based), suggesting that these FA show more variable calibration statistics (CV and R^2) than other FA. We also investigated calibration curve type as another potential source of the large slope differences. However, changing the calibration type from quadratic to linear did not change the number of FA within the 5% slope difference. Thus, we continue to use pure FA, dissolved in toluene, for our calibration curves with our established calibration curve types.

Since a multi-point calibration curve is included in every run, there is no additional calibration verification required. However, calibration verification is conducted at least every 6 months to verify an expanded calibration curve. For details, see Job Aid **"JA-4030-G-01-Calibration and Calibration Verification" found in Appendix B section A**.

RBC-based reference materials are not available for FA.

External proficiency testing programs currently do not exist for FA in RBC. An in-house proficiency testing program has been developed and is conducted at least twice a year. For details, see Job Aid **"JA-4030-G-02-Alternative In-House Proficiency Testing" found in Appendix B section A**.

Results from a series of in-house ruggedness testing experiments designed to assess accuracy changes when certain experimental parameters are varied are presented in **Appendix C.**

B. Instrument Calibration

1) Gas Chromatograph-Mass Spectrometer

The calibration of the mass spectrometer is scheduled on an annual basis as part of a preventative maintenance program and is performed by a qualified service engineer; it is also performed whenever the system is vented for routine maintenance.

The tuning and mass calibration of the quadrupole of the mass spectrometer is performed using PFDTD (Fluoroether E-3) and by running the instrument in CI autotune. Please refer to the User's Manual and the Job Aid "**JA-4030-I-02-GCMS Calibration, Startup and Shutdown Procedures" found in Appendix B section C** for additional details.

2) Hamilton Microlab Starlet Calibration Verification

Twice a year a Hamilton service engineer performs preventative maintenance including volume verification at 10 µL and 1000 µL.

A volume verification of the various steps of the method can also be performed either gravimetrically (e.g., using pre-weighed sample vessels) or photometrically (e.g., using a microplate reader and a suitable chromophore) by the user. Precision should be equal or better than that obtained using manual pipettes.

3) Pipettes (air displacement and positive displacement)

Pipettes are calibrated or calibration is verified on a semi-annual basis.

4) Balances

Balances are calibrated annually and verified as needed using calibrated weights.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

A. Preliminaries

1) Sample ID numbers must be scanned into the computer if they are barcoded.

2) Allow frozen RBC, quality control pools, and standards to reach ambient (+15°C to +30°C) temperature, then vortex each sample individually or as a set prior to aliquoting. Visually check each sample for unusual specimen color.

3) Set up Excel run sheet containing sample IDs prior to starting sample preparation. This will be used later to build a sequence to run the samples and also used to keep track of any problems that may occur during the sample preparation.

4) A typical run consists of 9 calibrators (includes a blank), 3 QC samples (first set), 38-75 patient samples, 3 QC samples (second set).

5) A brief description of the entire sample preparation process is shown below. For detailed sample preparation see Job Aid "JA-4030-G-03-Sample Preparation" found in Appendix B section A.

B. Sample Preparation

1) All steps are done in the fume hood except when items are in the Hamilton Microlab Starlet, oven, or Speedvac.

2) Using an air displacement pipette, add 100 µL of standards and reagent blank to 16- x 100-mm screw cap culture tube.

3) Using a positive displacement pipette, add 100 µL of RBC to 16- x 100-mm screw cap culture tube.

4) Add 100 µL of internal standard mixture to each 16- x 100-mm culture tube.

5) Add 2 mL of acetonitrile: 6N hydrochloric acid (90:10, v:v).

6) Cap each tube tightly using Teflon-lined caps.

7) Heat samples in oven for 45 minutes at 104°C.

8) Remove samples from oven and allow samples to cool.

9) Add 2mL of a solution of methanol:10N sodium hydroxide (90:10, v:v).

10) Recap tightly with the same teflon caps and heat in oven for 45 minutes at 104°C.

11) Remove samples from oven and allow samples to cool.

12) Re-acidify samples by addition of 350 µL of 6N HCl.

13) Proceed with Hamilton liquid-liquid extraction steps (step C).

C. Hamilton Liquid-Liquid Extraction

1) The Hamilton Microlab Starlet is used for a triple liquid-liquid extraction of the samples.

2) Hamilton preparation and function check

   a) Run daily maintenance function check

   b) Fill tip trays and hexane troughs

   c) Verify tip waste is not too full

3) A brief description of the triple extraction is as follows:

   a) 3 mL of hexane added to all samples

   b) after mixing 2 mL of the hexane extract is transferred to 13 x 100 mm culture tubes

   c) 2 mL of hexane added to all samples

   d) after mixing 2 mL of the hexane extract is transferred to 13 x 100 mm culture tubes

   e) 2 mL of hexane added to all samples

   f) after mixing 2 mL of the hexane extract is transferred to 13 x 100 mm culture tubes

4) Proceed with Sample Preparation for GCMS (step D).

   a) Note: Hexane extracts can be stored up to 5 days deep frozen (-50°C to -90°C). After storage, allow hexane extracts to come to room temperature prior to continuing with Sample Preparation for GCMS (step D).

D. Sample Preparation for GCMS

1) Dry down samples in the Speedvac (@ 45°C). It takes 45 minutes to dry down ~80 tubes each containing 6mL hexane.

2) Add 100 µL of prepared derivatizing solution (as shown in the table in section 6.a.3) to each tube and allow reaction to react for 15 minutes at room temperature. The derivatizing solution should be prepared within 15 minutes of use.

3) Reconstitute residue with 1.0 mL of hexane.

4) Transfer the reconstituted sample using a clean Pasteur pipette to a labeled GCMS autosampler vial containing a glass insert, then immediately cap. It is important to transfer from the top of the reconstituted sample to avoid the denser PFBBr at the bottom of the tube.

E. GCMS Instrument Preparation

An Agilent GCMS system is used to quantitate dietary FA in extracted RBC.

1) GC preparation and function checks

   a) Prior to each run

      1. Change septum

      2. Fill toluene and hexane wash vials with fresh solvent before each run

      3. Empty waste vials

      4. Verify syringe is moving freely

   b) Typically, the liner should be changed every 2-3 runs

   c) Load autosampler vials into appropriate positions according to sequence.

2) Typical Instrument Method (oven ramps, inlet temperatures, and split ratio are adjusted as needed)

   a) Oven: 230°C for 0 min, then 5°C/min to 234 °C for 7 min; then 1°C/min to 250°C for 3 min

   b) Front inlet: injector temperature: 240°C; initial pressure: ~47 psi; total flow: 105 mL/min; septum purge flow: 3 mL/min; gas saver: on at 2.0 min with a gas saver flow of 20.0 mL/min; gas type: Helium; typical split ratio: 50:1.

   c) MSD transfer line: initial temperature: 250°C.

   d) Injector: solvent A and B washes (pre and post injection): 5 each at 8 µL; sample washes: 3 at 8 µL; sample pumps: 2; injection volume: 1.0-µL; syringe size: 10.0-µL.

   e) 3.5 min solvent delay

   f) EMV mode relative

   g) CI flow rate: 40

   h) Capillary column: TG-POLAR; maximum temperature: 275°C; nominal length: 60.0 m; nominal diameter: 0.25 mm; nominal film thickness: 0.25 µm; mode: constant flow at 2 mL/min

   i) Outlet pressure: vacuum

j) Table of Selected Ion Monitoring (SIM) masses. Prior to starting the next run, verify the GCMS time segments don't need to be updated. For detailed instructions on verifying time segments see Job Aid "**JA-4030-I-03-GCMS Time Segment Assignments" found in Appendix B section C**.

	No.	Fatty Acid	Analyte code	SIM (M/Z)	Internal Standard used for quantitation
Segment 1	1	Myristic-d27	MR1_IS_N	254.5	
	2	Myristic	MR1P	227.4	MR1_IS_N
	3	Palmitic-d31	PM1_IS_N	286.6	
	4	Palmitic	PM1P	255.45	PM1_IS_N
	5	Palmitoleic-d14	PL1_IS_N	267.4	
	6	Palmitoleic	PL1P	253.45	PL1_IS_N
Segment 2	7	Stearic-d35	ST1_IS_N	318.6	
	8	Stearic	ST1P	283.4	ST1_IS_N
	9	13C-Oleic	OL1_IS_N	299.5	
	10	Oleic	OL1P	281.5	OL1_IS_N
Segment 3	11	13C- Linoleic	LNA_IS_N	297.5	
	12	Linoleic	LNAP	279.4	LNA_IS_N
	13	*alpha*-linolenic-d14	ALN_IS_N	291.4	
	14	*gamma*-Linolenic	GLAP	277.4	ALN_IS_N
	15	*alpha*-Linolenic	ALNP	277.4	ALN_IS_N
	16	Arachidic-d39	AR1_IS_N	350.3	
	17	Arachidic	AR1P	311.4	AR1_IS_N
Segment 4	18	11-Eicosenoic	EN1P	309.4	AR1_IS_N
	19	11,14-Eicosadienoic	ED1P	307.4	AR1_IS_N
	20	*homo-gamma*-Linolenic	HGLP	305.4	ALN_IS_N
	21	Arachidonic-d8	ARA_IS_N	311.4	
	22	Arachidonic	ARAP	303.4	ARA_IS_N
Segment 5	23	Docosanoic-d4	DA1_IS_N	343.5	
	24	Docosanoic	DA1P	339.4	DA1_IS_N
	25	Eicosapentaenoic-d5	EPA_IS_N	306.4	
	26	Eicosapentaenoic	EPAP	301.4	EPA_IS_N
Segment 6	27	Docosahexaenoic-d5	DHA_IS_N	332.4	
	28	Docosatetraenoic	DTAP	331.4	DHA_IS_N
	29	Docosapentaenoic n-6	DP6P	329.4	DHA_IS_N
	30	Docosapentaenoic n-3	DP3P	329.4	DHA_IS_N
	31	Docosahexaenoic	DHAP	327.4	DHA_IS_N
	32	Tetracosanoic-d4	LG1_IS_N	371.5	
	33	Tetracosanoic	LG1P	367.4	LG1_IS_N
	34	Nervonic	NR1P	365.4	LG1_IS_N

F. Processing and Reporting a Run

1) The Agilent MassHunter software is used to review/process a run. A LIMS database is used for additional levels of data review by the analyst, project lead, QA officer, and supervisor and for data reporting. For complete instructions see Job Aid **"JA-4030-DR-02-Processing and Reporting a Run" found in Appendix B section D**.

a) Reviewing the chromatography

- When the run is finished acquiring the data, the data is reviewed in MassHunter Quantitative Analysis. Chromatograms for each fatty acid and stable isotope labeled standard are checked for retention times, peak shape, peak separation, intensity and/or potential interferences.

b) Quantitation and integration of the completed data file

- Generate a batch table using auto integration.
- Review integrations and make any necessary integration corrections either using the manual or auto integration option. Auto integration is preferred over manual integration.

- Print the results for each analyte as a PDF to allow future review and documentation (routine procedure) or print hardcopies (exception).
- Save the results in an excel file to import into the LIMS database.
- Import the results file into the LIMS database for further data review.

c) A LIMS database is used for additional levels of data review by the analyst, project lead, QA officer, and supervisor and for data reporting. For complete instructions see Job Aid **"JA-4030-DR-03-STARLIMS Data Review and Criteria" found in Appendix B section D**.

G. Calculations

The MassHunter software performs all calculations using linear or quadratic regression of the peak area response of the extracted calibration solutions versus their nominal concentrations. Fatty acid molar concentrations in unknown samples are calculated using the regression parameters. Calculations are based on the single analysis of five standard concentrations according to the following formula: Concentration = Response factor (amount/area ratio) x peak area ratio x multiplier (dilution factor).

The following table lists calibration curve type and weighting associated with each analyte:

	Fatty Acid	Analyte Code (μM)	Curve Type	Weighting
1	*alpha*-Linolenic	ALN	linear	$1/x^2$
2	Arachidic	AR1	linear	$1/x^2$
3	Arachidonic	ARA	linear	$1/x^2$
4	Docosanoic	DA1	linear	$1/x^2$
5	Docosahexaenoic	DHA	linear	$1/x^2$
6	Docosapentaenoic n-3	DP3	linear	$1/x^2$
7	Docosapentaenoic n-6	DP6	linear	$1/x^2$
8	Docosatetraenoic	DTA	quadratic	$1/x^2$
9	11-14, Eicosadienoic	ED1	quadratic	$1/x^2$
10	11-Eicosenoic	EN1	quadratic	$1/x^2$
11	Eicosapentaenoic	EPA	quadratic	$1/x^2$
12	*gamma*-Linolenic	GLA	linear	$1/x^2$
13	*homo-gamma*-Linolenic	HGL	quadratic	$1/x^2$
14	Lignoceric	LG1	linear	$1/x^2$
15	Linoleic	LNA	linear	$1/x^2$
16	Myristic	MR1	quadratic	$1/x^2$
17	Nervonic	NR1	quadratic	$1/x^2$
18	Oleic	OL1	linear	$1/x^2$
19	Palmitoleic	PL1	quadratic	$1/x^2$
20	Palmitic	PM1	quadratic	$1/x^2$
21	Stearic	ST1	quadratic	$1/x^2$

Molar concentrations (μM) are converted to weight concentrations (mg/L) using the following calculation: [FA (μM) * FA molecular weight (g/mol)]/1000.

The weight concentrations are used to calculate wt % of total for each fatty acid as follows:

(1) Calculate total FA: 21 individual fatty acid weight concentrations (mg/L) are summed

(2) Calculate individual FA wt %: divide each FA (n=21) by the total FA to create 21 FA weight percentages [individual FA (mg/L)/total FA (mg/L)]

(3) Omega-3 Index is calculated for each sample as follows: The weight percentages of eicosapentaenoic and docosahexaenoic acid are summed

Note: The weight percentages for each fatty acid and the Omega-3 Index are reported for this method.

H. System Maintenance (other than daily maintenance)

(1) Agilent GCMS

Preventative maintenance is performed annually by an authorized Agilent service engineer. For GCMS instrument calibration, startup and shutdown procedures see Job Aid **"JA-4030-I-02-GCMS Calibration, Startup and Shutdown Procedures" found in Appendix B section C**.

Additional GCMS related system maintenance consists of:

a) Trim the column: as needed (~ every 2 months).

b) Source cleaning: as needed (determined by running an NCI autotune to look for elevated background peaks).

c) Replacement of the gas tanks: helium tank approximately every 3 months or when the tank pressure falls below ~500PSI and the methane tank approximately once per year or when the tank pressure falls below ~500PSI.

(2) Hamilton Microlab Starlet

Preventative maintenance is performed twice per year by an authorized Hamilton service engineer.

I. CDC Modifications

This document represents the first official method for the CDC lab for measuring 21 Fatty Acids in RBC.

9. Reportable Range of Results (AMR – Analytical Measurement Range)

This method uses molar concentrations (µM) to calculate weight concentrations (mg/L) of each fatty acid. The weight concentrations are then converted to weight percent of each fatty acid which are reported. The range of weight concentrations for each fatty acid should be between the lowest and highest standards whose approximate values are shown in the table below. An unknown with a weight concentration exceeding the upper limit is re-analyzed after appropriate dilution with a smaller RBC volume.

	Fatty Acid	Analyte Code	Lower Limit (mg/L)	Upper Limit (mg/L)
1	*alpha*-Linolenic	ALN	0.908	71.0
2	Arachidic	AR1	0.281	10.9
3	Arachidonic	ARA	21.3	555
4	Docosanoic	DA1	1.87	48.7
5	Docosahexaenoic	DHA	2.59	336.6
6	Docosapentaenoic n-3	DP3	2.47	107
7	Docosapentaenoic n-6	DP6	0.526	68.3
8	Docosatetraenoic	DTA	2.95	64.0
9	11,14-Eicosadienoic	ED1	0.302	11.8
10	11-Eicosenoic	EN1	0.310	12.1
11	Eicosapentaenoic	EPA	1.08	28.1
12	*gamma*-Linolenic	GLA	0.409	31.9
13	*homo-gamma*-Linolenic	HGL	1.42	55.4
14	Lignoceric	LG1	3.26	70.4
15	Linoleic	LNA	11.6	302
16	Myristic	MR1	1.16	30.0
17	Nervonic	NR1	4.29	92.9
18	Oleic	OL1	12.0	312
19	Palmitoleic	PL1	3.21	83.0
20	Palmitic	PM1	22.4	874
21	Stearic	ST1	7.54	294

Note: There is no known maximum acceptable dilution for the majority of FA. When possible, avoid small volume pipetting and minimize use of serial dilutions when generating diluted samples.

10. Quality Control (QC) Procedures

A. Blind Quality Controls

Blind QC specimens can be inserted into the mix of patient specimens. These QC specimens are often prepared at two levels that would be encountered in patient samples; the labels used are identical to those used for patient samples. One blind QC specimen randomly selected for concentration is included at a randomly selected location in every 20 patient specimens analyzed. Alternatively, open label blind QC specimens can be used where the analyst knows that the sample is a blind QC but does not know what pool the sample is from. Open label blind QCs are only used if one can choose from at least 6 different pools and the analyte concentrations are similar to those found in patient samples. After a run is completed, used blind QC are removed from the run, marked with a black dot on the cap to indicate that the vial has been thawed, and returned to the blind QC box. This helps to identify which vials have been used. If a run needs to be repeated, the same blind QC can be inserted as in the initial run.

The use of blind QCs is optional but encouraged. Blind QCs are used in this method as a supplementary tool to assist in monitoring accuracy, precision, and aid in detecting errors; they are not used as part of the primary control procedures to determine if a run is out of control.

B. Bench Quality Controls

Bench QC specimens are prepared from a minimum of two pools that represent low and high levels of key FA (Palmitic, Oleic, Linoleic, Stearic and Arachidonic). This assay typically uses three RBC pools that represent low, medium, and high levels. Generally, a vial of each pool is thawed before every assay and duplicate aliquots of each QC pool are prepared for analysis in the same manner as patient samples. QC samples are analyzed as part of each run (placed at the beginning and end of each run).

The results from these pools are checked after each run using a multi-rule quality control system **[7]** based their characterization data, namely: the pool mean; the pooled within-run standard deviation associated with individual QC results measured in the same run (S_w); the standard deviation associated with individual QC results (S_i); and the standard deviation associated with run mean QC results (S_m). QC rules have been designed to accommodate the use of 1–3 different QC pools during a run, the use of 1–2 measurements of each pool per run, and as many instruments as needed. These QC rules are described in the DLS Policies and Procedures Manual and a relevant selection applicable to this assay is shown below. The system is declared “in control” if all individual QC results are within 2S limits; the run is accepted. If not, then the rules shown below are applied and the run is rejected if any condition is met; the run is declared “out of control”:

1) One QC result per pool (due to accidental loss of duplicate)

   a) If one of the three QC run **individual** results is outside a 2 S_i limit, reject run if:
      1. 1 3S Rule—Run result is outside a 3 S_i limit or
      2. 2 2S Rule—Two or more of the three run results are outside the same 2 S_i limit
      3. 10 X-bar Rule—Current and previous nine run results are on the same side of the characterization mean or
      4. R 4S Rule—Two consecutive standardized run results differ by more than 4 S_i

2) Two QC results per pool (all three pools have duplicate results)

   a) If one of the three QC run **means** is outside a 2 S_m limit, reject run if:
      1. 1 3S Rule—Run mean is outside a 3 S_m limit or
      2. 2 2S Rule—Two or more of the three run means are outside the same 2 S_m limit or
      3. 10 X-bar Rule—Current and previous nine run means are on the same side of the characterization mean

   b) If one of the six QC **individual** results is outside a 2 S_i limit, reject run if:

      1. Outlier—One individual result is beyond the characterization mean ± 4 S_i or
      2. R 4S Rule—Two or more of the within-run ranges in the same run exceed 4 S_w (i.e., 95 percent range limit)

Abbreviations:
S_i = Standard deviation of individual results (the limits are not shown on the chart unless run results are actually single measurements)
S_m = Standard deviation of the run means (the limits are shown on the chart)
S_w = Within-run standard deviation (the limits are not shown on the chart)

A QC program written in SAS is available from the DLS Quality Assurance Officer and should be used to apply these rules to QC data and generate Shewhart QC charts. No results for a given analyte are to be reported from an analytical run that has been declared “out of control” for that analyte as assessed by internal (bench) QC.

The initial limits are established by analyzing pool material in 20 consecutive runs. The SAS QC program is used to monitor the QC performance over time for potential shifts, trending, or changes in assay precision. For assays performed routinely, quarterly statistics (mean, SD, CV) are calculated for each pool and compared to the characterization target values. For assays performed infrequently, statistics are calculated at least annually. As more QC data become available (covering multiple lots of reagents, multiple analysts, etc.), the initial QC limits can be reevaluated and updated. QC limits can also be reevaluated and updated as a result of a non-conforming event when the assay shows a higher than expected out of control rate and the root cause investigation does not reveal a correctable course of action to bring the assay back into control. This needs to be documented by a CAPA in STARLIMS.

While a study is in progress, QC results are stored in the STARLIMS database. For runs that are not imported into the database (e.g., R&D, troubleshooting, research-type runs), QC results are stored electronically in the analyte-specific folder on the DLS network. At the conclusion of studies, complete QC records are prepared and submitted as a study QC report in STARLIMS for review by the laboratory chief, branch chief, and a DLS statistician.

C. Sample QC Criteria

Sample QC is a set of criteria used to evaluate the quality of individual test results within a run, and to evaluate the quality of the calibration curves associated with a run. In addition to the sample QC criteria set forth in the DLS Policies and Procedures Manual that pertain to the reportable range of concentration results and calibration curves, sample QC criteria are also established for method-specific concentration and non-concentration data associated with an individual test result.

The method-specific concentration and non-concentration parameters identified for sample QC evaluation, along with their associated thresholds and flagging protocols (‘Pass’, ‘Check’, ‘Warn’, ‘Fail’) are maintained and updated in the LIMS database, and sample QC assessment is performed and documented as part of the run review process. A sample QC result flagged as ‘Fail’ should not be reported. A sample QC result flagged ‘Warn’ or ‘Check’ should be reviewed both by the analyst and supervisor to determine if the quality of the result is suitable for reporting. Results that are flagged during sample QC evaluation may also be assigned one of a series of standard comment codes available in the LIMS database to identify the nature of the sample QC flag.

For details on how the sample QC criteria are used, see **“JA-4030-DR-03-STARLIMS Data Review and Criteria” in Appendix B, section D**. At a minimum, the following parameters are subject to sample QC evaluation in this method. Additional parameters may also be included as needed:

- One or more of the three highest level calibrators exceed a 15% difference from their target value; run is repeated
- Calibration curve R^2 is <0.985 (‘Fail’); run is repeated
- Blank (mg/L) is > lowest standard specified in test manager (mg/L) (‘Fail’); run is repeated
- Individual ISTD area is not within 3SD from the mean ISTD of the run (calculated from QC and unknowns) (‘Warn’); sample analysis is repeated
- Final concentration (mg/L) is <LOD (mg/L) (‘Warn’); result is reviewed to determine whether it is a code 37 or sample analysis needs to be repeated
- Diluted instrument result (mg/L) is <LOD (mg/L) (‘Fail’); result is coded no reportable (code 97)

- Final concentration (mg/L) is > highest calibrator specified in test manager (mg/L) ('Fail'); sample analysis is repeated with diluted sample

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

For initial steps to investigate QC failures, refer to "**JA-4030-DR-04-Out-of-Control Corrective Action" found in Appendix B, section D**. The following steps are provided as a general guideline for identifying possible problems resulting in "out of control" values for QC materials. If needed the troubleshooting process should be done in consultation with the supervisor and may involve additional experiments beyond what is indicated below.

(A) Look for sample preparation errors, e.g., analyst forgot or under-pipetted the isotope-labeled standard or derivatizing agent; or any sample preparation problems, e.g. vial evaporation during heating.

(B) Check the calibration of the pipettes.

(C) Check to make sure that the hardware is functioning properly. Make sure the MS is tuned properly, and the gas velocity is as required. Check the autosampler to make sure the injections are being made as programmed.

(D) If the steps outlined above do not result in correction of the "out of control" values for QC materials, discuss problem with supervisor.

(E) Do not report analytical results for runs not in statistical control.

12. Limitations of Method; Interfering Substances and Conditions

(A) The most common causes of imprecision are intermittently inaccurate micropipettors and pipetting errors.

(B) Stock standards, stable isotope labeled standards and specimens should be mixed thoroughly by vortexing before pipetting.

(C) Handling stocks and internal standards in stepwise sequential manner will minimize the chances of cross-contaminations.

(D) Changing of gloves after preparations of stock and working standards and internal standards is recommended to avoid any contamination.

(E) Interference testing was performed on this method as part of its method validation and is documented in **Appendix A**. This method has also undergone a series of in-house ruggedness testing experiments designed to assess how much method accuracy changes when certain experimental parameters are varied. A total of five parameters judged to most likely affect the accuracy of the method have been identified and tested. Testing generally consisted of performing replicate measurements on a test specimen with the selected parameter set at a value substantially lower and higher than that specified in this method while holding all other experimental variables constant. **The ruggedness testing findings for this method are presented in Appendix C**. Please refer to the *DLS Policies and Procedures Manual* for further information on ruggedness testing.

13. Reference Ranges (Normal Values)

The reference ranges from a small in-house pilot study (n=149) are tabulated below.

	Fatty Acid	Analyte Code	Geomean (wt %)	Max (wt %)	Min (wt %)
1	*alpha*-Linolenic	ALNP	0.15%	0.30%	0.08%
2	Arachidic	AR1P	0.32%	0.54%	0.18%
3	Arachidonic	ARAP	16.8%	20.6%	11.3%
4	Docosanoic	DA1P	1.70%	2.93%	1.06%
5	Docosahexaenoic	DHAP	3.04%	7.19%	1.70%
6	Docosapentaenoic n-3	DP3P	2.18%	2.97%	1.53%
7	Docosapentaenoic n-6	DP6P	0.79%	1.23%	0.47%
8	Docosatetraenoic	DTAP	4.24%	5.59%	2.45%
9	11,14-Eicosadienoic	ED1P	0.26%	0.41%	0.15%
10	11-Eicosenoic	EN1P	0.17%	0.33%	0.10%
11	Eicosapentaenoic	EPAP	0.40%	1.18%	0.21%
12	*gamma*-Linolenic	GLAP	0.06%	0.18%	0.02%
13	*homo-gamma*-Linolenic	HGLP	1.72%	3.06%	0.86%
14	Lignoceric	LG1P	5.37%	6.78%	4.13%
15	Linoleic	LNAP	10.2%	14.7%	7.40%
16	Myristic	MR1P	0.31%	0.52%	0.14%
17	Nervonic	NR1P	4.57%	6.48%	3.06%
18	Oleic	OL1P	10.8%	14.1%	8.09%
19	Palmitoleic	PL1P	0.34%	1.06%	0.13%
20	Palmitic	PM1P	21.7%	24.9%	18.8%
21	Stearic	ST1P	14.2%	15.9%	12.9%
		n-3 Index	3.45%	8.27%	1.97%

14. Critical Call Results (“Panic Values”)

There are no known critical call values for fatty acids.

15. Specimen Storage and Handling during Testing

Specimens are allowed to reach room temperature during preparation. The unused portion of the patient specimen is returned to deep frozen storage (-50°C to -90°C), typically ≤-60°C) as soon as possible. Once the derivatized samples have been completed, they are placed into the autosampler tray. If necessary, derivatized samples can be stored deep frozen (-50°C to -90°C), typically ≤-60°C, for a few days or weeks until chromatographed, but must be brought to room temperature prior to injection.

16. Alternate Methods for Performing Test of Storing Specimens if Test System Fails

Because the analysis of FA is inherently complex and challenging, there are no acceptable alternative methods of analysis. If the analytical system fails, we recommend that the extracted and/or derivatized specimens be stored deep frozen (-50°C to -90°C), typically ≤-60°C, until the analytical system is restored to functionality. All specimens should be brought to room temperature prior to chromatographic analysis.

17. Test Result Reporting System; Protocol for Reporting Critical Calls (If Applicable)

Test results are reported to the collaborating agency at a frequency and using a method determined by the study coordinator. Generally, data from this analysis are compiled with results from other analyses and sent to the responsible person at the collaborating agency as an Excel file, either through electronic mail or via FTP through the internet. Data are transmitted via the CLIA Director after review by the Lab Supervisor, Branch Chief, and a CDC Statistician.

For NHANES 1999+, data are transmitted electronically on a periodic basis to the contractor managing the NHANES-IT system, who in turn transfers the results to NCHS. Abnormal values are confirmed by the analyst, and codes for missing data are entered by the analyst and are transmitted as part of the data file. NCHS makes arrangements for the abnormal report notifications to the NCHS Survey Physician.

For smaller studies, electronic copies of a data report are sent; a hard copy of the data report may also be sent if requested.

18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

This protocol does not involve referral of specimens for testing the analytes of this method at another laboratory.

A STARLIMS database is used to track specimens and store results for all studies.

We recommend that records, including related QA\QC data, be maintained for 10 years after completion of studies. Only numerical identifiers should be used (e.g., Sample ID); all personal identifiers should be available only to the medical supervisor or project coordinator. Residual RBC from these analyses for non-NHANES studies are retained for at least 1 year after results have been reported and may then be returned or discarded at the request of the principal investigator. Very little residual material will be available after NHANES analyses are completed, however residual RBC is retained for at least 2 years after results have been publicly released; at that point, samples with sufficient volume (>0.2 mL) are returned to NHANES and samples with insufficient volume may be routinely autoclaved.

The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the specimens are stored deep frozen (-50°C to -90°C), typically ≤-60°C. The specimen ID is read by a barcode reader attached to the computer used to prepare the electronic specimen table for the analytical system. When the analyses are completed, the export file containing the electronic copy of the results is loaded in the LIMS database, and the analytical results are linked to the LIMS database by ID number. The analyst is responsible for keeping records of specimens prepared incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies. In general, these are documented using codes in the LIMS.

19. Method Performance Documentation

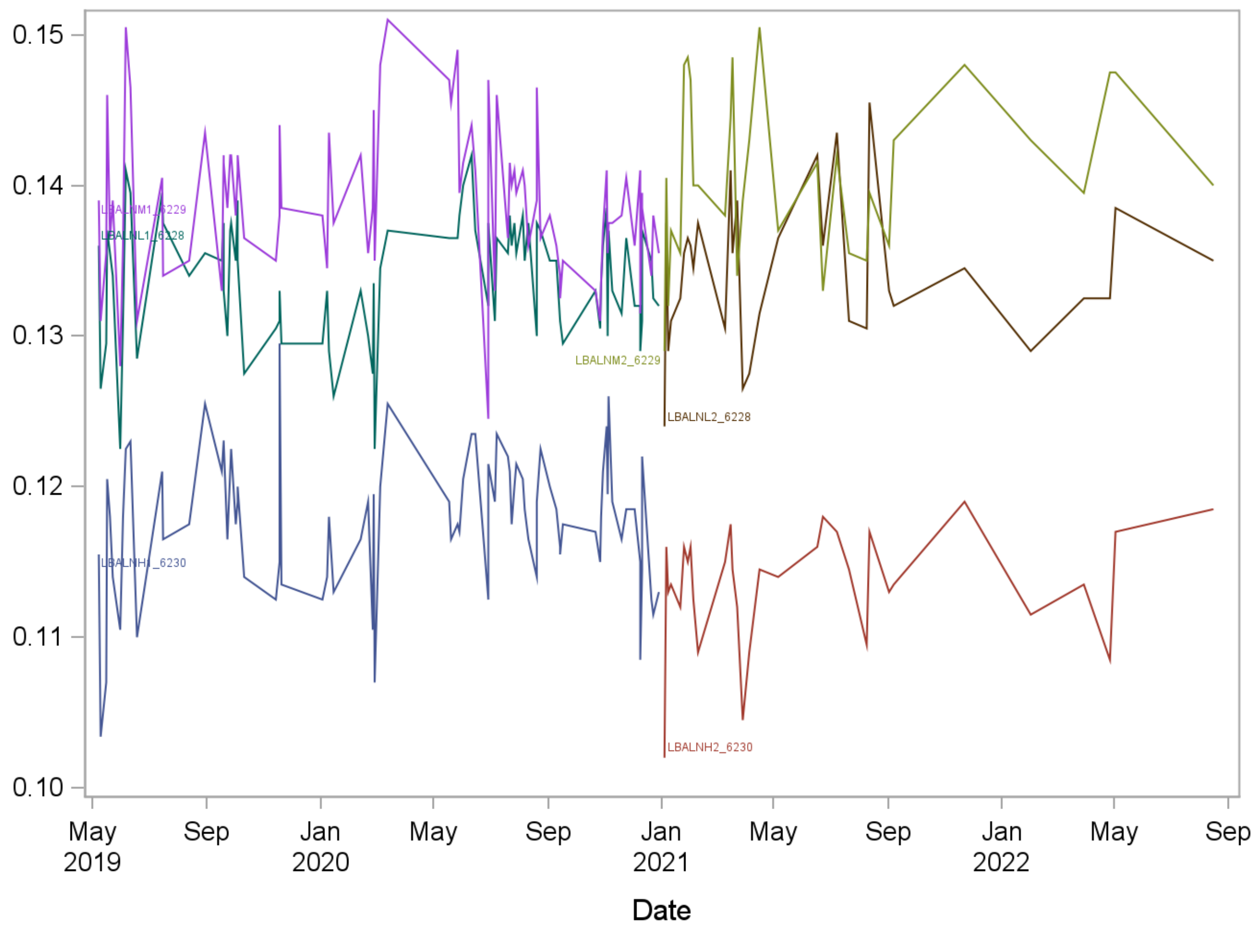
Method performance documentation for this method including accuracy, precision, sensitivity, specificity and stability is provided in **Appendix A** of this method documentation. **The approval of this procedure by the Branch Chief and CLIA Director denote that the method performance is fit for the intended use of the method.**

20. Summary Statistics and QC Graphs

Please see following pages.

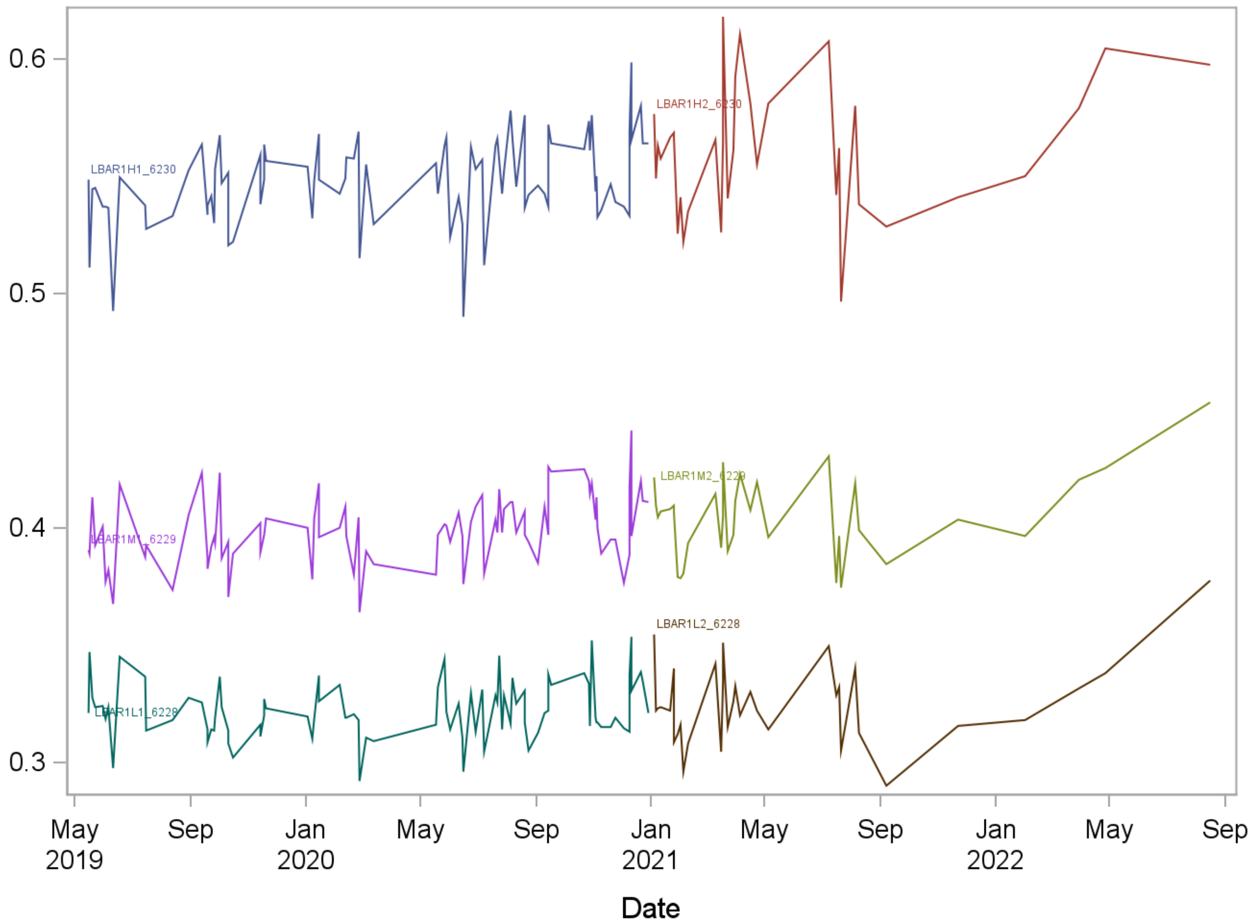
2019-2020 Summary Statistics and QC Chart LBXPAN (alpha-Linolenic acid (C18:3n-3) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBALNH1_6230	85	08MAY19	29DEC20	0.11778	0.00464	3.9
LBALNH2_6230	32	04JAN21	16AUG22	0.11352	0.00388	3.4
LBALNL1_6228	85	08MAY19	29DEC20	0.13384	0.00395	3.0
LBALNL2_6228	32	04JAN21	16AUG22	0.13430	0.00488	3.6
LBALNM1_6229	85	08MAY19	29DEC20	0.13885	0.00501	3.6
LBALNM2_6229	32	04JAN21	16AUG22	0.14072	0.00555	3.9



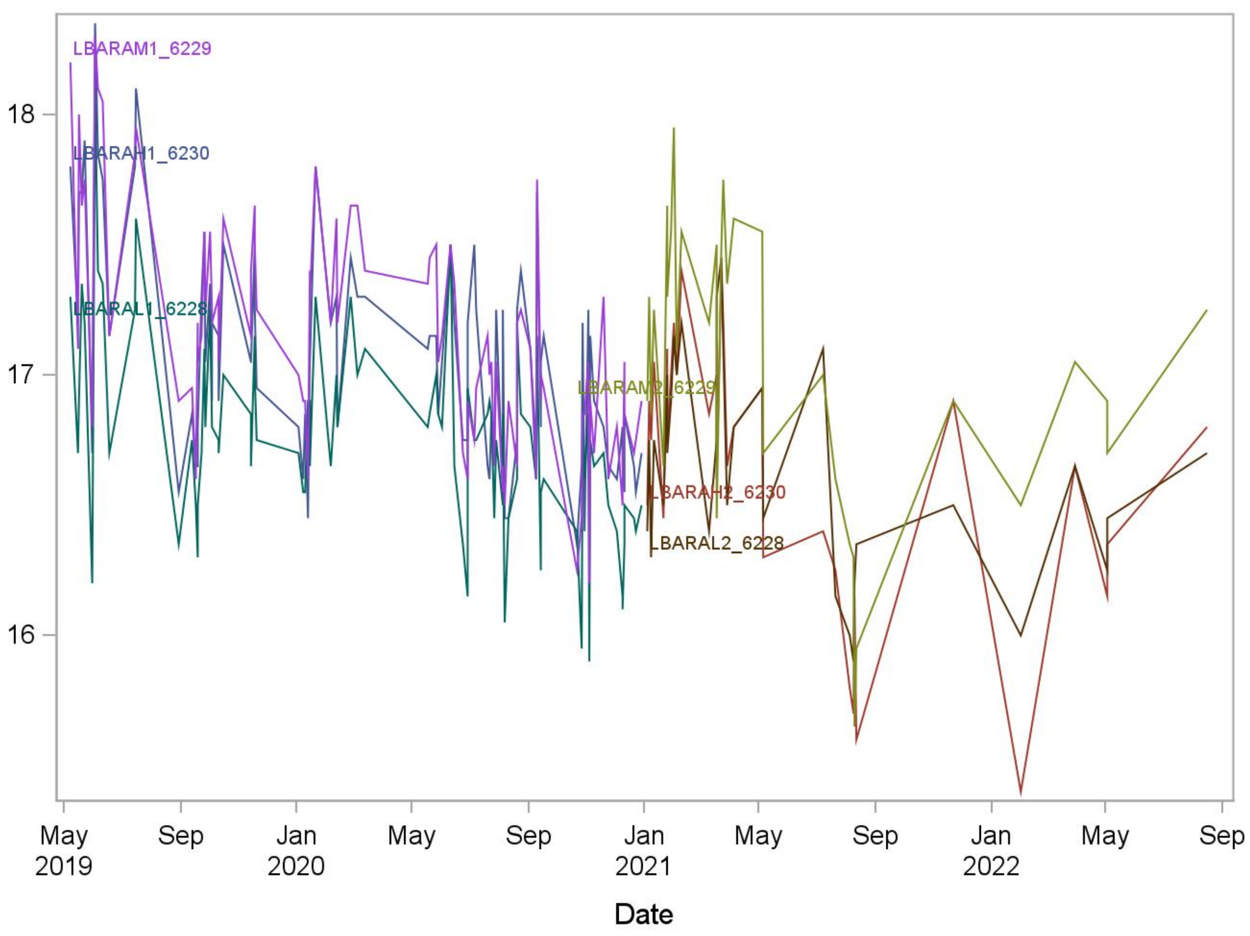
2019-2020 Summary Statistics and QC Chart
LBXP1A (Arachidic acid (C20:0) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBAR1H1_6230	88	16MAY19	29DEC20	0.54799	0.01847	3.4
LBAR1H2_6230	34	04JAN21	16AUG22	0.56156	0.02821	5.0
LBAR1L1_6228	88	16MAY19	29DEC20	0.32214	0.01200	3.7
LBAR1L2_6228	34	04JAN21	16AUG22	0.32513	0.01771	5.4
LBAR1M1_6229	88	16MAY19	29DEC20	0.39911	0.01477	3.7
LBAR1M2_6229	34	04JAN21	16AUG22	0.40475	0.01775	4.4



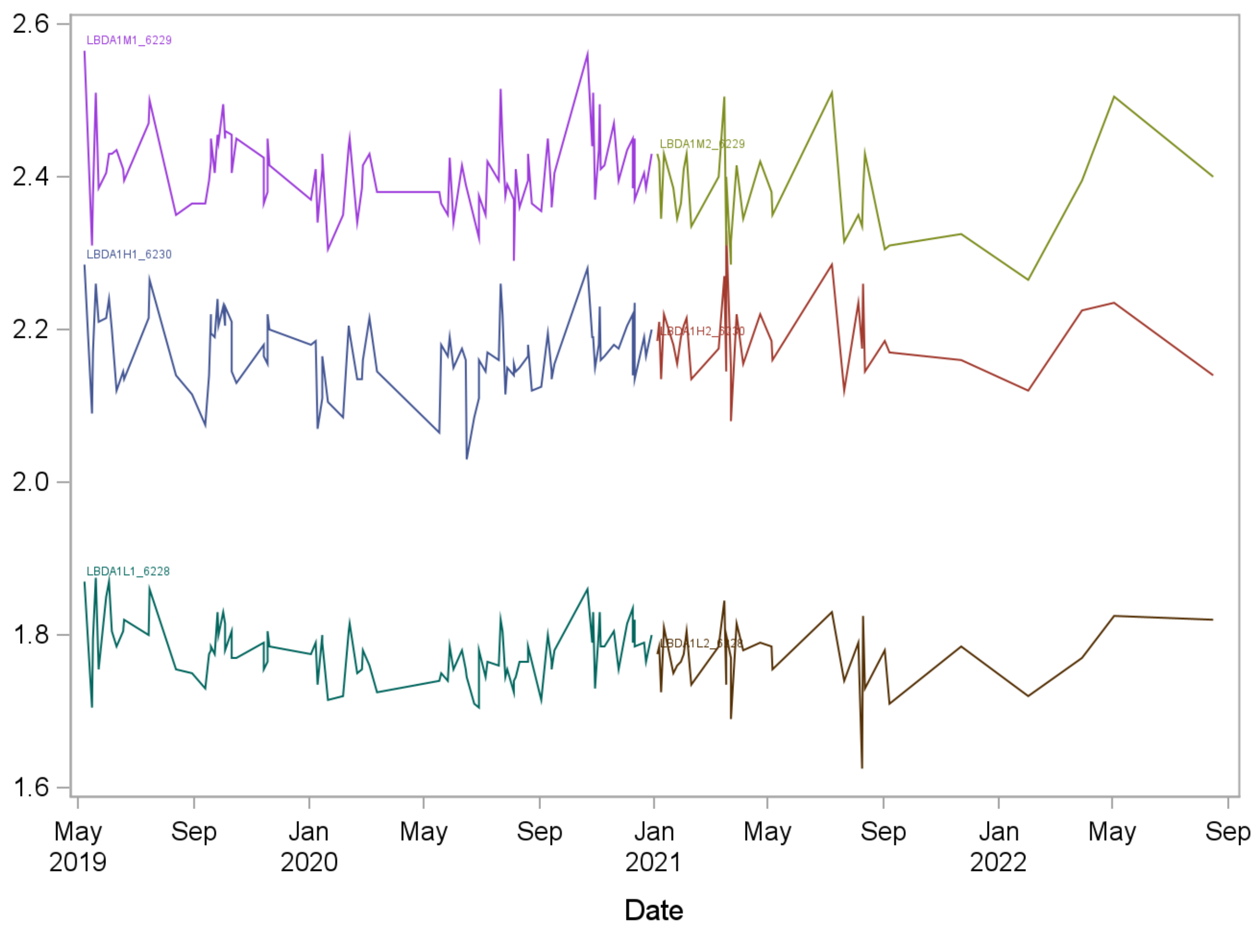
2019-2020 Summary Statistics and QC Chart
LBXPRA (Arachidonic acid (C20:4n-6) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBARAH1_6230	92	08MAY19	29DEC20	17.05870	0.42255	2.5
LBARAH2_6230	32	04JAN21	16AUG22	16.62500	0.50959	3.1
LBARAL1_6228	92	08MAY19	29DEC20	16.75870	0.37340	2.2
LBARAL2_6228	32	04JAN21	16AUG22	16.64375	0.40076	2.4
LBARAM1_6229	92	08MAY19	29DEC20	17.13533	0.44373	2.6
LBARAM2_6229	32	04JAN21	16AUG22	17.02500	0.53941	3.2



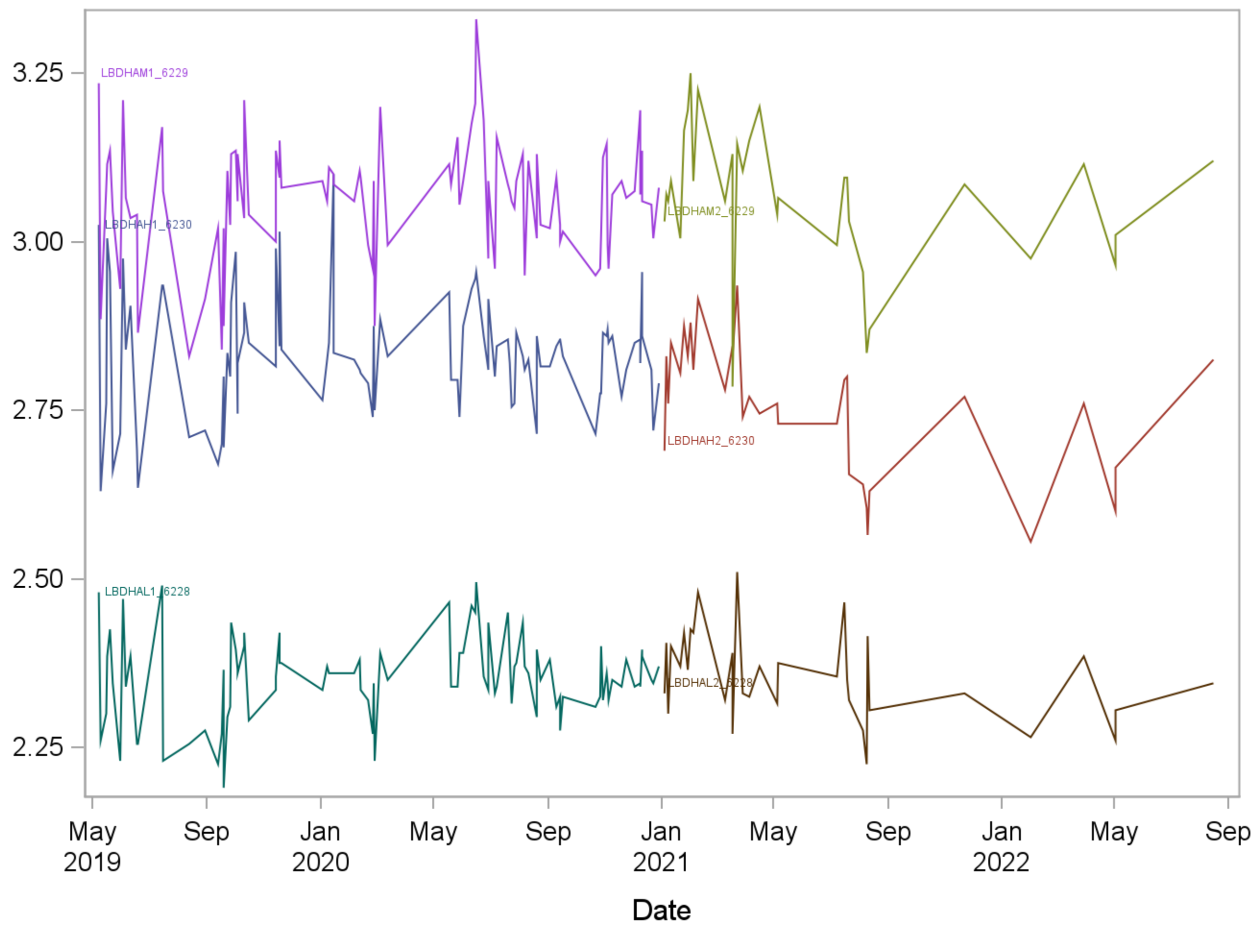
2019-2020 Summary Statistics and QC Chart
LBXPDA (Docosanoic acid (C22:0) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBDA1H1_6230	95	08MAY19	29DEC20	2.16968	0.04838	2.2
LBDA1H2_6230	34	04JAN21	16AUG22	2.18750	0.05231	2.4
LBDA1L1_6228	95	08MAY19	29DEC20	1.77979	0.03781	2.1
LBDA1L2_6228	34	04JAN21	16AUG22	1.77015	0.04497	2.5
LBDA1M1_6229	95	08MAY19	29DEC20	2.40874	0.05157	2.1
LBDA1M2_6229	34	04JAN21	16AUG22	2.37779	0.06121	2.6



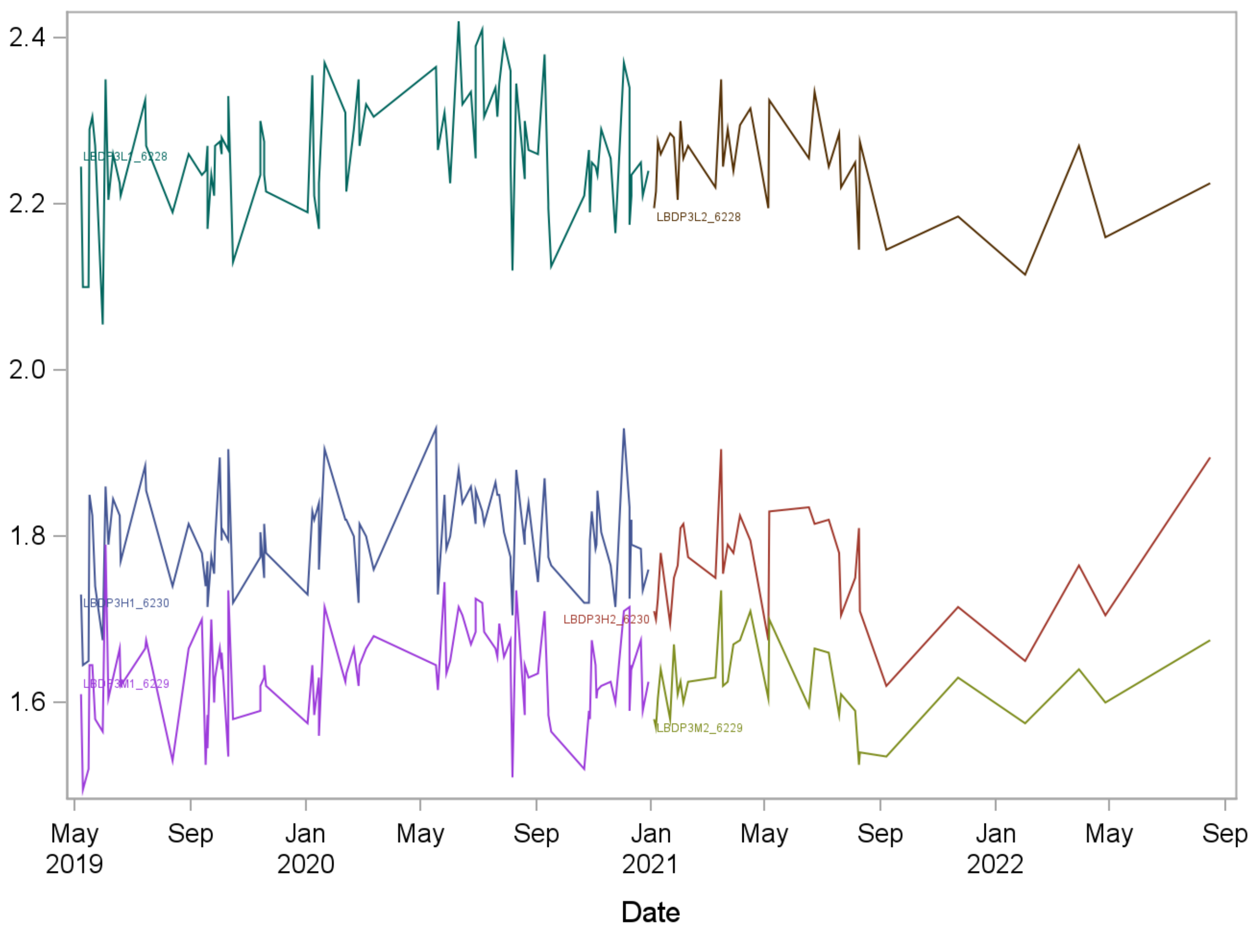
2019-2020 Summary Statistics and QC Chart
LBXPHA (Docosahexaenoic acid (C22:6n-3) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBDHAH1_6230	95	08MAY19	29DEC20	2.83137	0.08822	3.1
LBDHAH2_6230	33	04JAN21	16AUG22	2.75682	0.09882	3.6
LBDHAL1_6228	95	08MAY19	29DEC20	2.35247	0.06106	2.6
LBDHAL2_6228	33	04JAN21	16AUG22	2.35515	0.06589	2.8
LBDHAM1_6229	95	08MAY19	29DEC20	3.06342	0.08907	2.9
LBDHAM2_6229	33	04JAN21	16AUG22	3.05606	0.11087	3.6



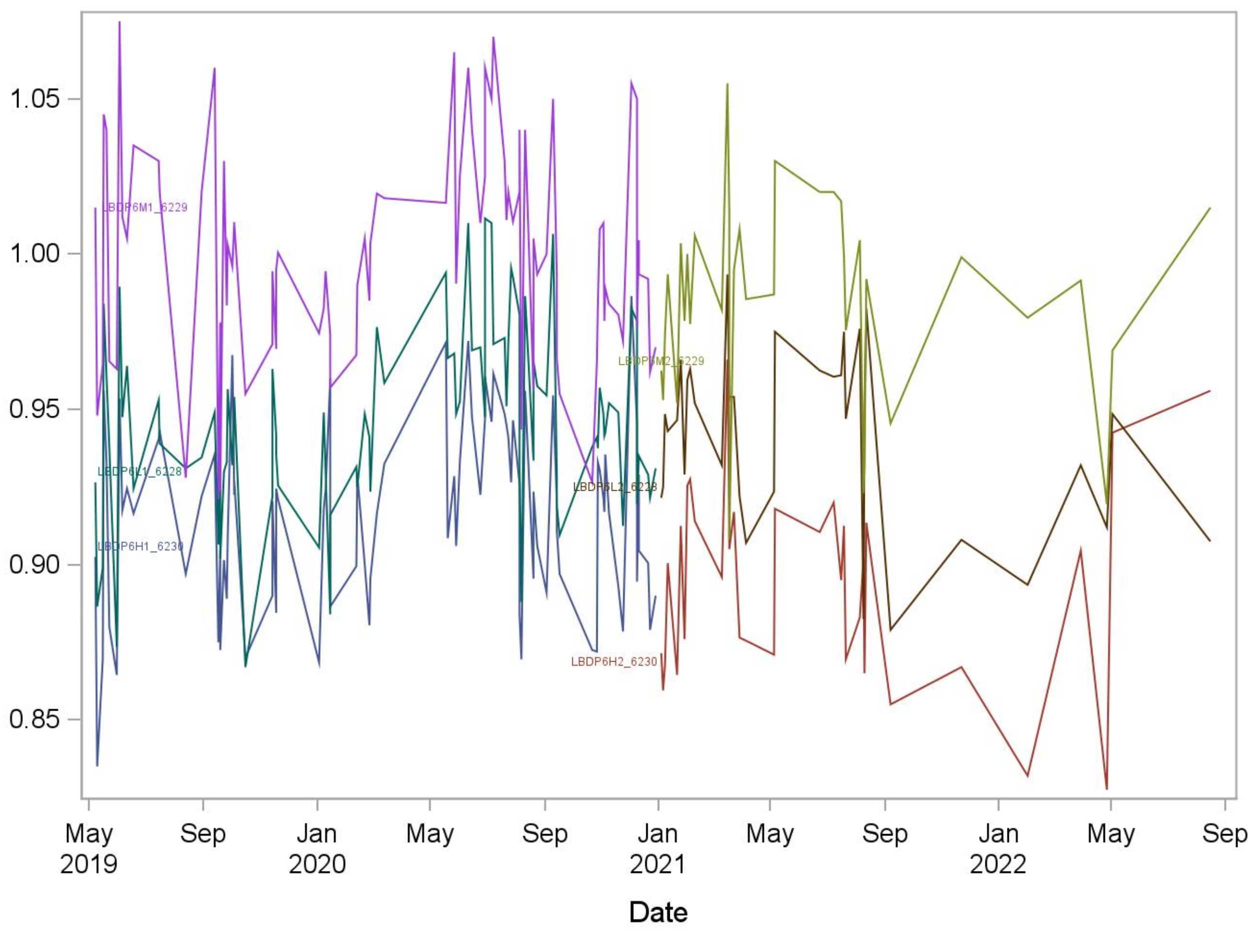
2019-2020 Summary Statistics and QC Chart
LBXPD3 (Docosapentaenoic acid 3 (C22:5n-3) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBDP3H1_6230	93	08MAY19	29DEC20	1.79688	0.05745	3.2
LBDP3H2_6230	33	04JAN21	16AUG22	1.76379	0.06451	3.7
LBDP3L1_6228	93	08MAY19	29DEC20	2.26177	0.07173	3.2
LBDP3L2_6228	33	04JAN21	16AUG22	2.24621	0.05667	2.5
LBDP3M1_6229	93	08MAY19	29DEC20	1.63441	0.05719	3.5
LBDP3M2_6229	33	04JAN21	16AUG22	1.62121	0.04937	3.0



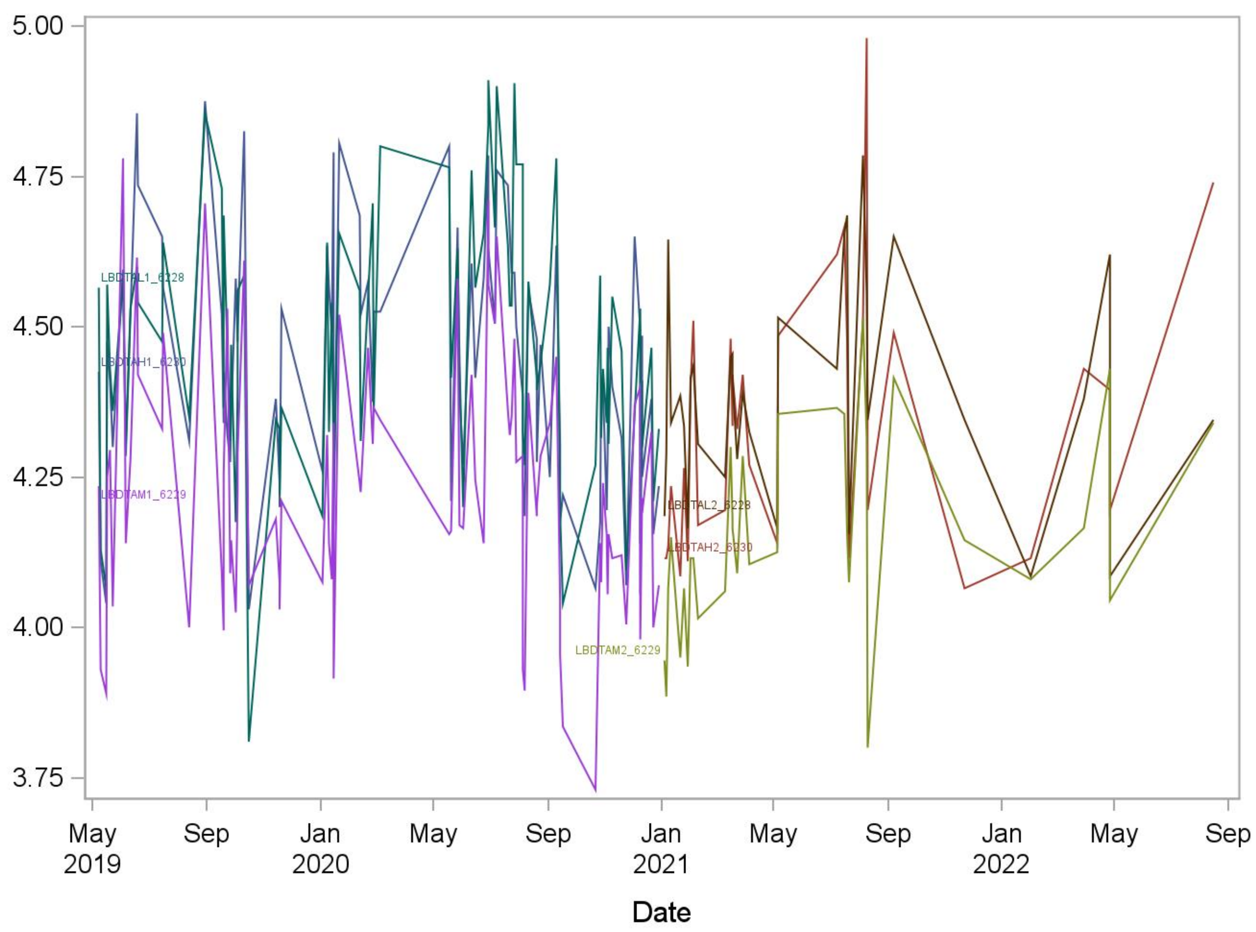
2019-2020 Summary Statistics and QC Chart
LBXPD6 (Docosapentaenoic acid 6 (C22:5n-6) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBDP6H1_6230	90	08MAY19	29DEC20	0.91583	0.02967	3.2
LBDP6H2_6230	35	04JAN21	16AUG22	0.89464	0.03188	3.6
LBDP6L1_6228	90	08MAY19	29DEC20	0.94524	0.02993	3.2
LBDP6L2_6228	35	04JAN21	16AUG22	0.94126	0.02812	3.0
LBDP6M1_6229	90	08MAY19	29DEC20	1.00029	0.03484	3.5
LBDP6M2_6229	35	04JAN21	16AUG22	0.98476	0.03272	3.3



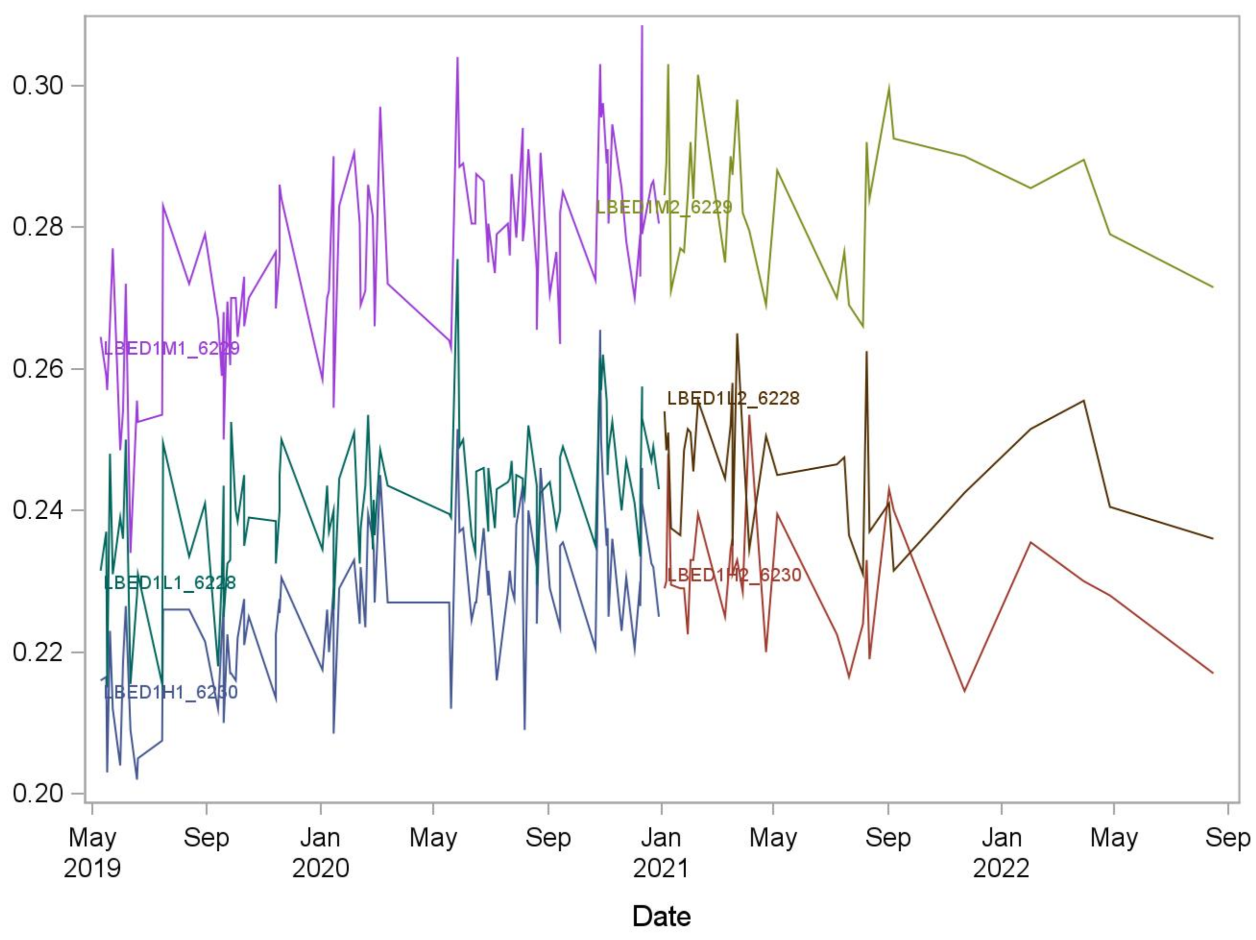
2019-2020 Summary Statistics and QC Chart LBXPTA (Docosatetraenoic acid (C22:4n-6) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBDTAH1_6230	90	08MAY19	29DEC20	4.43817	0.20766	4.7
LBDTAH2_6230	33	04JAN21	16AUG22	4.33955	0.21737	5.0
LBDTAL1_6228	90	08MAY19	29DEC20	4.46211	0.21624	4.8
LBDTAL2_6228	33	04JAN21	16AUG22	4.38970	0.17709	4.0
LBDTAM1_6229	90	08MAY19	29DEC20	4.23267	0.21140	5.0
LBDTAM2_6229	33	04JAN21	16AUG22	4.15470	0.16853	4.1



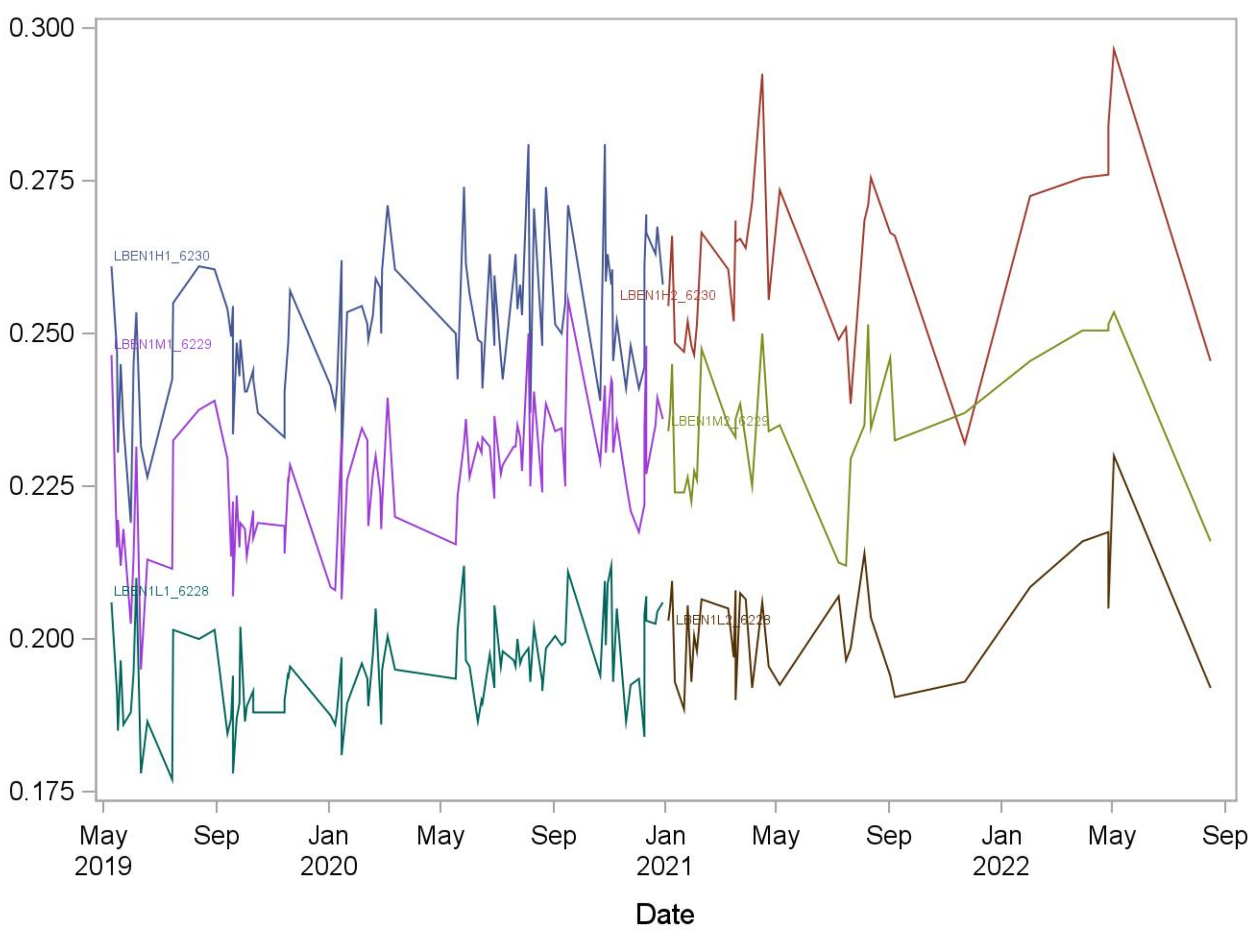
2019-2020 Summary Statistics and QC Chart
LBXPED (11,14-Eicosadienoic acid (C20:2n6) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBED1H1_6230	96	10MAY19	29DEC20	0.22657	0.01120	4.9
LBED1H2_6230	32	04JAN21	16AUG22	0.23017	0.00910	4.0
LBED1L1_6228	96	10MAY19	29DEC20	0.24136	0.00978	4.1
LBED1L2_6228	32	04JAN21	16AUG22	0.24606	0.00883	3.6
LBED1M1_6229	96	10MAY19	29DEC20	0.27582	0.01314	4.8
LBED1M2_6229	32	04JAN21	16AUG22	0.28389	0.00994	3.5



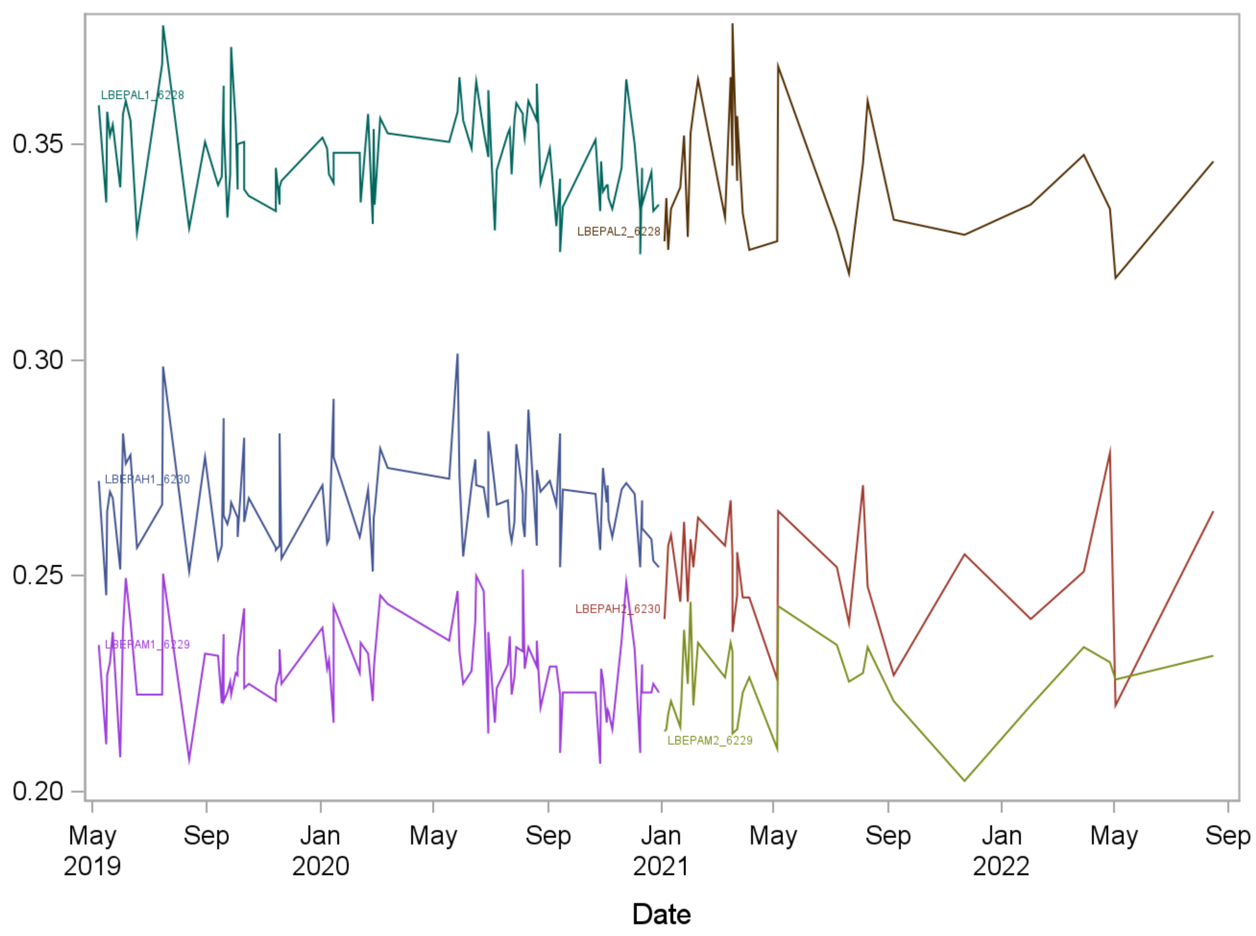
2019-2020 Summary Statistics and QC Chart LBXP1E (11-Eicosenoic acid (C20:1n-9) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBEN1H1_6230	94	10MAY19	29DEC20	0.25151	0.01179	4.7
LBEN1H2_6230	35	04JAN21	16AUG22	0.26221	0.01438	5.5
LBEN1L1_6228	94	10MAY19	29DEC20	0.19505	0.00780	4.0
LBEN1L2_6228	35	04JAN21	16AUG22	0.20220	0.00919	4.5
LBEN1M1_6229	94	10MAY19	29DEC20	0.22660	0.01087	4.8
LBEN1M2_6229	35	04JAN21	16AUG22	0.23500	0.01119	4.8



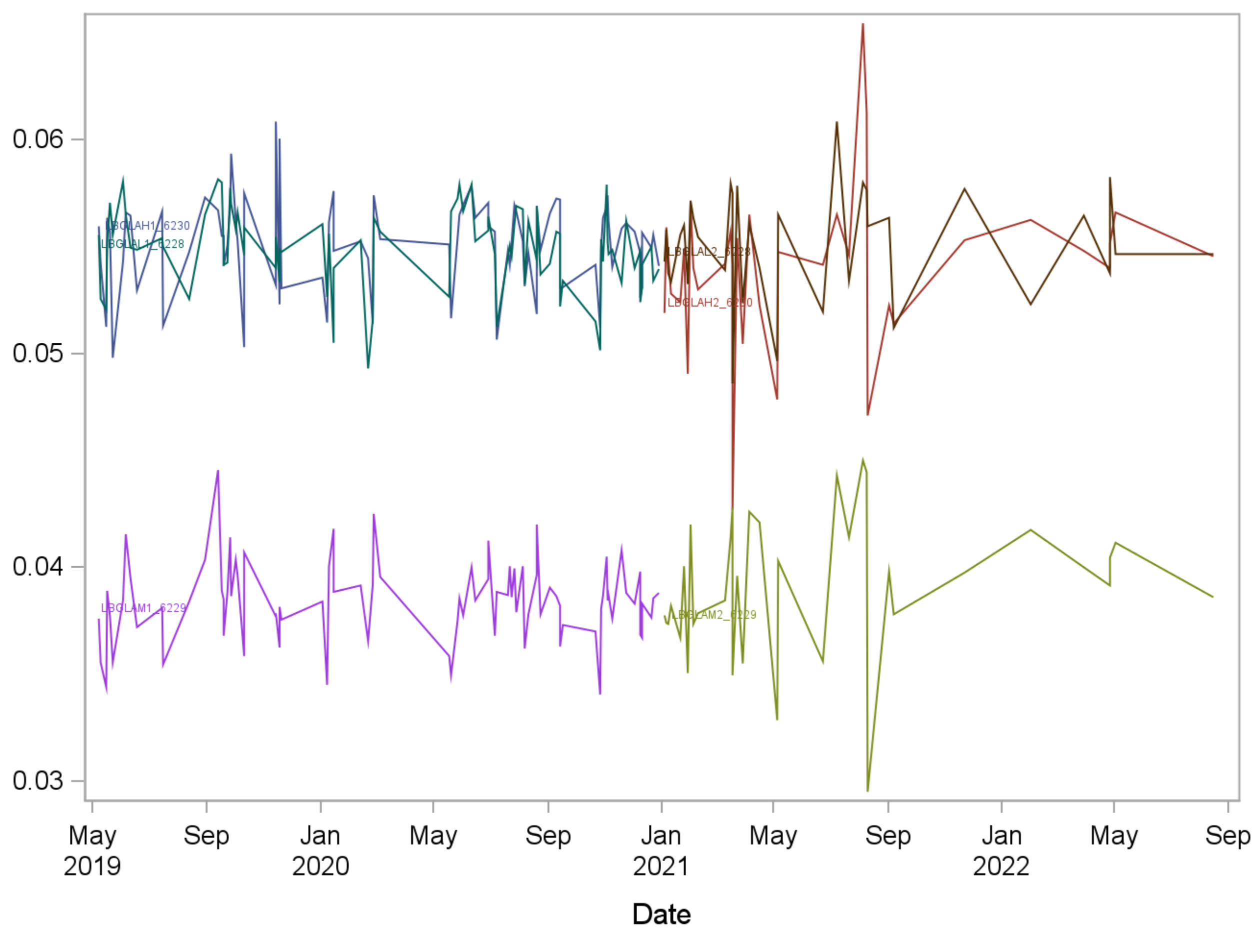
2019-2020 Summary Statistics and QC Chart LBXPPE (Eicosapentaenoic acid (C20:5n-3) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBEPAH1_6230	92	08MAY19	29DEC20	0.26691	0.01080	4.0
LBEPAH2_6230	31	04JAN21	16AUG22	0.25074	0.01332	5.3
LBEPAL1_6228	92	08MAY19	29DEC20	0.34739	0.01105	3.2
LBEPAL2_6228	31	04JAN21	16AUG22	0.34181	0.01524	4.5
LBEPAM1_6229	92	08MAY19	29DEC20	0.22846	0.01014	4.4
LBEPAM2_6229	31	04JAN21	16AUG22	0.22474	0.00988	4.4



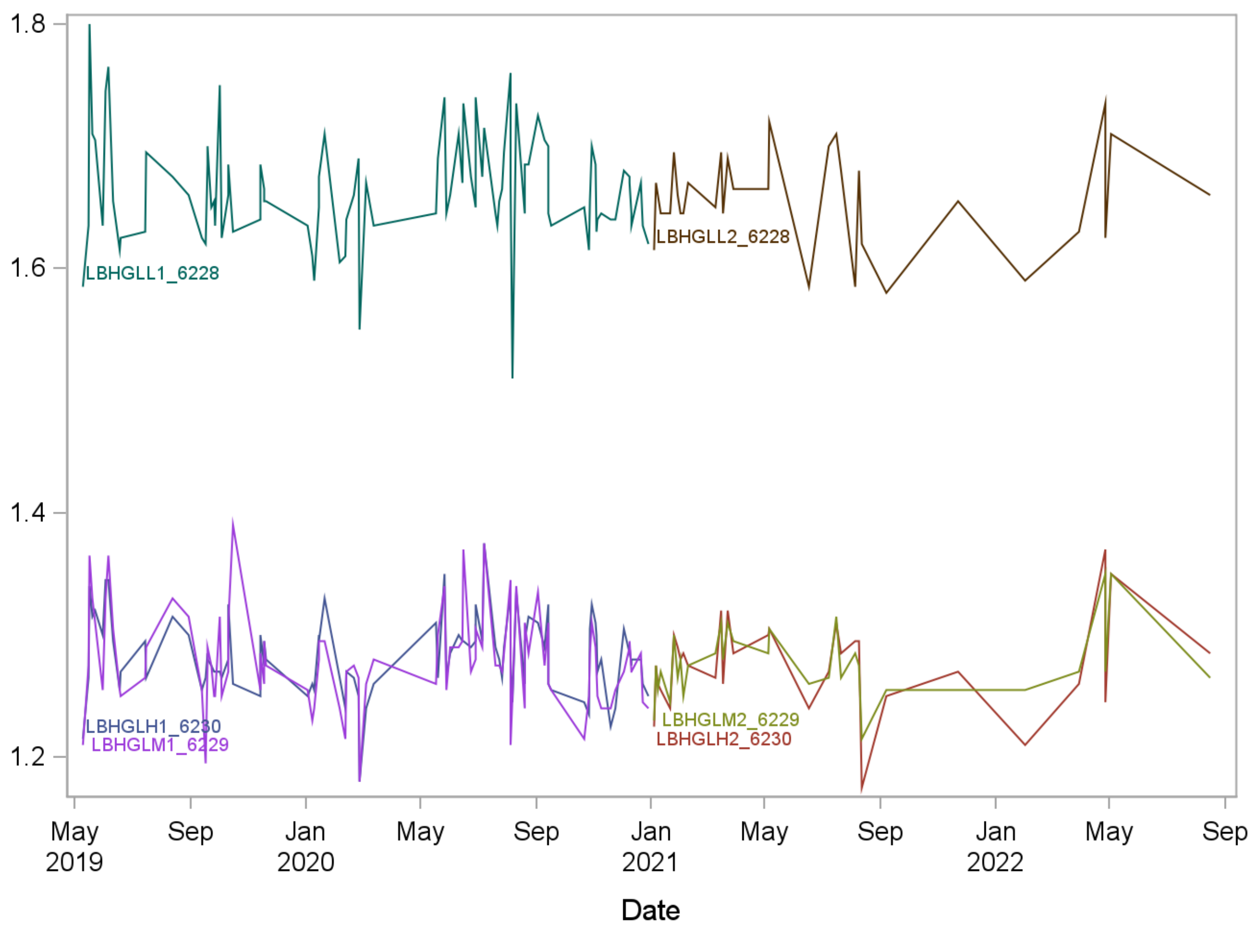
2019-2020 Summary Statistics and QC Chart LBXPLG (gamma-Linolenic acid (C18:3n-6) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBGLAH1_6230	85	08MAY19	29DEC20	0.05509	0.00214	3.9
LBGLAH2_6230	35	04JAN21	16AUG22	0.05397	0.00385	7.1
LBGLAL1_6228	85	08MAY19	29DEC20	0.05487	0.00188	3.4
LBGLAL2_6228	35	04JAN21	16AUG22	0.05510	0.00260	4.7
LBGLAM1_6229	85	08MAY19	29DEC20	0.03842	0.00187	4.9
LBGLAM2_6229	35	04JAN21	16AUG22	0.03911	0.00331	8.5



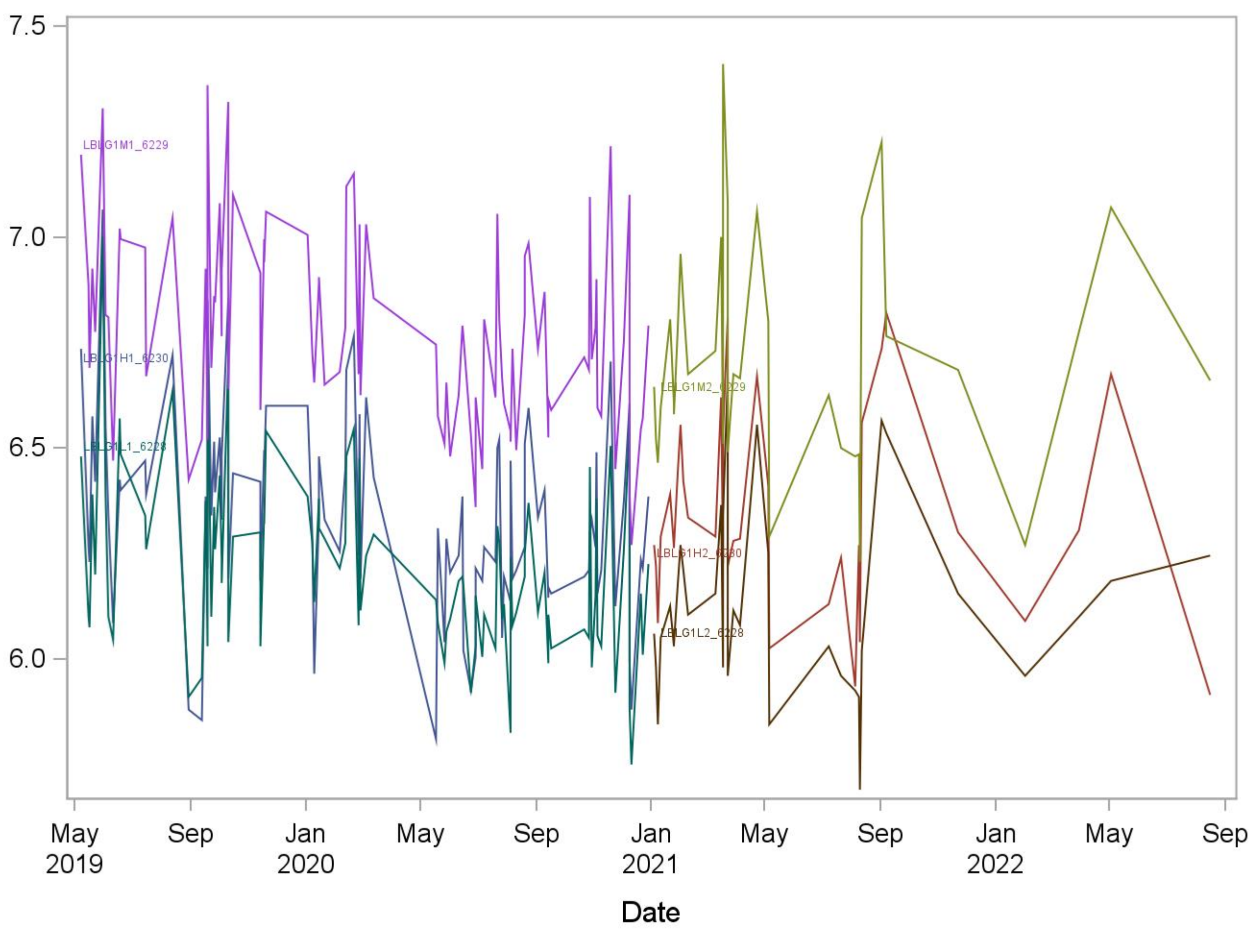
2019-2020 Summary Statistics and QC Chart
LBXPGH (Homo-gamma-Linolenic acid (C20:3n-6) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBHGLH1_6230	94	10MAY19	29DEC20	1.28319	0.03173	2.5
LBHGLH2_6230	32	04JAN21	16AUG22	1.27656	0.03826	3.0
LBHGLL1_6228	94	10MAY19	29DEC20	1.66340	0.04509	2.7
LBHGLL2_6228	32	04JAN21	16AUG22	1.65703	0.03996	2.4
LBHGLM1_6229	94	10MAY19	29DEC20	1.27883	0.03869	3.0
LBHGLM2_6229	32	04JAN21	16AUG22	1.27703	0.02945	2.3



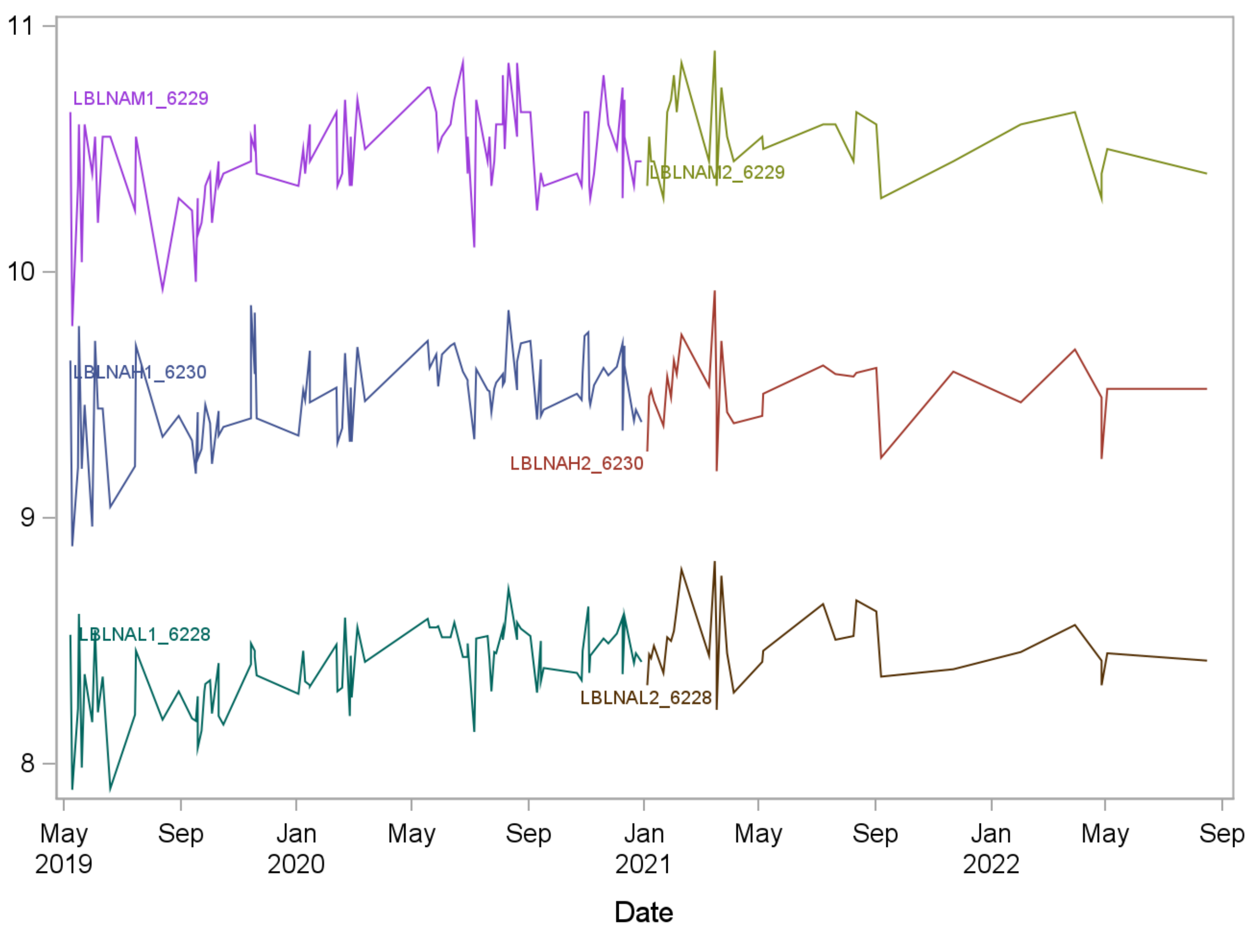
2019-2020 Summary Statistics and QC Chart LBXP1G (Tetracosanoic acid (C24:0) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBLG1H1_6230	95	08MAY19	29DEC20	6.33626	0.23191	3.7
LBLG1H2_6230	35	04JAN21	16AUG22	6.34071	0.23379	3.7
LBLG1L1_6228	95	08MAY19	29DEC20	6.21737	0.21198	3.4
LBLG1L2_6228	35	04JAN21	16AUG22	6.12800	0.21825	3.6
LBLG1M1_6229	95	08MAY19	29DEC20	6.77905	0.23119	3.4
LBLG1M2_6229	35	04JAN21	16AUG22	6.71429	0.26806	4.0



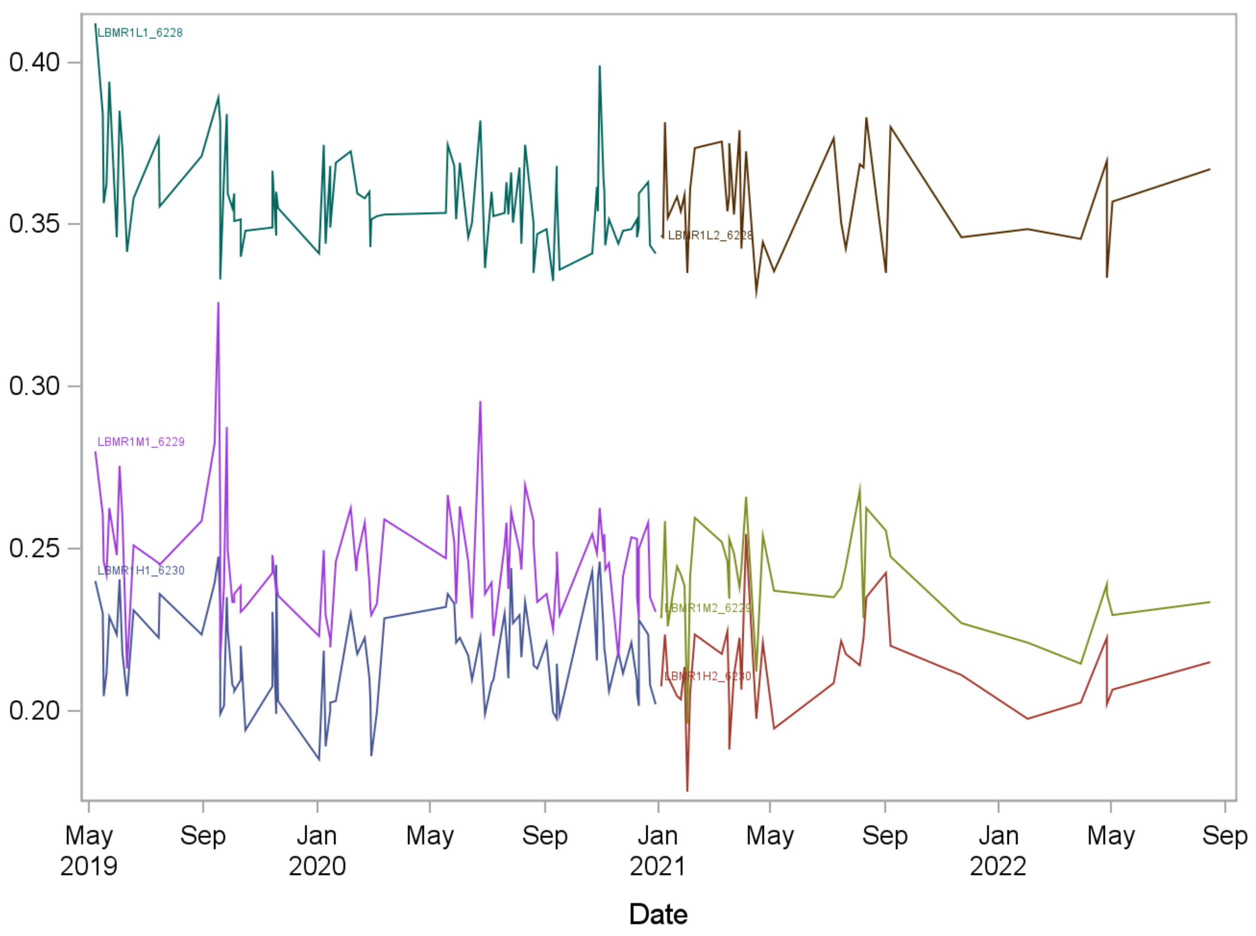
2019-2020 Summary Statistics and QC Chart
LBXPNL (Linoleic acid (C18:2n-6) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBLNAH1_6230	93	08MAY19	29DEC20	9.49495	0.18533	2.0
LBLNAH2_6230	32	04JAN21	16AUG22	9.51563	0.15372	1.6
LBLNAL1_6228	93	08MAY19	29DEC20	8.39376	0.16192	1.9
LBLNAL2_6228	32	04JAN21	16AUG22	8.49047	0.14187	1.7
LBLNAM1_6229	93	08MAY19	29DEC20	10.46570	0.20262	1.9
LBLNAM2_6229	32	04JAN21	16AUG22	10.54063	0.15680	1.5



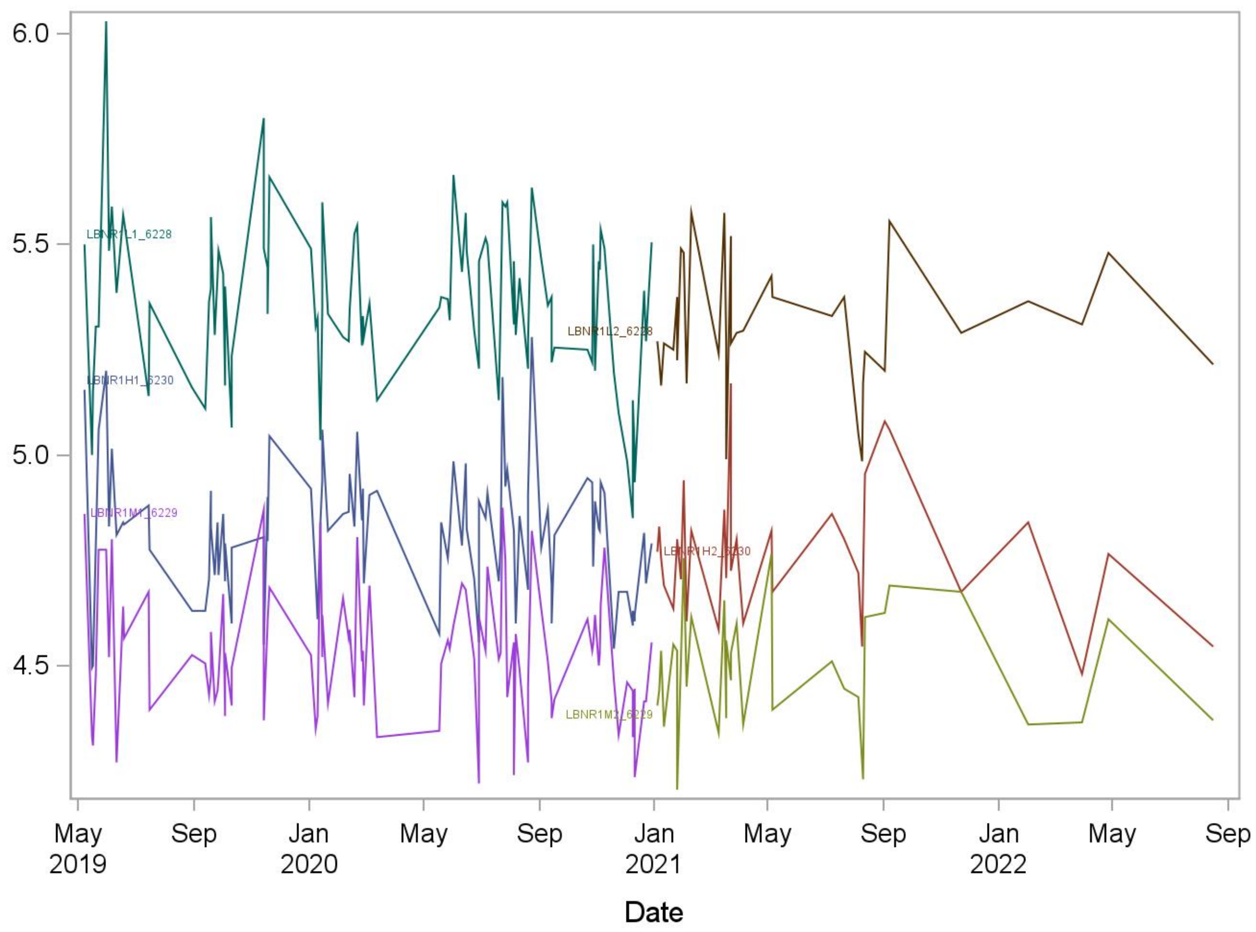
2019-2020 Summary Statistics and QC Chart
LBXPMR (Myristic acid (C14:0) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBMR1H1_6230	91	08MAY19	29DEC20	0.21732	0.01455	6.7
LBMR1H2_6230	36	04JAN21	16AUG22	0.21276	0.01471	6.9
LBMR1L1_6228	91	08MAY19	29DEC20	0.35840	0.01524	4.3
LBMR1L2_6228	36	04JAN21	16AUG22	0.35715	0.01544	4.3
LBMR1M1_6229	91	08MAY19	29DEC20	0.24664	0.01772	7.2
LBMR1M2_6229	36	04JAN21	16AUG22	0.24001	0.01537	6.4



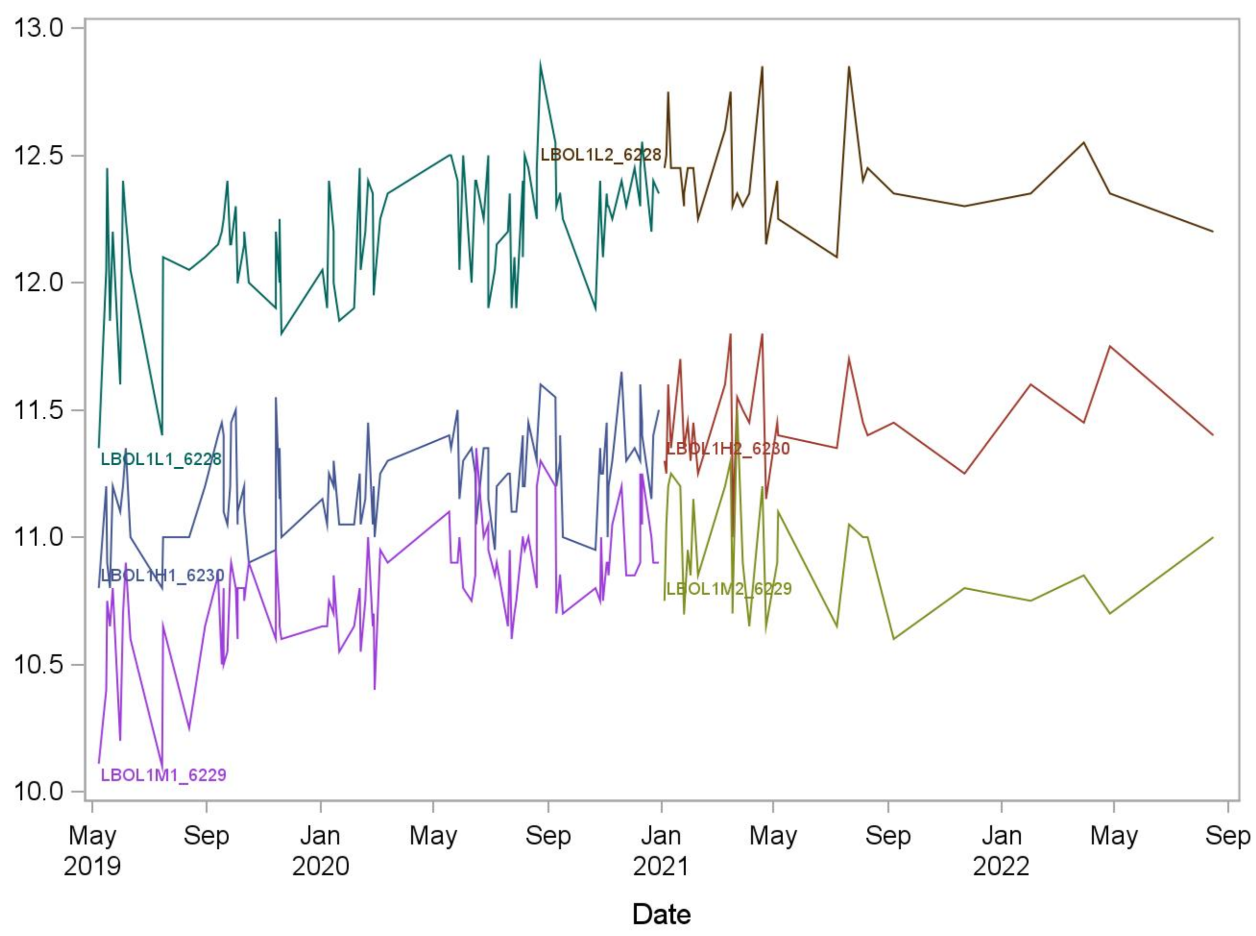
2019-2020 Summary Statistics and QC Chart LBXPNR (15-Tetracosenoic acid (C24:1n9) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBNR1H1_6230	96	08MAY19	29DEC20	4.81359	0.15439	3.2
LBNR1H2_6230	34	04JAN21	16AUG22	4.76588	0.15339	3.2
LBNR1L1_6228	96	08MAY19	29DEC20	5.35932	0.19109	3.6
LBNR1L2_6228	34	04JAN21	16AUG22	5.30279	0.15132	2.9
LBNR1M1_6229	96	08MAY19	29DEC20	4.53031	0.15212	3.4
LBNR1M2_6229	34	04JAN21	16AUG22	4.48794	0.14358	3.2



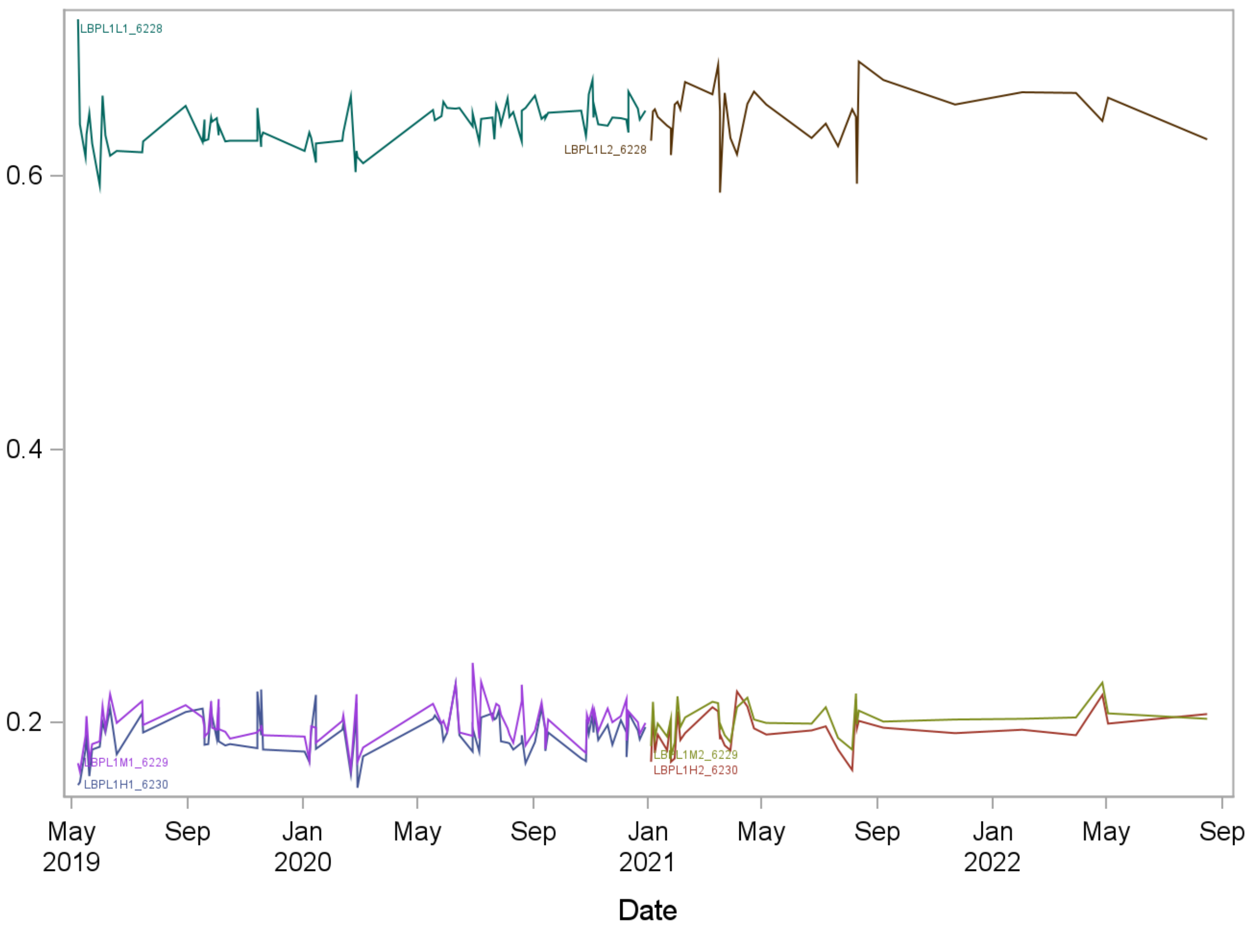
2019-2020 Summary Statistics and QC Chart LBXPOL (Oleic acid (C18:1n-9) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBOL1H1_6230	95	08MAY19	29DEC20	11.22263	0.18847	1.7
LBOL1H2_6230	32	04JAN21	16AUG22	11.44063	0.18684	1.6
LBOL1L1_6228	95	08MAY19	29DEC20	12.20158	0.24445	2.0
LBOL1L2_6228	32	04JAN21	16AUG22	12.41875	0.18173	1.5
LBOL1M1_6229	95	08MAY19	29DEC20	10.80116	0.23295	2.2
LBOL1M2_6229	32	04JAN21	16AUG22	10.93438	0.23191	2.1



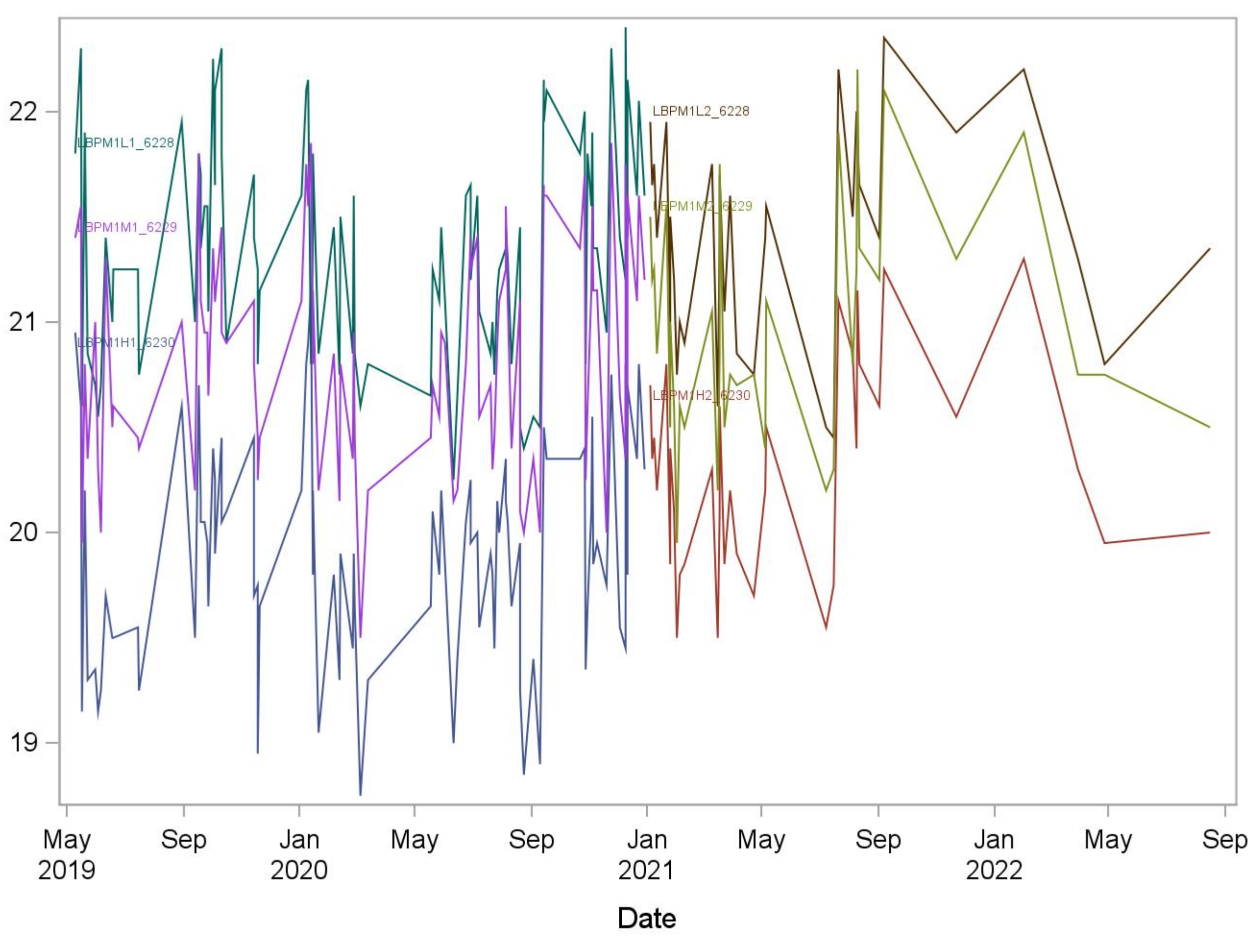
2019-2020 Summary Statistics and QC Chart
LBXPPL (Palmitoleic acid (C16:1n-7) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBPL1H1_6230	87	08MAY19	29DEC20	0.19141	0.01533	8.0
LBPL1H2_6230	35	04JAN21	16AUG22	0.19397	0.01432	7.4
LBPL1L1_6228	87	08MAY19	29DEC20	0.63776	0.01650	2.6
LBPL1L2_6228	35	04JAN21	16AUG22	0.64451	0.02131	3.3
LBPL1M1_6229	87	08MAY19	29DEC20	0.19869	0.01466	7.4
LBPL1M2_6229	35	04JAN21	16AUG22	0.20110	0.01241	6.2



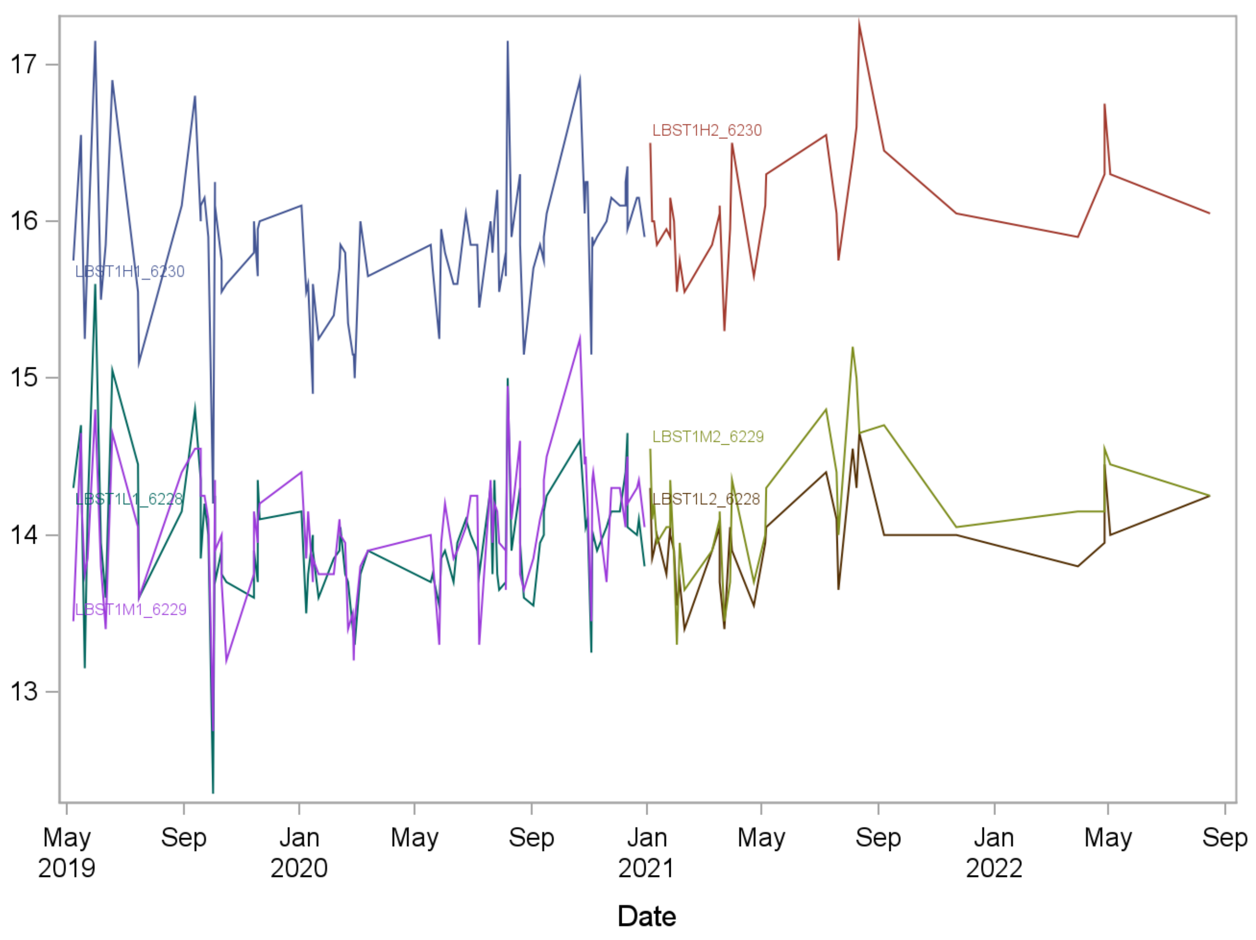
2019-2020 Summary Statistics and QC Chart
LBXPPM (Palmitic acid (C16:0) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBPM1H1_6230	94	10MAY19	29DEC20	19.90745	0.52604	2.6
LBPM1H2_6230	35	04JAN21	16AUG22	20.29714	0.49704	2.4
LBPM1L1_6228	94	10MAY19	29DEC20	21.34096	0.52374	2.5
LBPM1L2_6228	35	04JAN21	16AUG22	21.40429	0.50721	2.4
LBPM1M1_6229	94	10MAY19	29DEC20	20.86064	0.53626	2.6
LBPM1M2_6229	35	04JAN21	16AUG22	20.97286	0.56273	2.7



2019-2020 Summary Statistics and QC Chart
LBXPST (Stearic acid (C18:0) (%))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
LBST1H1_6230	90	08MAY19	29DEC20	15.84833	0.46085	2.9
LBST1H2_6230	33	04JAN21	16AUG22	16.10303	0.38870	2.4
LBST1L1_6228	90	08MAY19	29DEC20	13.96833	0.42608	3.1
LBST1L2_6228	33	04JAN21	16AUG22	13.97121	0.30208	2.2
LBST1M1_6229	90	08MAY19	29DEC20	14.03389	0.40895	2.9
LBST1M2_6229	33	04JAN21	16AUG22	14.18182	0.41586	2.9



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Acknowledgements

We gratefully acknowledge David Scully for who performed validation of this method.

Appendix A: Method Performance Documentation

A. Accuracy using Spike Recovery

The accuracy of spike recovery of the 21 analytes in two QC pools is shown below.

1) *alpha*-Linolenic acid (C18:3n, ALN)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**ALN**

		Sample 1: BRH1587886					Sample 2: BRH1586627						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		0.653	0.636	0.641			0.745	0.726	0.745			
	2	0	0.593	0.645			0.0	0.701	0.716			106.1	4.2
	3		0.650	0.671				0.734	0.847				
Sample + Spike 1	1		102	102	101	109.1		101	107	103	111.2		
	2	92.2	100	103			92.2	101	100				
	3		98.7	102				107	103				
Sample + Spike 2	1		60.0	61.6	60.1	107.6		60.6	61.6	59.5	106.2		
	2	55.3	59.1	59.5			55.3	59.9	57.5				
	3		59.2	61.4				59.1	58.3				
Sample + Spike 3	1		28.4	27.8	28.3	100.1		28.8	30.0	29.1	102.5		
	2	27.7	28.2	28.7			27.7	28.3	30.3				
	3		28.2	28.7				28.5	28.5				

Sample prepared with 50uL RBC with and without spike (as opposed to 100uL aliquots per method)

2) Arachidic acid (C20:0, AR1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**AR1**

		Sample 1: BRH1587886					Sample 2: BRH1586627						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		1.8	1.9	1.9			1.8	1.8	1.8			
	2	0	1.9	2.0			0	1.7	1.6			101.0	3.8
	3		1.7	1.8				1.7	1.9				
Sample + Spike 1	1		18	17	17	103.4		17	18	18	107.3		
	2	14.9	15	19			14.9	17	17				
	3		16	19				19	18				
Sample + Spike 2	1		10	12	11	100.6		10	11	11	99.4		
	2	8.91	10	12			8.91	9	10				
	3		10	11				12	11				
Sample + Spike 3	1		6	6	6	96.2		6	7	6	99.4		
	2	4.47	6	6			4.47	6	6				
	3		6	6				6	6				

Sample prepared with 50uL RBC with and without spike (as opposed to 100uL aliquots per method)

3) Arachidonic acid (C20:4n-6, ARA)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**ARA**

	Replicate	Sample 1: BRH1587886					Sample 2: BRH1586627						
		Spike concentration	Measured concentration			Recovery (%)	Spike concentration	Measured concentration			Recovery (%)	Mean recovery (%)	SD (%)
			Day 1	Day 2	Mean			Day 1	Day 2	Mean			
Sample	1		76.6	77.2	76.3			84.2	83.5	83.7			
	2	0	72.9	78.7			0	81.4	83.7			99.1	2.9
	3		75.5	77.2				82.6	87.0				
Sample + Spike 1	1		425	425	428	100.3		440	450	445	103.1		
	2	350	427	434			350	444	428				
	3		422	433				458	448				
Sample + Spike 2	1		278	296	284	99.0		297	299	292	98.9		
	2	210	279	286			210	294	280				
	3		281	286				292	286				
Sample + Spike 3	1		174	172	175	94.0		189	189	188	99.1		
	2	105	176	178			105	194	187				
	3		176	175				184	185				

Sample prepared with 50uL RBC with and without spike (as opposed to 100uL aliquots per method)

4) Docosanoic acid (C22:0, DA1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**DA1**

	Replicate	Sample 1: BRH1587886					Sample 2: BRH1586627						
		Spike concentration	Measured concentration			Recovery (%)	Spike concentration	Measured concentration			Recovery (%)	Mean recovery (%)	SD (%)
			Day 1	Day 2	Mean			Day 1	Day 2	Mean			
Sample	1		11.0	11.1	11.0			8.91	8.42	8.58			
	2	0	10.6	11.5			0	8.38	8.75			95.5	3.6
	3		10.6	10.9				8.38	8.63				
Sample + Spike 1	1		41.9	42.9	42.6	95.7		41.0	41.1	41.3	98.9		
	2	33.0	41.4	44.1			33.0	40.9	39.3				
	3		41.1	44.0				43.5	41.6				
Sample + Spike 2	1		26.7	31.8	29.5	93.6		27.1	28.1	27.7	96.4		
	2	19.8	28.7	30.6			19.8	24.6	26.6				
	3		29.0	30.2				32.5	27.3				
Sample + Spike 3	1		19.7	19.2	19.8	89.3		18.5	18.4	18.4	98.9		
	2	9.91	20.1	20.2			9.91	19.3	18.2				
	3		20.1	19.5				18.4	17.5				

Sample prepared with 50uL RBC with and without spike (as opposed to 100uL aliquots per method)

5) Docosahexaenoic acid (C22:6n-3, DHA)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**DHA**

		Sample 1: BRH1587886					Sample 2: BRH1586627						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		17.1	17.7	17.3			18.9	19.2	18.9			
	2	0	16.3	18.6			0	18.3	18.9			92.2	3.1
	3		16.5	17.4				18.7	19.5				
Sample + Spike 1	1		142	136	137	90.7		141	153	147	96.4		
	2	132	136	138			132	143	142				
	3		132	141				150	150				
Sample + Spike 2	1		90.6	93.8	90.5	92.1		93.3	96.3	94.3	94.8		
	2	79.5	87.1	91.6			79.5	98.5	92.2				
	3		90.2	89.7				90.2	95.2				
Sample + Spike 3	1		52.6	48.5	52.0	87.4		55.1	58.1	55.5	92.0		
	2	39.8	53.3	51.3			39.8	56.1	54.1				
	3		53.3	53.2				54.9	54.9				

Sample prepared with 50uL RBC with and without spike (as opposed to 100uL aliquots per method)

6) Docosapentaenoic acid (C22:5n-3, DP3)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**DP3**

		Sample 1: BRH1587886					Sample 2: BRH1586627						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		9.54	9.84	9.69			10.3	10.5	10.5			
	2	0	8.86	10.61			0	10.7	10.7			94.7	2.5
	3		9.20	10.11				10.2	10.7				
Sample + Spike 1	1		49.2	47.7	48.9	93.3		51.5	51.5	51.9	98.5		
	2	42.0	50.8	48.7			42.0	51.8	52.5				
	3		48.2	48.5				52.4	51.7				
Sample + Spike 2	1		33.5	34.2	33.7	95.5		35.3	34.9	34.7	95.8		
	2	25.2	34.3	34.5			25.2	35.2	33.3				
	3		33.4	32.6				34.7	34.6				
Sample + Spike 3	1		21.4	20.2	21.2	91.2		23.3	22.7	22.4	94.1		
	2	12.6	21.6	20.7			12.6	23.1	21.9				
	3		21.8	21.3				21.8	21.3				

Sample prepared with 50uL RBC with and without spike (as opposed to 100uL aliquots per method)

7) Docosapentaenoic acid (C22:5n-6, DP6)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**DP6**

	Replicate	Sample 1: BRH1587886					Sample 2: BRH1586627						
		Spike concentration	Measured concentration			Recovery (%)	Spike concentration	Measured concentration			Recovery (%)	Mean recovery (%)	SD (%)
			Day 1	Day 2	Mean			Day 1	Day 2	Mean			
Sample	1	0	3.79	3.71	3.77		0	4.34	4.52	4.41		92.9	2.7
	2		3.44	4.01				4.27	4.34				
	3		3.65	3.99				4.24	4.73				
Sample + Spike 1	1	24.8	26.5	25.5	26.1	90.3	24.8	28.0	28.8	28.6	97.4		
	2		26.8	26.3				28.4	28.7				
	3		25.4	26.3				28.4	29.1				
Sample + Spike 2	1	14.9	17.6	17.5	17.5	92.1	14.9	18.9	18.7	18.5	94.5		
	2		17.5	18.1				18.6	17.9				
	3		16.8	17.4				18.2	18.5				
Sample + Spike 3	1	7.44	10.4	10.2	10.5	90.7	7.44	11.5	11.6	11.3	92.3		
	2		10.6	10.5				11.5	11.2				
	3		10.6	10.8				10.9	10.9				

Sample prepared with 50uL RBC with and without spike (as opposed to 100uL aliquots per method)

8) Docosatetraenoic acid (C22:4n-6, DTA)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**DTA**

	Replicate	Sample 1: BRH1587886					Sample 2: BRH1586627						
		Spike concentration	Measured concentration			Recovery (%)	Spike concentration	Measured concentration			Recovery (%)	Mean recovery (%)	SD (%)
			Day 1	Day 2	Mean			Day 1	Day 2	Mean			
Sample	1	0	20.1	18.8	19.4		0	17.6	17.7	17.6		91.6	2.0
	2		18.5	20.7				18.1	17.2				
	3		19.3	18.9				17.2	17.7				
Sample + Spike 1	1	25.8	41.0	41.3	42.1	88.0	25.8	41.7	40.8	41.5	93.0		
	2		44.1	43.0				41.7	43.3				
	3		41.6	41.3				40.9	40.9				
Sample + Spike 2	1	15.5	33.0	34.8	33.7	92.5	15.5	33.2	31.6	32.1	93.7		
	2		34.0	35.2				31.9	30.8				
	3		32.4	32.7				32.8	32.0				
Sample + Spike 3	1	7.75	26.6	25.3	26.5	91.4	7.75	25.7	24.6	24.6	90.9		
	2		27.6	25.3				26.5	24.0				
	3		27.7	26.2				24.1	22.8				

Sample prepared with 50uL RBC with and without spike (as opposed to 100uL aliquots per method)

9) Eicosadienoic acid (C20:2n-6, ED1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**ED1**

	Replicate	Sample 1: BRH1587886					Sample 2: BRH1586627						
		Spike concentration	Measured concentration Day 1	Measured concentration Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Measured concentration Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		1.28	1.39	1.30			1.23	1.38	1.31			
	2	0	1.28	1.34			0	1.32	1.33			93.1	3.2
	3		1.21	1.31				1.26	1.37				
Sample + Spike 1	1		15.5	13.7	14.5	88.5		14.9	15.0	15.1	92.2		
	2	14.9	13.7	14.4			14.9	15.6	13.9				
	3		14.7	15.0				16.5	14.6				
Sample + Spike 2	1		9.84	9.61	9.51	91.7		10.17	9.73	9.72	93.9		
	2	8.95	9.52	9.37			8.95	9.95	9.61				
	3		9.33	9.37				9.60	9.26				
Sample + Spike 3	1		5.42	5.42	5.50	93.8		5.67	5.83	5.70	98.1		
	2	4.47	5.35	5.53			4.47	5.86	5.76				
	3		5.62	5.66				5.41	5.70				

Sample spike ran at 50uL RBC as opposed to 100uL aliquots per method

10) Eicosenoic acid (C20:1n-9, EN1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**EN1**

	Replicate	Sample 1: BRH1587886					Sample 2: BRH1586627						
		Spike concentration	Measured concentration Day 1	Measured concentration Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Measured concentration Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		0.849	1.018	0.941			0.784	0.916	0.854			
	2	0	0.905	1.025			0	0.787	0.909			93.0	3.2
	3		0.882	0.969				0.803	0.923				
Sample + Spike 1	1		16.1	14.0	14.9	88.3		15.0	15.2	15.3	91.5		
	2	15.8	14.3	14.8			15.8	15.9	14.2				
	3		15.2	15.0				17.1	14.7				
Sample + Spike 2	1		9.93	9.96	9.70	92.1		10.3	9.67	9.79	94.1		
	2	9.50	9.64	9.58			9.50	9.95	9.40				
	3		9.53	9.52				9.97	9.50				
Sample + Spike 3	1		5.41	5.38	5.41	94.0		5.43	5.76	5.50	97.8		
	2	4.75	5.26	5.44			4.75	5.65	5.60				
	3		5.53	5.44				5.24	5.31				

Sample spike ran at 50uL RBC as opposed to 100uL aliquots per method

11) Eicosapentaenoic acid (C20:5n-3, EPA)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**EPA**

		Sample 1: BRH1587886					Sample 2: BRH1586627						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		1.73	1.72	1.84			2.17	2.27	2.26			
	2	0	1.75	1.94			0	2.25	2.28			91.9	6.1
	3		1.96	1.92				2.34	2.27				
Sample + Spike 1	1		51.1	53.7	52.6	84.4		51.8	56.6	53.9	85.8		
	2	60.2	51.6	55.1			60.2	51.5	55.3				
	3		50.1	54.3				52.8	55.6				
Sample + Spike 2	1		34.4	36.0	35.4	93.2		34.1	36.8	34.9	90.6		
	2	36.0	33.7	37.0			36.0	34.3	35.2				
	3		34.4	36.8				33.5	35.4				
Sample + Spike 3	1		19.1	19.7	19.4	97.2		20.0	20.9	20.3	99.9		
	2	18.0	18.6	19.7			18.0	19.9	21.1				
	3		19.2	19.8				19.3	20.5				

Sample spike ran at 50uL RBC as opposed to 100uL aliquots per method

12) *gamma*-Linolenic acid (C18:3n-6, GLA)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**GLA**

		Sample 1: BRH1587886					Sample 2: BRH1586627						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		0.189	0.243	0.215			0.340	0.393	0.373			
	2	0	0.164	0.233			0	0.324	0.390			103.1	1.9
	3		0.189	0.272				0.344	0.445				
Sample + Spike 1	1		44.1	43.2	43.5	103.5		44.4	45.1	44.6	105.8		
	2	41.8	43.7	43.5			41.8	45.1	42.6				
	3		42.7	43.5				46.0	44.1				
Sample + Spike 2	1		26.4	26.2	26.1	103.5		27.4	26.9	26.2	103.6		
	2	25.0	26.3	25.5			25.0	26.2	25.3				
	3		25.7	26.4				26.4	25.3				
Sample + Spike 3	1		12.8	12.7	12.8	100.6		13.3	13.2	13.0	101.4		
	2	12.5	13.1	12.7			12.5	13.0	13.2				
	3		12.7	12.9				12.9	12.7				

Sample spike ran at 50uL RBC as opposed to 100uL aliquots per method

13) *homo-gamma*-Linolenic acid (C20:3n-6, HGL)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**HGL**

		Sample 1: BRH1587886					Sample 2: BRH1586627						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		6.41	6.20	6.17			7.51	7.61	7.41			
	2	0	5.73	6.38			0	7.07	7.45			94.2	2.2
	3		5.99	6.31				7.08	7.76				
Sample + Spike 1	1		69.6	70.8	70.7	90.7		74.5	75.0	73.7	93.2		
	2	71.1	71.3	71.3			71.1	73.1	72.0				
	3		69.2	71.9				73.8	73.7				
Sample + Spike 2	1		45.3	47.5	46.5	94.6		49.8	48.8	47.7	94.5		
	2	42.6	46.9	46.5			42.6	46.2	46.3				
	3		44.5	48.1				48.1	46.8				
Sample + Spike 3	1		25.7	26.5	26.4	95.0		28.4	28.5	28.2	97.3		
	2	21.3	26.4	27.0			21.3	28.5	28.6				
	3		26.0	26.9				26.8	28.0				

Sample spike ran at 50uL RBC as opposed to 100uL aliquots per method

14) Lignoceric acid (C24:0, LG1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**LG1**

		Sample 1: BRH1587886					Sample 2: BRH1586627						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		26.7	28.1	26.9			29.8	29.5	29.3			
	2	0	25.7	27.8			0	28.9	29.5			100.2	9.7
	3		26.0	27.3				28.6	29.2				
Sample + Spike 1	1		49.0	52.8	51.6	95.6		56.3	56.8	56.5	105.7		
	2	25.8	50.2	53.9			25.8	56.4	54.2				
	3		49.7	54.0				58.0	57.6				
Sample + Spike 2	1		37.1	46.3	41.3	93.0		45.3	45.3	45.4	104.6		
	2	15.5	39.1	43.3			15.5	42.1	44.8				
	3		39.6	42.8				49.3	45.7				
Sample + Spike 3	1		33.3	33.9	33.7	87.8		37.4	38.2	38.1	114.2		
	2	7.74	32.5	35.0			7.74	41.2	37.3				
	3		33.0	34.7				36.9	37.5				

Sample spike ran at 50uL RBC as opposed to 100uL aliquots per method

15) Linoleic acid (C18:2n-6, LNA)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**LNA**

		Sample 1: BRH1587886					Sample 2: BRH1586627						
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		40.4	39.8	39.8			46.6	46.8	46.4			
	2	0	38.4	40.6			0	44.7	46.4			99.8	1.7
	3		39.5	40.2				46.2	48.0				
Sample + Spike 1	1		1236	1258	1247	99.7		1269	1313	1282	102.0		
	2	1211	1231	1265			1211	1259	1253				
	3		1229	1260				1307	1292				
Sample + Spike 2	1		762	795	773	100.9		781	797	770	99.6		
	2	727	759	781			727	772	744				
	3		758	784				772	757				
Sample + Spike 3	1		388	388	392	97.0		409	417	408	99.4		
	2	363	392	394			363	402	416				
	3		391	399				398	403				

Sample spike ran at 50uL RBC as opposed to 100uL aliquots per method

16) Myristic acid (C14:0, MR1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**MR1**

		Sample 1: BRH1587886					Sample 2: BRH1586627						
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		1.41	1.23	1.45			1.84	1.70	1.73			
	2	0	1.49	1.75			0	1.47	1.40			87.2	5.5
	3		1.45	1.37				1.77	2.21				
Sample + Spike 1	1		121	85	101	80.9		115	86	103	82.3		
	2	123	114	86			123	118	83				
	3		115	85				128	87				
Sample + Spike 2	1		68.5	61.2	65.4	86.6		71.8	58.9	65.5	86.4		
	2	73.8	70.8	60.0			73.8	68.1	56.1				
	3		72.1	59.5				80.5	57.8				
Sample + Spike 3	1		37.4	33.0	35.3	92.1		38.9	35.9	36.7	95.1		
	2	36.8	36.6	33.6			36.8	37.4	36.1				
	3		37.2	34.2				37.9	33.9				

Sample spike ran at 50uL RBC as opposed to 100uL aliquots per method

17) Nervonic acid (C24:1n-9, NR1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**NR1**

		Sample 1: BRH1587886					Sample 2: BRH1586627						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		21.1	20.8	20.7			20.8	19.9	20.4			
	2	0	20.6	20.9			0	20.7	20.2			97.0	5.6
	3		20.6	20.5				19.9	20.7				
Sample + Spike 1	1		53.8	57.6	55.9	96.3		55.5	58.1	57.1	100.7		
	2	36.5	53.3	58.6			36.5	56.6	55.3				
	3		52.5	59.5				57.5	59.6				
Sample + Spike 2	1		38.9	43.9	41.1	93.0		43.0	42.5	42.0	99.0		
	2	21.9	41.5	41.8			21.9	40.0	41.9				
	3		38.9	41.6				43.2	41.6				
Sample + Spike 3	1		31.2	29.9	30.5	88.6		31.6	30.4	31.8	104.2		
	2	11.0	30.4	30.1			11.0	35.7	31.3				
	3		30.7	30.4				31.0	30.7				

Sample spike ran at 50uL RBC as opposed to 100uL aliquots per method

18) Oleic acid (C18:1n-9, OL1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**OL1**

		Sample 1: BRH1587886					Sample 2: BRH1586627						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		50.8	47.6	48.6			50.9	48.9	50.0			
	2	0	47.3	49.1			0	49.1	49.1			102.8	2.3
	3		48.3	48.5				50.7	51.2				
Sample + Spike 1	1		1378	1360	1359	103.6		1406	1367	1389	105.9		
	2	1264	1358	1363			1264	1400	1306				
	3		1330	1363				1462	1391				
Sample + Spike 2	1		805	853	837	103.9		844	827	827	102.4		
	2	758	847	840			758	793	780				
	3		837	839				915	801				
Sample + Spike 3	1		441	406	424	99.1		449	434	436	101.9		
	2	379	438	416			379	448	434				
	3		429	414				436	416				

Sample spike ran at 50uL RBC as opposed to 100uL aliquots per method

19) Palmitoleic acid (C16:1n-7, PL1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**PL1**

		Sample 1: BRH1587886					Sample 2: BRH1586627						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		1.02	0.871	0.978			1.60	1.57	1.61			
	2	0	0.898	1.06			0	1.52	1.57			91.3	4.9
	3		1.02	1.00				1.67	1.76				
Sample + Spike 1	1		287	268	275	85.2		275	279	279	86.2		
	2	322	279	272			322	280	267				
	3		278	269				297	276				
Sample + Spike 2	1		182	184	180	92.8		180	180	177	91.0		
	2	193	177	178			193	183	168				
	3		181	179				180	172				
Sample + Spike 3	1		94.6	90.0	93.5	95.7		96.5	98.0	95.6	97.2		
	2	96.7	94.6	93.2			96.7	94.2	97.7				
	3		94.3	94.1				94.1	93.1				

Sample spike ran at 50uL RBC as opposed to 100uL aliquots per method

20) Palmitic acid (C16:0, PM1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**PM1**

		Sample 1: BRH1587886					Sample 2: BRH1586627						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		88.0	97.3	93.5			107	112	109			
	2	0	87.2	104.3			0	99.3	110			91.8	6.8
	3		86.1	98.2				105	120				
Sample + Spike 1	1		1114	1115	1112	82.8		1227	1123	1163	85.7		
	2	1230	1118	1105			1230	1218	1090				
	3		1095	1125				1184	1134				
Sample + Spike 2	1		705	802	773	92.2		792	791	793	92.7		
	2	738	777	791			738	704	749				
	3		769	797				943	776				
Sample + Spike 3	1		452	436	448	96.1		483	497	483	101.5		
	2	369	445	450			369	487	490				
	3		452	454				485	460				

Sample spike ran at 50uL RBC as opposed to 100uL aliquots per method

21) Stearic acid (C18:0, ST1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**ST1**

		Sample 1: BRH1587886					Sample 2: BRH1586627						
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	58.9	65.8	63.9		0	69.3	72.4	68.7		96.3	2.2
	2		59.2	74.5				60.0	66.8				
	3		57.5	67.5				65.8	77.8				
Sample + Spike 1	1	409	495	419	450	94.4	409	493	419	465	96.9		
	2		468	424				505	408				
	3		473	421				539	426				
Sample + Spike 2	1	245	277	306	297	94.9	245	310	292	304	96.1		
	2		297	298				279	276				
	3		306	295				375	293				
Sample + Spike 3	1	123	181	174	180	94.8	123	196	193	192	100.4		
	2		181	181				194	189				
	3		181	182				196	182				

Sample spike ran at 50uL RBC as opposed to 100uL aliquots per method

B. Precision

The method precision of the 21 analytes in the low QC and high QC is shown below for data from 2021.

1) *alpha*-Linolenic acid (C18:3n, ALNP)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	ALNP					

Quality material 1: Low Pool						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	0.123	0.125	0.124	0.000	0.000	0.031
2	0.137	0.138	0.138	0.000	0.000	0.038
3	0.130	0.129	0.130	0.000	0.000	0.034
4	0.131	0.127	0.129	0.000	0.000	0.033
5	0.131	0.131	0.131	0.000	0.000	0.034
6	0.132	0.133	0.133	0.000	0.000	0.035
7	0.133	0.132	0.133	0.000	0.000	0.035
8	0.134	0.137	0.136	0.000	0.000	0.037
9	0.135	0.135	0.135	0.000	0.000	0.036
10	0.137	0.136	0.137	0.000	0.000	0.037
Grand sum	2.65	Grand mean	0.132			
	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)		
Within Run	0.000017	0.000002	0.001304	0.99		
Between Run	0.000303	0.000034	0.003999	3.02		
Total	0.000320		0.004206	3.18		

Quality material 2: High pool						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	0.101	0.103	0.102	0.000	0.000	0.020808
2	0.112	0.120	0.116	0.000	0.000	0.026912
3	0.113	0.113	0.113	0.000	0.000	0.025538
4	0.114	0.112	0.113	0.000	0.000	0.025538
5	0.114	0.113	0.114	0.000	0.000	0.025765
6	0.118	0.117	0.118	0.000	0.000	0.027613
7	0.114	0.110	0.112	0.000	0.000	0.025088
8	0.119	0.113	0.116	0.000	0.000	0.026912
9	0.108	0.110	0.109	0.000	0.000	0.023762
10	0.115	0.115	0.115	0.000	0.000	0.026450
Grand sum	2.25	Grand mean	0.113			
	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev		
Within Run	0.00006	0.00001	0.00255	2.26		
Between Run	0.00036	0.00004	0.00409	3.63		
Total	0.00042		0.00482	4.27		

2) Arachidic acid (C20:0, AR1P)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**AR1P**					

Quality material 1: Low Pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	0.363	0.346	0.355	0.0001	0.0001	0.2513
2	0.320	0.324	0.322	0.0000	0.0000	0.2074
3	0.327	0.315	0.321	0.0000	0.0000	0.2061
4	0.322	0.324	0.323	0.0000	0.0000	0.2087
5	0.314	0.333	0.324	0.0001	0.0001	0.2093
6	0.334	0.310	0.322	0.0001	0.0001	0.2074
7	0.310	0.334	0.322	0.0001	0.0001	0.2074
8	0.330	0.350	0.340	0.0001	0.0001	0.2312
9	0.332	0.285	0.309	0.0006	0.0006	0.1903
10	0.312	0.312	0.312	0.0000	0.0000	0.1947
Grand sum	6.50	**Grand mean**	0.325			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.0023	0.0002	0.0151	4.66		
Between Run	0.0032	0.0004	0.0079	2.42		
Total	0.0055		0.0170	**5.25**		

Quality material 2: High pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	0.581	0.572	0.577	0.0000	0.0000	0.665
2	0.541	0.557	0.549	0.0001	0.0001	0.603
3	0.616	0.516	0.566	0.0025	0.0025	0.641
4	0.555	0.571	0.563	0.0001	0.0001	0.634
5	0.556	0.559	0.558	0.0000	0.0000	0.622
6	0.556	0.550	0.553	0.0000	0.0000	0.612
7	0.553	0.580	0.567	0.0002	0.0002	0.642
8	0.570	0.567	0.569	0.0000	0.0000	0.646
9	0.610	0.523	0.567	0.0019	0.0019	0.642
10	0.517	0.534	0.526	0.0001	0.0001	0.552
Grand sum	11.2	**Grand mean**	0.559			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	0.0096	0.0010	0.0310	5.55		
Between Run	0.0037	0.0004	0.0000	0.00		
Total	0.0133		0.0310	**5.55**		

3) Arachidonic acid (C20:4n-6, ARAP)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**ARAP**					

Quality material 1: Low Pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	16.3	16.5	16.4	0.0100	0.0100	538
2	16.6	16.9	16.8	0.0225	0.0225	561
3	16.5	16.6	16.6	0.0025	0.0025	548
4	16.3	16.3	16.3	0.0000	0.0000	531
5	16.7	16.8	16.8	0.0025	0.0025	561
6	17.1	17.1	17.1	0.0000	0.0000	585
7	16.5	16.5	16.5	0.0000	0.0000	545
8	17.1	16.9	17.0	0.0100	0.0100	578
9	16.5	16.9	16.7	0.0400	0.0400	558
10	16.9	16.9	16.9	0.0000	0.0000	571
Grand sum	334	**Grand mean**	16.7			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.175	0.017	0.13	0.79		
Between Run	1.215	0.135	0.24	1.45		
Total	1.390		0.28	**1.65**		

Quality material 2: High pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	16.5	16.5	16.5	0.0000	0.0000	545
2	16.7	17.1	16.9	0.0400	0.0400	571
3	16.8	16.6	16.7	0.0100	0.0100	558
4	16.9	16.6	16.8	0.0225	0.0225	561
5	17.1	17.0	17.1	0.0025	0.0025	581
6	17.2	17.4	17.3	0.0100	0.0100	599
7	16.5	16.4	16.5	0.0025	0.0025	541
8	17.2	17.0	17.1	0.0100	0.0100	585
9	16.7	16.8	16.8	0.0025	0.0025	561
10	17.0	17.0	17.0	0.0000	0.0000	578
Grand sum	337	**Grand mean**	16.9			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	0.200	0.020	0.141	0.84		
Between Run	1.310	0.146	0.251	1.49		
Total	1.510		0.288	**1.71**		

4) Docosanoic acid (C22:0, DA1P)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**DA1P**					

Quality material 1: Low Pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	1.77	1.78	1.78	0.00002	0.00003	6.30
2	1.76	1.81	1.79	0.00063	0.00062	6.37
3	1.75	1.79	1.77	0.00040	0.00040	6.27
4	1.72	1.73	1.73	0.00003	0.00002	5.95
5	1.80	1.82	1.81	0.00010	0.00010	6.55
6	1.78	1.80	1.79	0.00010	0.00010	6.41
7	1.75	1.75	1.75	0.00000	0.00000	6.13
8	1.76	1.76	1.76	0.00000	0.00000	6.20
9	1.73	1.74	1.74	0.00002	0.00003	6.02
10	1.74	1.79	1.77	0.00063	0.00062	6.23
Grand sum	35.3	**Grand mean**	1.77			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.004	0.000	0.020	1.11		
Between Run	0.012	0.001	0.022	1.22		
Total	0.016		0.029	**1.65**		

Quality material 2: High pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	2.15	2.22	2.19	0.0012	0.0012	9.55
2	2.18	2.24	2.21	0.0009	0.0009	9.77
3	2.13	2.17	2.15	0.0004	0.0004	9.25
4	2.15	2.12	2.14	0.0002	0.0002	9.12
5	2.21	2.23	2.22	0.0001	0.0001	9.86
6	2.19	2.23	2.21	0.0004	0.0004	9.77
7	2.19	2.17	2.18	0.0001	0.0001	9.50
8	2.14	2.17	2.16	0.0002	0.0002	9.29
9	2.15	2.16	2.16	0.0000	0.0000	9.29
10	2.18	2.20	2.19	0.0001	0.0001	9.59
Grand sum	44	**Grand mean**	2.18			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	0.007	0.001	0.027	1.25		
Between Run	0.015	0.002	0.022	1.01		
Total	0.023		0.035	**1.61**		

5) Docosahexaenoic acid (C22:6n-3, DHAP)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**DHAP**					

Quality material 1: Low Pool						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	2.34	2.32	2.33	0.00010	0.00010	10.9
2	2.42	2.39	2.41	0.00022	0.00023	11.6
3	2.37	2.34	2.36	0.00023	0.00023	11.1
4	2.32	2.28	2.30	0.00040	0.00040	10.6
5	2.40	2.40	2.40	0.00000	0.00000	11.5
6	2.37	2.39	2.38	0.00010	0.00010	11.3
7	2.39	2.35	2.37	0.00040	0.00040	11.2
8	2.42	2.42	2.42	0.00000	0.00000	11.7
9	2.34	2.41	2.38	0.00123	0.00123	11.3
10	2.39	2.34	2.37	0.00062	0.00063	11.2
Grand sum	47.4	Grand mean	2.37			
	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)		
Within Run	0.007	0.001	0.026	1.08		
Between Run	0.023	0.003	0.031	1.30		
Total	0.030		0.040	**1.69**		

Quality material 2: High pool						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	2.69	2.69	2.69	0.00000	0.00000	14.5
2	2.80	2.86	2.83	0.00090	0.00090	16.0
3	2.77	2.80	2.79	0.00023	0.00022	15.5
4	2.77	2.75	2.76	0.00010	0.00010	15.2
5	2.85	2.85	2.85	0.00000	0.00000	16.2
6	2.90	2.86	2.88	0.00040	0.00040	16.6
7	2.80	2.81	2.81	0.00002	0.00003	15.7
8	2.88	2.87	2.88	0.00002	0.00002	16.5
9	2.80	2.88	2.84	0.00160	0.00160	16.1
10	2.82	2.84	2.83	0.00010	0.00010	16.0
Grand sum	56	Grand mean	2.81			
	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev		
Within Run	0.007	0.001	0.03	0.92		
Between Run	0.060	0.007	0.05	1.94		
Total	0.066		0.06	**2.15**		

6) Docosapentaenoic acid (C22:5n-3, DP3P)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**DP3P**					

Quality material 1: Low Pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	2.20	2.19	2.20	0.0000	0.0000	9.6
2	2.29	2.14	2.22	0.0056	0.0056	9.8
3	2.22	2.16	2.19	0.0009	0.0009	9.6
4	2.31	2.24	2.28	0.0012	0.0012	10.4
5	2.30	2.22	2.26	0.0016	0.0016	10.2
6	2.23	2.25	2.24	0.0001	0.0001	10.0
7	2.35	2.22	2.29	0.0042	0.0042	10.4
8	2.33	2.23	2.28	0.0025	0.0025	10.4
9	2.28	2.10	2.19	0.0081	0.0081	9.6
10	2.22	2.19	2.21	0.0002	0.0002	9.7
Grand sum	44.7	**Grand mean**	2.23			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.049	0.005	0.070	3.14		
Between Run	0.027	0.003	0.000	0.00		
Total	0.076		0.070	**3.14**		

Quality material 2: High pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	1.73	1.69	1.71	0.0004	0.0004	5.85
2	1.73	1.67	1.70	0.0009	0.0009	5.78
3	1.82	1.73	1.78	0.0020	0.0020	6.30
4	1.70	1.75	1.73	0.0006	0.0006	5.95
5	1.83	1.73	1.78	0.0025	0.0025	6.34
6	1.77	1.73	1.75	0.0004	0.0004	6.13
7	1.72	1.67	1.70	0.0006	0.0006	5.75
8	1.75	1.75	1.75	0.0000	0.0000	6.13
9	1.60	1.64	1.62	0.0004	0.0004	5.25
10	1.78	1.75	1.77	0.0002	0.0002	6.23
Grand sum	35	**Grand mean**	1.73			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	0.016	0.002	0.04	2.33		
Between Run	0.042	0.005	0.04	2.27		
Total	0.058		0.06	**3.25**		

7) Docosapentaenoic acid (C22:5n-6, DP6P)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	wt %
Analyte:	**DP6P**

Quality material 1: Low Pool

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	0.923	0.920	0.922	0.0000	0.0000	1.70
2	0.959	0.891	0.925	0.0012	0.0012	1.71
3	0.944	0.930	0.937	0.0000	0.0000	1.76
4	0.952	0.945	0.949	0.0000	0.0000	1.80
5	0.958	0.928	0.943	0.0002	0.0002	1.78
6	0.956	0.949	0.953	0.0000	0.0000	1.81
7	0.975	0.918	0.947	0.0008	0.0008	1.79
8	0.986	0.946	0.966	0.0004	0.0004	1.87
9	0.899	0.835	0.867	0.0010	0.0010	1.50
10	0.938	0.920	0.929	0.0001	0.0001	1.73
Grand sum	18.7	Grand mean	0.934			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.008	0.001	0.03	2.94
Between Run	0.013	0.001	0.02	2.01
Total	0.021		0.03	3.56

Quality material 2: High pool

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	0.892	0.851	0.872	0.0004	0.0004	1.52
2	0.865	0.854	0.860	0.0000	0.0000	1.48
3	0.925	0.896	0.911	0.0002	0.0002	1.66
4	0.841	0.894	0.868	0.0007	0.0007	1.51
5	0.911	0.890	0.901	0.0001	0.0001	1.62
6	0.903	0.893	0.898	0.0000	0.0000	1.61
7	0.877	0.852	0.865	0.0002	0.0002	1.49
8	0.944	0.881	0.913	0.0010	0.0010	1.67
9	0.782	0.790	0.786	0.0000	0.0000	1.24
10	0.878	0.874	0.876	0.0000	0.0000	1.53
Grand sum	17.5	Grand mean	0.875			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	0.005	0.001	0.02	2.64
Between Run	0.024	0.003	0.03	3.77
Total	0.030		0.04	4.60

8) Docosatetraenoic acid (C22:4n-6, DTAP)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**DTAP**					

Quality material 1: Low Pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	4.29	4.08	4.19	0.011	0.011	35.0
2	4.59	3.99	4.29	0.090	0.090	36.8
3	4.28	4.07	4.18	0.011	0.011	34.9
4	4.68	4.61	4.65	0.001	0.001	43.2
5	4.55	4.13	4.34	0.044	0.044	37.7
6	4.30	4.26	4.28	0.000	0.000	36.6
7	4.63	4.14	4.39	0.060	0.060	38.5
8	4.60	4.07	4.34	0.070	0.070	37.6
9	4.70	4.20	4.45	0.063	0.063	39.6
10	4.29	4.04	4.17	0.016	0.016	34.7
Grand sum	86.5	**Grand mean**	4.33			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.732	0.073	0.271	6.26		
Between Run	0.386	0.043	0.000	0.00		
Total	1.118		0.271	**6.26**		

Quality material 2: High pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	4.27	3.96	4.12	0.024	0.024	33.9
2	4.31	3.92	4.12	0.038	0.038	33.9
3	4.50	4.09	4.30	0.042	0.042	36.9
4	3.96	4.31	4.14	0.031	0.031	34.2
5	4.42	4.05	4.24	0.034	0.034	35.9
6	4.28	4.10	4.19	0.008	0.008	35.1
7	4.19	3.98	4.09	0.011	0.011	33.4
8	4.53	4.00	4.27	0.070	0.070	36.4
9	4.21	4.23	4.22	0.000	0.000	35.6
10	4.22	4.00	4.11	0.012	0.012	33.8
Grand sum	83.5	**Grand mean**	4.18			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	0.541	0.054	0.233	5.57		
Between Run	0.099	0.011	0.000	0.00		
Total	0.640		0.233	**5.57**		

9) Eicosadienoic acid (C20:2n-6, ED1P)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	wt %
Analyte:	ED1P

Quality material 1: Low Pool

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	0.252	0.256	0.254	0.000004	0.000004	0.13
2	0.239	0.258	0.249	0.000090	0.000090	0.12
3	0.244	0.210	0.227	0.000289	0.000289	0.10
4	0.249	0.253	0.251	0.000004	0.000004	0.13
5	0.228	0.247	0.238	0.000090	0.000090	0.11
6	0.248	0.257	0.253	0.000020	0.000020	0.13
7	0.225	0.248	0.237	0.000132	0.000132	0.11
8	0.230	0.267	0.249	0.000342	0.000342	0.12
9	0.238	0.210	0.224	0.000196	0.000196	0.10
10	0.252	0.251	0.252	0.000000	0.000000	0.13
Grand sum	4.86	Grand mean	0.243			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.0023	0.0002	0.0153	6.29
Between Run	0.0022	0.0002	0.0023	0.93
Total	0.0045		0.0155	6.36

Quality material 2: High pool

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	0.228	0.230	0.229	0.000001	0.000001	0.10
2	0.225	0.235	0.230	0.000025	0.000025	0.11
3	0.232	0.198	0.215	0.000289	0.000289	0.09
4	0.242	0.254	0.248	0.000036	0.000036	0.12
5	0.225	0.234	0.230	0.000020	0.000020	0.11
6	0.218	0.234	0.226	0.000064	0.000064	0.10
7	0.223	0.235	0.229	0.000036	0.000036	0.10
8	0.220	0.238	0.229	0.000081	0.000081	0.10
9	0.252	0.194	0.223	0.000841	0.000841	0.10
10	0.217	0.228	0.223	0.000030	0.000030	0.10
Grand sum	4.56	Grand mean	0.228			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	0.0028	0.0003	0.0169	7.40
Between Run	0.0013	0.0001	0.0000	0.00
Total	0.0041		0.0169	7.40

10) Eicosenoic acid (C20:1n-9, EN1P)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	EN1P					

Quality material 1: Low Pool						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	0.203	0.203	0.203	0.000000	0.000000	0.082
2	0.197	0.214	0.206	0.000072	0.000072	0.084
3	0.194	0.166	0.180	0.000196	0.000196	0.065
4	0.207	0.212	0.210	0.000006	0.000006	0.088
5	0.187	0.199	0.193	0.000036	0.000036	0.074
6	0.190	0.192	0.191	0.000001	0.000001	0.073
7	0.181	0.196	0.189	0.000056	0.000056	0.071
8	0.197	0.214	0.206	0.000072	0.000072	0.084
9	0.198	0.177	0.188	0.000110	0.000110	0.070
10	0.191	0.195	0.193	0.000004	0.000004	0.074
Grand sum	3.91	Grand mean	0.196			
	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)		
Within Run	0.001	0.000	0.011	5.38		
Between Run	0.002	0.000	0.006	3.14		
Total	0.003		0.012	6.23		

Quality material 2: High pool						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	0.253	0.256	0.255	0.000002	0.000002	0.13
2	0.256	0.265	0.261	0.000020	0.000020	0.14
3	0.261	0.220	0.241	0.000420	0.000420	0.12
4	0.265	0.267	0.266	0.000001	0.000001	0.14
5	0.245	0.252	0.249	0.000012	0.000012	0.12
6	0.247	0.259	0.253	0.000036	0.000036	0.13
7	0.242	0.252	0.247	0.000025	0.000025	0.12
8	0.246	0.258	0.252	0.000036	0.000036	0.13
9	0.286	0.225	0.256	0.000930	0.000930	0.13
10	0.250	0.246	0.248	0.000004	0.000004	0.12
Grand sum	5.05	Grand mean	0.253			
	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev		
Within Run	0.0030	0.0003	0.0172	6.83		
Between Run	0.0009	0.0001	0.0000	0.00		
Total	0.0039		0.0172	6.83		

11) Eicosapentaenoic acid (C20:5n-3, EPAP)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**EPAP**					

Quality material 1: Low Pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	0.331	0.324	0.328	0.000012	0.000012	0.21
2	0.334	0.341	0.338	0.000012	0.000012	0.23
3	0.335	0.334	0.335	0.000000	0.000000	0.22
4	0.331	0.320	0.326	0.000030	0.000030	0.21
5	0.342	0.328	0.335	0.000049	0.000049	0.22
6	0.330	0.321	0.326	0.000020	0.000020	0.21
7	0.342	0.338	0.340	0.000004	0.000004	0.23
8	0.357	0.347	0.352	0.000025	0.000025	0.25
9	0.342	0.346	0.344	0.000004	0.000004	0.24
10	0.323	0.334	0.329	0.000030	0.000030	0.22
Grand sum	6.70	**Grand mean**	0.335			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.0004	0.0000	0.0061	1.83		
Between Run	0.0014	0.0002	0.0075	2.25		
Total	0.0017		0.0097	**2.90**		

Quality material 2: High pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	0.245	0.235	0.240	0.00003	0.00003	0.12
2	0.244	0.252	0.248	0.00002	0.00002	0.12
3	0.251	0.253	0.252	0.00000	0.00000	0.13
4	0.254	0.260	0.257	0.00001	0.00001	0.13
5	0.268	0.251	0.260	0.00007	0.00007	0.13
6	0.242	0.242	0.242	0.00000	0.00000	0.12
7	0.248	0.240	0.244	0.00002	0.00002	0.12
8	0.267	0.258	0.263	0.00002	0.00002	0.14
9	0.263	0.253	0.258	0.00003	0.00003	0.13
10	0.241	0.247	0.244	0.00001	0.00001	0.12
Grand sum	5.01	**Grand mean**	0.251			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	0.0004	0.0000	0.0062	2.48		
Between Run	0.0012	0.0001	0.0069	2.74		
Total	0.0016		0.0093	**3.70**		

12) *gamma*-Linolenic acid (C18:3n-6, GLAP)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**GLAP**					

Quality material 1: Low Pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	0.0553	0.0533	0.0543	0.000001	0.000001	0.0059
2	0.0563	0.0553	0.0558	0.000000	0.000000	0.0062
3	0.0531	0.0547	0.0539	0.000001	0.000001	0.0058
4	0.0539	0.0536	0.0538	0.000000	0.000000	0.0058
5	0.0522	0.0545	0.0534	0.000001	0.000001	0.0057
6	0.0524	0.0551	0.0538	0.000002	0.000002	0.0058
7	0.0557	0.0554	0.0556	0.000000	0.000000	0.0062
8	0.0567	0.0553	0.0560	0.000000	0.000000	0.0063
9	0.0545	0.0529	0.0537	0.000001	0.000001	0.0058
10	0.0538	0.0527	0.0533	0.000000	0.000000	0.0057
Grand sum	1.09	**Grand mean**	0.054			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.00001	0.00000	0.0011	2.10		
Between Run	0.00002	0.00000	0.0007	1.22		
Total	0.00003		0.0013	**2.43**		

Quality material 2: High pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	0.0519	0.0519	0.0519	0.000000	0.000000	0.005
2	0.0557	0.0561	0.0559	0.000000	0.000000	0.006
3	0.0520	0.0521	0.0521	0.000000	0.000000	0.005
4	0.0543	0.0550	0.0547	0.000000	0.000000	0.006
5	0.0508	0.0548	0.0528	0.000004	0.000004	0.006
6	0.0537	0.0550	0.0544	0.000000	0.000000	0.006
7	0.0509	0.0539	0.0524	0.000002	0.000002	0.005
8	0.0550	0.0548	0.0549	0.000000	0.000000	0.006
9	0.0524	0.0519	0.0522	0.000000	0.000000	0.005
10	0.0496	0.0485	0.0491	0.000000	0.000000	0.005
Grand sum	1.06	**Grand mean**	0.0530			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	0.0000	0.0000	0.0012	2.27		
Between Run	0.0001	0.0000	0.0018	3.38		
Total	0.0001		0.0022	**4.07**		

13) *homo-gamma*-Linolenic acid (C20:3n-6, HGLP)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**HGLP**					

Quality material 1: Low Pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	1.63	1.60	1.62	0.0002	0.0002	5.22
2	1.69	1.65	1.67	0.0004	0.0004	5.58
3	1.65	1.55	1.60	0.0025	0.0025	5.12
4	1.67	1.65	1.66	0.0001	0.0001	5.51
5	1.67	1.62	1.65	0.0006	0.0006	5.41
6	1.65	1.59	1.62	0.0009	0.0009	5.25
7	1.67	1.62	1.65	0.0006	0.0006	5.41
8	1.72	1.67	1.70	0.0006	0.0006	5.75
9	1.74	1.73	1.74	0.0000	0.0000	6.02
10	1.67	1.65	1.66	0.0001	0.0001	5.51
Grand sum	33.1	**Grand mean**	1.65			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.012	0.001	0.035	2.12		
Between Run	0.029	0.003	0.031	1.89		
Total	0.041		0.047	**2.84**		

Quality material 2: High pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	1.23	1.22	1.23	0.00002	0.00003	3.00
2	1.27	1.28	1.28	0.00002	0.00003	3.25
3	1.27	1.21	1.24	0.00090	0.00090	3.08
4	1.26	1.26	1.26	0.00000	0.00000	3.18
5	1.26	1.25	1.26	0.00003	0.00002	3.15
6	1.26	1.25	1.26	0.00003	0.00002	3.15
7	1.27	1.21	1.24	0.00090	0.00090	3.08
8	1.30	1.30	1.30	0.00000	0.00000	3.38
9	1.30	1.31	1.31	0.00003	0.00002	3.41
10	1.31	1.27	1.29	0.00040	0.00040	3.33
Grand sum	25.3	**Grand mean**	1.26			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	0.0047	0.0005	0.0216	1.71		
Between Run	0.0132	0.0015	0.0224	1.77		
Total	0.0179		0.0311	**2.46**		

14) Lignoceric acid (C24:0, LG1P)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**LG1P**					

Quality material 1: Low Pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	6.06	6.06	6.06	0.00000	0.00000	73.4
2	5.92	6.04	5.98	0.00360	0.00360	71.5
3	6.22	6.15	6.19	0.00122	0.00123	76.5
4	5.90	5.79	5.85	0.00302	0.00303	68.3
5	6.03	6.07	6.05	0.00040	0.00040	73.2
6	6.26	6.27	6.27	0.00002	0.00002	78.5
7	6.11	6.14	6.13	0.00022	0.00022	75.0
8	6.03	6.03	6.03	0.00000	0.00000	72.7
9	6.04	6.03	6.04	0.00002	0.00002	72.8
10	6.16	6.19	6.18	0.00023	0.00022	76.3
Grand sum	122	**Grand mean**	6.08			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.018	0.002	0.04	0.69		
Between Run	0.254	0.028	0.12	1.89		
Total	0.272		0.12	**2.02**		

Quality material 2: High pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	6.18	6.36	6.27	0.0081	0.0081	78.6
2	6.18	6.26	6.22	0.0016	0.0016	77.4
3	6.27	6.33	6.30	0.0009	0.0009	79.4
4	6.11	6.06	6.09	0.0006	0.0006	74.1
5	6.25	6.33	6.29	0.0016	0.0016	79.1
6	6.45	6.52	6.49	0.0012	0.0012	84.1
7	6.35	6.43	6.39	0.0016	0.0016	81.7
8	6.21	6.32	6.27	0.0030	0.0030	78.5
9	6.30	6.23	6.27	0.0012	0.0012	78.5
10	6.38	6.50	6.44	0.0036	0.0036	82.9
Grand sum	126	**Grand mean**	6.30			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	0.047	0.005	0.07	1.09		
Between Run	0.236	0.026	0.10	1.65		
Total	0.283		0.12	**1.97**		

15) Linoleic acid (C18:2n-6, LNAP)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**LNAP**					

Quality material 1: Low Pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	8.31	8.33	8.32	0.0001	0.0001	138
2	8.37	8.52	8.45	0.0056	0.0056	143
3	8.32	8.38	8.35	0.0009	0.0009	139
4	8.45	8.41	8.43	0.0004	0.0004	142
5	8.42	8.54	8.48	0.0036	0.0036	144
6	8.40	8.50	8.45	0.0025	0.0025	143
7	8.35	8.39	8.37	0.0004	0.0004	140
8	8.48	8.55	8.52	0.0012	0.0012	145
9	8.46	8.73	8.60	0.0182	0.0182	148
10	8.50	8.50	8.50	0.0000	0.0000	145
Grand sum	169	**Grand mean**	8.45			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.066	0.007	0.08	0.96		
Between Run	0.124	0.014	0.06	0.71		
Total	0.190		0.10	**1.20**		

Quality material 2: High pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	9.23	9.31	9.27	0.002	0.002	172
2	9.40	9.59	9.50	0.009	0.009	180
3	9.28	9.38	9.33	0.003	0.003	174
4	9.55	9.49	9.52	0.001	0.001	181
5	9.44	9.51	9.48	0.001	0.001	180
6	9.41	9.59	9.50	0.008	0.008	181
7	9.40	9.35	9.38	0.001	0.001	176
8	9.48	9.67	9.58	0.009	0.009	183
9	9.49	9.77	9.63	0.020	0.020	185
10	9.38	9.60	9.49	0.012	0.012	180
Grand sum	189	**Grand mean**	9.47			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	0.13	0.01	0.11	1.20		
Between Run	0.22	0.02	0.08	0.80		
Total	0.35		0.14	**1.44**		

16) Myristic acid (C14:0, MR1P)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**MR1P**					

Quality material 1: Low Pool						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	0.356	0.337	0.347	0.00009	0.00009	0.24
2	0.355	0.337	0.346	0.00008	0.00008	0.24
3	0.368	0.348	0.358	0.00010	0.00010	0.26
4	0.381	0.382	0.382	0.00000	0.00000	0.29
5	0.360	0.344	0.352	0.00006	0.00006	0.25
6	0.353	0.346	0.350	0.00001	0.00001	0.24
7	0.350	0.367	0.359	0.00007	0.00007	0.26
8	0.347	0.361	0.354	0.00005	0.00005	0.25
9	0.338	0.351	0.345	0.00004	0.00004	0.24
10	0.353	0.365	0.359	0.00004	0.00004	0.26
Grand sum	7	Grand mean	0.355			
	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)		
Within Run	0.0011	0.0001	0.01	2.95		
Between Run	0.0021	0.0002	0.01	2.20		
Total	0.0032		0.01	3.68		

Quality material 2: High pool						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	0.200	0.215	0.208	0.00006	0.00006	0.09
2	0.224	0.207	0.216	0.00007	0.00007	0.09
3	0.215	0.215	0.215	0.00000	0.00000	0.09
4	0.214	0.233	0.224	0.00009	0.00009	0.10
5	0.212	0.209	0.211	0.00000	0.00000	0.09
6	0.210	0.193	0.202	0.00007	0.00007	0.08
7	0.215	0.194	0.205	0.00011	0.00011	0.08
8	0.200	0.207	0.204	0.00001	0.00001	0.08
9	0.176	0.132	0.154	0.00048	0.00048	0.05
10	0.220	0.207	0.214	0.00004	0.00004	0.09
Grand sum	4	Grand mean	0.205			
	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev		
Within Run	0.0019	0.0002	0.01	6.7		
Between Run	0.0066	0.0007	0.02	8.02		
Total	0.0084		0.02	10.4		

17) Nervonic acid (C24:1n-9, NR1P)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**NR1P**					

Quality material 1: Low Pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	5.35	5.19	5.27	0.006	0.006	56
2	5.13	5.32	5.23	0.009	0.009	55
3	5.42	4.82	5.12	0.090	0.090	52
4	5.17	5.16	5.17	0.000	0.000	53
5	5.19	5.34	5.27	0.006	0.006	55
6	5.67	5.85	5.76	0.008	0.008	66
7	5.14	5.36	5.25	0.012	0.012	55
8	5.35	5.40	5.38	0.001	0.001	58
9	5.32	5.13	5.23	0.009	0.009	55
10	5.58	5.40	5.49	0.008	0.008	60
Grand sum	106	**Grand mean**	5.31			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.30	0.03	0.17	3.25		
Between Run	0.64	0.07	0.14	2.69		
Total	0.93		0.22	**4.22**		

Quality material 2: High pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	4.81	4.73	4.77	0.002	0.002	46
2	4.79	4.87	4.83	0.002	0.002	47
3	4.71	4.50	4.61	0.011	0.011	42
4	4.75	4.76	4.76	0.000	0.000	45
5	4.65	4.73	4.69	0.002	0.002	44
6	4.94	5.10	5.02	0.006	0.006	50
7	4.57	4.70	4.64	0.004	0.004	43
8	4.75	4.84	4.80	0.002	0.002	46
9	4.99	4.61	4.80	0.036	0.036	46
10	4.70	4.71	4.71	0.000	0.000	44
Grand sum	95	**Grand mean**	4.76			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	0.13	0.01	0.11	2.39		
Between Run	0.25	0.03	0.08	1.78		
Total	0.38		0.14	**2.98**		

18) Oleic acid (C18:1n-9, OL1P)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**OL1P**					

Quality material 1: Low Pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	12.2	12.7	12.5	0.063	0.063	310
2	12.2	12.8	12.5	0.090	0.090	313
3	12.5	12.6	12.6	0.003	0.002	315
4	12.8	12.7	12.8	0.003	0.003	325
5	12.4	12.5	12.5	0.002	0.003	310
6	12.4	12.7	12.6	0.023	0.022	315
7	12.4	12.5	12.5	0.002	0.003	310
8	12.3	12.3	12.3	0.000	0.000	303
9	12.2	12.5	12.4	0.023	0.023	305
10	12.5	12.4	12.5	0.003	0.002	310
Grand sum	250	**Grand mean**	12.5			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.420	0.042	0.205	1.64		
Between Run	0.272	0.030	0.000	0.00		
Total	0.692		0.205	**1.64**		

Quality material 2: High pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	11.4	11.2	11.3	0.010	0.010	255
2	11.1	11.4	11.3	0.023	0.023	253
3	11.4	11.7	11.6	0.023	0.022	267
4	11.7	11.5	11.6	0.010	0.010	269
5	11.3	11.4	11.4	0.003	0.002	258
6	11.3	11.4	11.4	0.003	0.002	258
7	11.5	11.9	11.7	0.040	0.040	274
8	11.3	11.4	11.4	0.003	0.002	258
9	11.2	11.5	11.4	0.023	0.023	258
10	11.4	11.5	11.5	0.002	0.003	262
Grand sum	229	**Grand mean**	11.4			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	0.28	0.03	0.17	1.45		
Between Run	0.38	0.04	0.09	0.76		
Total	0.66		0.19	**1.64**		

19) Palmitoleic acid (C16:1n-7, PL1P)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**PL1P**					

Quality material 1: Low Pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	0.624	0.628	0.626	0.00000	0.00000	0.784
2	0.645	0.650	0.648	0.00001	0.00001	0.839
3	0.645	0.649	0.647	0.00000	0.00000	0.837
4	0.650	0.648	0.649	0.00000	0.00000	0.842
5	0.641	0.646	0.644	0.00001	0.00001	0.828
6	0.640	0.632	0.636	0.00002	0.00002	0.809
7	0.633	0.641	0.637	0.00002	0.00002	0.812
8	0.630	0.640	0.635	0.00003	0.00003	0.806
9	0.633	0.598	0.616	0.00031	0.00031	0.758
10	0.650	0.655	0.653	0.00001	0.00001	0.852
Grand sum	12.8	**Grand mean**	0.639			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.0	0.00	0.01	1.38		
Between Run	0.0	0.00	0.01	1.51		
Total	0.0		0.01	**2.05**		

Quality material 2: High pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	0.166	0.176	0.171	0.00002	0.00003	0.058
2	0.207	0.210	0.209	0.00000	0.00000	0.087
3	0.173	0.184	0.179	0.00003	0.00003	0.064
4	0.178	0.177	0.178	0.00000	0.00000	0.063
5	0.192	0.190	0.191	0.00000	0.00000	0.073
6	0.199	0.196	0.198	0.00000	0.00000	0.078
7	0.180	0.179	0.180	0.00000	0.00000	0.064
8	0.203	0.202	0.203	0.00000	0.00000	0.082
9	0.186	0.156	0.171	0.00023	0.00023	0.058
10	0.174	0.173	0.174	0.00000	0.00000	0.060
Grand sum	3.70	**Grand mean**	0.185			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	0.001	0.000	0.008	4.09		
Between Run	0.003	0.000	0.013	6.85		
Total	0.004		0.015	**7.98**		

20) Palmitic acid (C16:0, PM1P)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**PM1P**					

Quality material 1: Low Pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	22.0	21.9	22.0	0.0025	0.0025	964
2	21.7	21.6	21.7	0.0025	0.0025	937
3	21.2	21.3	21.3	0.0025	0.0025	903
4	21.6	21.9	21.8	0.0225	0.0225	946
5	21.4	21.4	21.4	0.0000	0.0000	916
6	20.9	20.6	20.8	0.0225	0.0225	861
7	21.9	22.0	22.0	0.0025	0.0025	964
8	20.8	21.2	21.0	0.0400	0.0400	882
9	21.8	21.2	21.5	0.0900	0.0900	925
10	21.1	21.3	21.2	0.0100	0.0100	899
Grand sum	429	**Grand mean**	21.4			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.39	0.04	0.20	0.92		
Between Run	2.86	0.32	0.37	1.74		
Total	3.25		0.42	**1.97**		

Quality material 2: High pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	20.6	20.8	20.7	0.010	0.010	857
2	20.4	20.3	20.4	0.002	0.003	828
3	19.8	19.8	19.8	0.000	0.000	784
4	20.4	20.5	20.5	0.003	0.003	836
5	20.2	20.2	20.2	0.000	0.000	816
6	19.7	19.5	19.6	0.010	0.010	768
7	20.9	20.7	20.8	0.010	0.010	865
8	19.8	19.9	19.9	0.003	0.002	788
9	20.8	20.0	20.4	0.160	0.160	832
10	20.1	20.1	20.1	0.000	0.000	808
Grand sum	405	**Grand mean**	20.2			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	0.395	0.040	0.20	0.98		
Between Run	2.763	0.307	0.37	1.81		
Total	3.158		0.42	**2.06**		

21) Stearic acid (C18:0, ST1P)

Method name:	Fatty acids in RBC					
Method #:	4030					
Matrix:	RBC					
Units:	wt %					
Analyte:	**ST1P**					

Quality material 1: Low Pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	14.4	14.2	14.3	0.010	0.010	409
2	14.0	13.7	13.9	0.023	0.023	384
3	14.3	15.1	14.7	0.160	0.160	432
4	13.8	14.0	13.9	0.010	0.010	386
5	14.0	14.0	14.0	0.000	0.000	392
6	13.8	13.5	13.7	0.023	0.023	373
7	13.7	13.8	13.8	0.003	0.003	378
8	14.0	14.1	14.1	0.003	0.002	395
9	13.7	14.3	14.0	0.090	0.090	392
10	13.8	14.0	13.9	0.010	0.010	386
Grand sum	280	**Grand mean**	14.0			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.66	0.07	0.26	1.83		
Between Run	1.62	0.18	0.24	1.70		
Total	2.28		0.35	**2.50**		

Quality material 2: High pool						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	16.4	16.6	16.5	0.010	0.010	545
2	16.3	15.7	16.0	0.090	0.090	512
3	16.5	17.1	16.8	0.090	0.090	564
4	16.0	16.0	16.0	0.000	0.000	512
5	15.8	15.9	15.9	0.003	0.002	502
6	15.8	15.6	15.7	0.010	0.010	493
7	16.0	15.9	16.0	0.003	0.002	509
8	15.8	16.0	15.9	0.010	0.010	506
9	15.8	16.5	16.2	0.122	0.123	522
10	16.1	15.9	16.0	0.010	0.010	512
Grand sum	322	**Grand mean**	16.1			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	0.70	0.07	0.26	1.64		
Between Run	1.93	0.21	0.27	1.67		
Total	2.63		0.38	**2.34**		

C. Stability

The freeze/thaw, bench-top, processed sample, and long-term stability of the 21 analytes in the low QC and high QC is shown below. Analyte codes without a suffix P are used when weight concentrations are displayed (mg/L).

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions	
Describe condition:	QC vials thawed three times and re-frozen at -80°C (3 freeze-thaw cycles)
Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)	
Describe condition:	QC vials stored on the bench top for 3 hours after removing from -80°C freezer
Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler	
Describe condition:	Processed samples stored at room temperature for 3 days
Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis	
Describe condition:	QC pools prepared stored continuously at -80°C for 2 months

1) *alpha*-Linolenic acid (C18:3n, ALN)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**ALN**

Quality material 1: Low pool	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	1.62	1.69	1.66	1.69	1.46	1.46	1.69	1.62
Replicate 2	1.71	1.66	1.64	1.61	1.47	1.47	1.61	1.60
Replicate 3		1.66		1.67	1.42	1.39	1.67	1.58
Mean	1.67	1.67	1.65	1.66	1.45	1.44	1.66	1.60
% difference from initial measurement	--	**0.2**	--	**0.5**	--	**-0.7**	--	**-3.4**

Quality material 2: High pool	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	1.34	1.48	1.31	1.32	1.03	1.10	1.32	1.32
Replicate 2	1.38	1.50	1.37	1.42	1.10	1.07	1.42	1.40
Replicate 3		1.43		1.35	1.11	1.08	1.35	1.27
Mean	1.36	1.47	1.34	1.36	1.08	1.09	1.36	1.33
% difference from initial measurement	--	**8.0**	--	**1.5**	--	**0.8**	--	**-2.5**

2) Arachidic acid (C20:0, AR1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**AR1**

Quality material 1: Low pool	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	3.32	3.45	3.24	3.46	3.63	3.42	3.46	3.54
Replicate 2	3.49	3.26	3.47	3.19	3.71	3.33	3.19	3.28
Replicate 3		3.52		3.54		3.29		3.52
Mean	3.41	3.41	3.36	3.39	3.67	3.35	3.32	3.45
% difference from initial measurement	--	**0.0**	--	**1.0**	--	**-8.8**	--	**3.7**

Quality material 2: High pool	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	4.94	5.07	4.78	4.73	5.21	4.84	4.73	4.98
Replicate 2	4.67	5.26	4.65	4.91	5.13	4.69	4.91	4.81
Replicate 3		5.42		4.91	5.20	4.68	4.91	5.38
Mean	4.81	5.25	4.72	4.85	5.18	4.74	4.85	5.06
% difference from initial measurement	--	**9.2**	--	**2.9**	--	**-8.6**	--	**4.2**

3) Arachidonic acid (C20:4n-6, ARA)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**ARA**

Quality material 1: Low pool	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	177	177	180	189	181	184	189	194
Replicate 2	178	176	190	186	185	185	186	190
Replicate 3		180		192	183	182	192	196
Mean	178	178	185	189	183	183	189	193
% difference from initial measurement	--	**0.1**	--	**2.3**	--	**0.1**	--	**2.2**

Quality material 2: High pool	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	155	171	158	165	161	158	165	170
Replicate 2	155	182	165	169	161	159	169	175
Replicate 3		167		166	162	160	166	174
Mean	155	173	162	167	161	159	167	173
% difference from initial measurement	--	**11.5**	--	**3.1**	--	**-1.3**	--	**3.7**

4) Docosanoic acid (C22:0, DA1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**DA1**

Quality material 1: Low pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	18.7	18.8	19.0	20.2	19.5	19.4	20.2	19.9
Replicate 2	18.5	18.5	20.0	19.2	19.5	19.2	19.2	19.5
Replicate 3		18.6		20.0	19.4	19.7	20.0	20.2
Mean	18.6	18.6	19.5	19.8	19.5	19.4	19.8	19.9
% difference from initial measurement	--	**-0.1**	--	**1.6**	--	**-0.3**	--	**0.5**

Quality material 2: High pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	19.1	20.7	19.4	19.8	19.3	19.8	19.8	20.3
Replicate 2	18.6	21.1	19.6	19.6	19.8	20.1	19.6	20.2
Replicate 3		20.0		19.8	19.9	19.8	19.8	20.2
Mean	18.9	20.6	19.5	19.8	19.6	19.9	19.8	20.2
% difference from initial measurement	--	**9.1**	--	**1.3**	--	**1.5**	--	**2.4**

5) Docosahexaenoic acid (C22:6n-3, DHA)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**DHA**

Quality material 1: Low pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	24.3	25.4	24.0	24.6	25.8	25.7	24.6	25.0
Replicate 2	24.5	23.1	25.0	24.9	25.9	26.3	24.9	25.1
Replicate 3		24.8		25.3	26.0	25.8	25.3	26.5
Mean	24.4	24.4	24.5	25.0	25.9	25.9	25.0	25.5
% difference from initial measurement	--	**0.2**	--	**2.0**	--	**0.0**	--	**2.3**

Quality material 2: High pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	25.5	27.5	24.0	25.7	27.1	27.1	25.7	27.4
Replicate 2	24.5	28.9	25.6	26.7	26.4	27.0	26.7	27.0
Replicate 3		26.6		25.5	26.7	27.0	25.5	25.8
Mean	25.0	27.7	24.8	26.0	26.7	27.0	26.0	26.8
% difference from initial measurement	--	**10.7**	--	**4.7**	--	**1.2**	--	**3.1**

6) Docosapentaenoic acid (C22:5n-3, DP3)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**DP3**

Quality material 1: Low pool	Initial measurement	Three freeze-thaw cycles		Initial measurement	Bench-top stability		Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	24.0	25.0		25.5	26.0		24.1	24.6		26.0	25.8
Replicate 2	24.3	24.5		25.2	24.3		24.3	24.8		24.3	25.0
Replicate 3		24.8			25.0		25.0	23.8		25.0	26.5
Mean	24.2	24.8		25.3	25.1		24.5	24.4		25.1	25.8
% difference from initial measurement	--	**2.4**		--	**-0.9**		--	**-0.3**		--	**2.7**

Quality material 2: High pool	Initial measurement	Three freeze-thaw cycles		Initial measurement	Bench-top stability		Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	16.1	17.7		16.4	16.8		16.9	16.5		16.8	18.3
Replicate 2	15.2	19.2		16.3	16.8		16.3	16.6		16.8	17.4
Replicate 3		17.5			16.2		16.4	16.6		16.2	17.2
Mean	15.7	18.1		16.3	16.6		16.5	16.6		16.6	17.6
% difference from initial measurement	--	**15.7**		--	**1.8**		--	**0.4**		--	**6.2**

7) Docosapentaenoic acid (C22:5n-6, DP6)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**DP6**

Quality material 1: Low pool	Initial measurement	Three freeze-thaw cycles		Initial measurement	Bench-top stability		Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	10.1	10.2		10.6	10.6		10.2	10.2		10.6	10.8
Replicate 2	10.4	10.1		10.3	10.3		10.3	10.3		10.3	10.9
Replicate 3		10.1			10.4		10.0	10.0		10.4	10.7
Mean	10.3	10.1		10.5	10.4		10.2	10.2		10.4	10.8
% difference from initial measurement	--	**-1.2**		--	**-0.3**		--	**-0.1**		--	**3.6**

Quality material 2: High pool	Initial measurement	Three freeze-thaw cycles		Initial measurement	Bench-top stability		Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	8.42	8.91		8.52	8.49		8.72	8.47		8.49	8.88
Replicate 2	8.37	9.59		8.32	8.79		8.52	8.54		8.79	9.07
Replicate 3		8.78			8.73		8.57	8.46		8.73	8.86
Mean	8.39	9.09		8.42	8.67		8.60	8.49		8.67	8.94
% difference from initial measurement	--	**8.3**		--	**2.9**		--	**-1.3**		--	**3.1**

8) Docosatetraenoic acid (C22:4n-6, DTA)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**DTA**

Quality material 1: Low pool	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	52.0	51.7	54.9	55.9	46.7	47.2	55.9	55.2
Replicate 2	51.9	51.7	52.1	51.2	47.5	47.4	51.2	53.1
Replicate 3		51.6		51.2	47.8	47.4	51.2	54.1
Mean	51.9	51.7	53.5	52.8	47.4	47.4	52.8	54.1
% difference from initial measurement	--	**-0.5**	--	**-1.4**	--	**0.0**	--	**2.6**

Quality material 2: High pool	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	42.4	45.5	45.1	44.8	41.5	40.5	44.8	47.5
Replicate 2	41.7	51.1	42.5	44.7	40.4	41.4	44.7	44.3
Replicate 3		45.9		44.2	41.0	41.0	44.2	45.5
Mean	42.0	47.5	43.8	44.5	41.0	41.0	44.5	45.8
% difference from initial measurement	--	**13.0**	--	**1.6**	--	**0.0**	--	**2.8**

9) Eicosadienoic acid (C20:2n-6, ED1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**ED1**

Quality material 1: Low pool	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	2.73	2.53	2.81	2.82	2.76	2.69	2.82	2.80
Replicate 2	2.78	2.69	2.91	2.77	2.87	2.66	2.77	2.76
Replicate 3		2.81		2.96	2.80	2.55	2.96	2.95
Mean	2.75	2.68	2.86	2.85	2.81	2.63	2.85	2.84
% difference from initial measurement	--	**-2.9**	--	**-0.4**	--	**-6.3**	--	**-0.5**

Quality material 2: High pool	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	2.07	2.32	2.14	2.09	2.08	2.05	2.09	2.10
Replicate 2	2.17	2.34	2.16	2.39	2.08	1.98	2.39	2.19
Replicate 3		2.36		2.19	2.09	2.03	2.19	2.23
Mean	2.12	2.34	2.15	2.22	2.09	2.02	2.22	2.17
% difference from initial measurement	--	**10.4**	--	**3.3**	--	**-3.2**	--	**-2.3**

10) Eicosenoic acid (C20:1n-9, EN1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**EN1**

Quality material 1: Low pool	Initial measurement	Three freeze-thaw cycles		Initial measurement	Bench-top stability		Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	2.11	1.98		2.12	2.15		2.18	2.15		2.15	2.07
Replicate 2	2.19	2.15		2.20	2.12		2.21	2.08		2.12	2.12
Replicate 3		2.21			2.28		2.20	2.07		2.28	2.19
Mean	2.15	2.11		2.16	2.18		2.20	2.10		2.18	2.13
% difference from initial measurement	--	**-1.7**		--	**1.1**		--	**-4.5**		--	**-2.6**

Quality material 2: High pool	Initial measurement	Three freeze-thaw cycles		Initial measurement	Bench-top stability		Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	2.11	2.45		2.17	2.10		2.29	2.21		2.10	2.12
Replicate 2	2.29	2.46		2.19	2.39		2.29	2.17		2.39	2.17
Replicate 3		2.46			2.20		2.30	2.16		2.20	2.10
Mean	2.20	2.46		2.18	2.23		2.29	2.18		2.23	2.13
% difference from initial measurement	--	**11.7**		--	**2.3**		--	**-4.9**		--	**-4.6**

11) Eicosapentaenoic acid (C20:5n-3, EPA)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**EPA**

Quality material 1: Low pool	Initial measurement	Three freeze-thaw cycles		Initial measurement	Bench-top stability		Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	4.18	3.88		4.05	4.00		4.02	3.94		4.00	4.21
Replicate 2	3.89	3.82		4.31	4.08		3.77	3.94		4.08	4.05
Replicate 3		3.93			4.10		4.11	3.94		4.10	4.23
Mean	4.03	3.88		4.18	4.06		3.97	3.94		4.06	4.16
% difference from initial measurement	--	**-3.8**		--	**-2.8**		--	**-0.7**		--	**2.6**

Quality material 2: High pool	Initial measurement	Three freeze-thaw cycles		Initial measurement	Bench-top stability		Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	2.72	2.93		2.67	2.78		2.65	2.63		2.78	2.75
Replicate 2	2.70	3.13		2.88	2.83		2.54	2.63		2.83	2.98
Replicate 3		2.86			2.80		2.65	2.62		2.80	2.72
Mean	2.71	2.97		2.78	2.80		2.61	2.63		2.80	2.82
% difference from initial measurement	--	**9.8**		--	**1.0**		--	**0.6**		--	**0.5**

12) *gamma*-Linolenic acid (C18:3n-6, GLA)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**GLA**

Quality material 1: Low pool	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	0.688	0.679	0.661	0.694	0.609	0.587	0.694	0.678
Replicate 2	0.683	0.687	0.686	0.692	0.580	0.615	0.692	0.664
Replicate 3		0.716		0.688	0.590	0.592	0.688	0.681
Mean	0.685	0.694	0.673	0.691	0.593	0.598	0.691	0.675
% difference from initial measurement	--	**1.3**	--	**2.6**	--	**0.9**	--	**-2.4**

Quality material 2: High pool	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	0.623	0.658	0.584	0.606	0.538	0.526	0.606	0.585
Replicate 2	0.626	0.677	0.615	0.629	0.527	0.505	0.629	0.567
Replicate 3		0.637		0.616	0.531	0.515	0.616	0.585
Mean	0.624	0.657	0.600	0.617	0.532	0.515	0.617	0.579
% difference from initial measurement	--	**5.3**	--	**2.8**	--	**-3.2**	--	**-6.1**

13) *homo-gamma*-Linolenic acid (C20:3n-6, HGL)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**HGL**

Quality material 1: Low pool	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	18.6	18.3	19.5	20.2	19.0	18.5	20.2	20.9
Replicate 2	18.8	18.3	20.0	19.2	19.0	18.4	19.2	20.2
Replicate 3		19.0		19.9	18.3	18.1	19.9	20.6
Mean	18.7	18.5	19.7	19.8	18.7	18.4	19.8	20.6
% difference from initial measurement	--	**-1.1**	--	**0.1**	--	**-2.1**	--	**4.0**

Quality material 2: High pool	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	12.0	13.3	12.8	12.9	12.4	12.2	12.9	13.2
Replicate 2	12.4	14.1	13.1	13.6	12.6	12.1	13.6	14.1
Replicate 3		13.7		13.1	12.6	12.0	13.1	13.8
Mean	12.2	13.7	13.0	13.2	12.5	12.1	13.2	13.7
% difference from initial measurement	--	**12.7**	--	**1.8**	--	**-3.5**	--	**3.7**

14) Lignoceric acid (C24:0, LG1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**LG1**

Quality material 1: Low pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	67.5	67.2	65.8	71.0	69.4	70.1	71.0	72.4
Replicate 2	66.3	65.4	70.7	69.9	69.4	70.1	69.9	71.6
Replicate 3		67.2		71.5	70.4	69.3	71.5	71.8
Mean	66.9	66.6	68.3	70.8	69.7	69.8	70.8	71.9
% difference from initial measurement	--	**-0.4**	--	**3.7**	--	**0.1**	--	**1.6**

Quality material 2: High pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	57.2	61.0	58.4	58.9	58.2	57.8	58.9	59.2
Replicate 2	57.6	63.7	59.1	59.5	58.4	58.1	59.5	64.1
Replicate 3		59.4		59.4	59.2	58.9	59.4	59.8
Mean	57.4	61.4	58.7	59.3	58.6	58.2	59.3	61.0
% difference from initial measurement	--	**7.0**	--	**0.9**	--	**-0.6**	--	**3.0**

15) Linoleic acid (C18:2n-6, LNA)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**LNA**

Quality material 1: Low pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	88.5	88.0	87.9	91.8	93.3	92.4	91.8	92.3
Replicate 2	87.9	86.5	90.6	90.7	93.3	93.2	90.7	90.3
Replicate 3		88.3		93.0	92.5	92.7	93.0	93.3
Mean	88.2	87.6	89.3	91.8	93.0	92.8	91.8	92.0
% difference from initial measurement	--	**-0.6**	--	**2.9**	--	**-0.3**	--	**0.1**

Quality material 2: High pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	84.1	91.3	84.5	86.9	87.3	88.6	86.9	87.7
Replicate 2	83.2	96.0	86.2	87.9	88.3	88.1	87.9	88.8
Replicate 3		90.0		87.2	89.0	89.2	87.2	88.7
Mean	83.6	92.4	85.3	87.3	88.2	88.6	87.3	88.4
% difference from initial measurement	--	**10.5**	--	**2.3**	--	**0.5**	--	**1.2**

16) Myristic acid (C14:0, MR1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**MR1**

Quality material 1: Low pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	3.82	4.07	3.73	3.78	4.01	3.86	3.78	3.72
Replicate 2	4.11	3.76	3.89	3.49	3.99	3.95	3.49	3.62
Replicate 3		4.05		3.55	4.20	4.07	3.55	3.60
Mean	3.96	3.96	3.81	3.61	4.07	3.96	3.61	3.65
% difference from initial measurement	--	**0.0**	--	**-5.4**	--	**-2.6**	--	**1.2**

Quality material 2: High pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	1.86	2.17	1.82	1.42	1.85	1.78	1.42	1.57
Replicate 2	2.11	2.3	1.48	1.62	1.40	1.37	1.62	1.81
Replicate 3		1.87		1.69	2.08	2.03	1.69	1.84
Mean	1.99	2.11	1.65	1.57	1.78	1.73	1.57	1.74
% difference from initial measurement	--	**6.2**	--	**-4.6**	--	**-2.8**	--	**10.5**

17) Nervonic acid (C24:1n-9, NR1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**NR1**

Quality material 1: Low pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	58.9	53.6	61.6	63.1	57.9	57.5	63.1	66.5
Replicate 2	56.5	55.7	62.6	62.1	56.5	57.2	62.1	65.5
Replicate 3		57.6		62.9	56.2	56.6	62.9	64.5
Mean	57.7	55.6	62.1	62.7	56.8	57.1	62.7	65.5
% difference from initial measurement	--	**-3.6**	--	**1.0**	--	**0.5**	--	**4.4**

Quality material 2: High pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	40.1	44.0	45.8	43.4	42.5	42.2	43.4	43.8
Replicate 2	43.8	46.1	45.2	47.8	42.2	41.8	47.8	52.4
Replicate 3		41.9		44.9	43.6	42.8	44.9	44.2
Mean	42.0	44.0	45.5	45.4	42.8	42.3	45.4	46.8
% difference from initial measurement	--	**4.9**	--	**-0.3**	--	**-1.1**	--	**3.0**

18) Oleic acid (C18:1n-9, OL1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**OL1**

Quality material 1: Low pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	132	130	134	133	142	140	133	137
Replicate 2	133	131	141	137	143	141	137	133
Replicate 3		133		141	139	140	141	139
Mean	132	131	137	137	141	140	137	136
% difference from initial measurement	--	**-1.0**	--	**-0.2**	--	**-0.9**	--	**-0.5**

Quality material 2: High pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	101	111	103	105	107	106	105	106
Replicate 2	98.9	116	105	108	104	104	108	107
Replicate 3		106		107	106	109	107	107
Mean	100	111	104	107	106	106	107	107
% difference from initial measurement	--	**10.8**	--	**2.5**	--	**0.2**	--	**0.0**

19) Palmitoleic acid (C16:1n-7, PL1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**PL1**

Quality material 1: Low pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	7.33	7.25	7.20	7.37	7.29	7.07	7.37	7.54
Replicate 2	7.37	7.22	7.27	7.22	7.23	7.01	7.22	7.34
Replicate 3		7.38		7.39	7.05	6.86	7.39	7.55
Mean	7.35	7.29	7.23	7.33	7.19	6.98	7.33	7.48
% difference from initial measurement	--	**-0.9**	--	**1.3**	--	**-2.9**	--	**2.1**

Quality material 2: High pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	2.18	2.37	1.91	1.91	1.47	1.41	1.91	1.63
Replicate 2	2.22	2.39	1.93	1.97	1.50	1.43	1.93	1.65
Replicate 3		2.36		1.97	1.52	1.44		1.65
Mean	2.20	2.37	1.92	1.95	1.49	1.43	1.92	1.65
% difference from initial measurement	--	**7.6**	--	**1.5**	--	**-4.5**	--	**-14.3**

20) Palmitic acid (C16:0, PM1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**PM1**

Quality material 1: Low pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	227	234	231	236	238	247	236	210
Replicate 2	234	223	235	229	238	253	229	203
Replicate 3		230		236	244	265	236	209
Mean	231	229	233	234	240	255	234	207
% difference from initial measurement	--	-0.7	--	0.3	--	6.0	--	-11.3

Quality material 2: High pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	174	190	177	174	181	187	174	155
Replicate 2	178	195	173	182	183	195	182	163
Replicate 3		182		179	190	204	179	158
Mean	176	189	175	178	185	195	178	159
% difference from initial measurement	--	7.3	--	1.8	--	5.9	--	-11.1

21) Stearic acid (C18:0, ST1)

Method name:	Fatty acids in RBC
Method #:	4030
Matrix:	RBC
Units:	mg/L
Analyte:	**ST1**

Quality material 1: Low pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	152	161	149	152	151	151	152	151
Replicate 2	156	149	157	147	151	150	147	146
Replicate 3		154		154	150	153	154	151
Mean	154	155	153	151	151	151	151	149
% difference from initial measurement	--	0.4	--	-1.1	--	0.4	--	-1.3

Quality material 2: High pool

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	149	161	143	142	139	142	142	144
Replicate 2	142	162	138	139	138	139	139	139
Replicate 3		154		142	144	146	142	142
Mean	145	159	140	141	140	142	141	142
% difference from initial measurement	--	9.3	--	0.7	--	1.3	--	0.3

D. LOD, specificity and fit for intended use

The LOD, specificity, and fit for intended use of the 21 analytes is summarized here:

Method name:	Fatty acids in RBC		
Method #:	4030		
Matrix:	RBC		
Units:	mg/L		

Analytes	**Limit of Detection (mg/L)**	**Interferences successfully checked in at least 50 human samples**	**Accuracy, precision, LOD, specificity and stability meet performance specifications for intended use**
alpha-Linolenic acid (C18:3n-3) (ALN)	0.03	yes	yes
Arachidic acid (C20:0) (AR1)	0.3	yes	yes
Arachidonic acid (C20:4n-6) (ARA)	0.08	yes	yes
Docosanoic acid (C22:0) (DA1)	0.1	yes	yes
Docosahexaenoic acid (C22:6n-3) (DHA)	0.2	yes	yes
Docosapentaenoic acid (C22:5n-3) (DP3)	0.07	yes	yes
Docosapentaenoic acid (C22:5n-6) (DP6)	0.08	yes	yes
Docosatetraenoic acid (C22:4n-6) (DTA)	0.05	yes	yes
Eicosadienoic acid (C20:2n-6) (ED1)	0.05	yes	yes
Eicosenoic acid (C20:1n-9) (EN1)	0.06	yes	yes
Eicosapentaenoic acid (C20:5n-3) (EPA)	0.4	yes	yes
gamma-Linolenic acid (C18:3n-6) (GLA)	0.07	yes	yes
homo-gamma-Linolenic acid (C20:3n-6) (HGL)	0.06	yes	yes
Lignoceric acid (C24:0) (LG1)	0.1	yes	yes
Linoleic acid (C18:2n-6) (LNA)	0.3	yes	yes
Myristic acid (C14:0) (MR1)	1	yes	yes
Nervonic acid (C24:1n-9) (NR1)	0.05	yes	yes
Oleic acid (C18:1n-9) (OL1)	0.3	yes	yes
Palmitoleic acid (C16:1n-7) (PL1)	0.05	yes	yes
Palmitic acid (C16:0) (PM1)	3	yes	yes
Stearic acid (C18:0) (ST1)	9	yes	yes

Appendix B: Job Aids

A. General

1) JA-4030-G-01-Calibration and Calibration Verification

❖ Calibration

The 21-analyte panel for the #4030 Fatty Acids in RBC method consists of 6 saturated, 4 monounsaturated and 11 polyunsaturated fatty acids. This assay is calibrated daily by using a five-point calibration curve (except for ALNP, GLAP, and HGLP, which use a six-point calibration curve). The calibration range and LOD for each analyte are listed in the table below.

No.	Fatty acids - Saturated	Analyte Code	LOD (mg/L)	Lower Limit (mg/L)	Upper Limit (mg/L)
1	Myristic acid (C14:0)	MR1	1	1.16	30
2	Palmitic acid (C16:0)	PM1	3	22.4	874
3	Stearic acid (C18:0)	ST1	9	7.54	294
4	Arachidic acid (C20:0)	AR1	0.3	0.281	10.9
5	Docosanoic acid (C22:0)	DA1	0.1	1.87	48.7
6	Lignoceric acid (C24:0)	LG1	0.1	3.26	70.4
No.	**Fatty acids - Monounsaturated**				
7	Palmitoleic acid (C16:1n-7)	PL1	0.05	3.21	83
8	Oleic acid (C18:1n-9)	OL1	0.3	12	312
9	Eicosenoic acid (C20:1n-9)	EN1	0.06	0.31	12.1
10	Nervonic acid (C24:1n-9)	NR1	0.05	4.29	92.9
No.	**Fatty acids - Polyunsaturated**				
11	Linoleic acid (C18:2n-6)	LNA	0.3	11.6	302
12	*alpha*-Linolenic acid (C18:3n-3)	ALN	0.03	0.908	71
13	*gamma*-Linolenic acid (C18:3n-6)	GLA	0.07	0.409	31.9
14	Eicosadienoic acid (C20:2n-6)	ED1	0.05	0.302	11.8
15	*homo-gamma*-Linolenic acid (C20:3n-6)	HGL	0.06	1.42	55.4
16	Arachidonic acid (C20:4n-6)	ARA	0.08	21.3	555
17	Eicosapentaenoic acid (C20:5n-3)	EPA	0.4	1.08	28.1
18	Docosatetraenoic acid (C22:4n-6)	DTA	0.05	2.95	64
19	Docosapentaenoic acid (C22:5n-3)	DP3	0.07	2.47	107
20	Docosapentaenoic acid (C22:5n-6)	DP6	0.08	0.526	68.3
21	Docosahexaenoic acid (C22:6n-3)	DHA	0.2	2.59	336.6

Reportable Range

This method uses molar concentrations (uM) to calculate weight concentrations (mg/L) of each fatty acid. The weight concentrations are then converted to weight percent of each fatty acid which are reported. The range of weight concentrations for each fatty acid should be between the lowest and highest standards whose approximate values are shown in the table above. Unknowns with weight concentrations exceeding the Upper limit are re-analyzed after appropriate dilution with smaller RBC volume.

- **Calibration Verification**

According to the updated CLIA regulations from 2003 (see also statement of the Joint Commission on Accreditation of Healthcare Organizations), the requirement for calibration verification is met if the test system's calibration procedure includes three or more levels of calibration materials, and includes a low, mid, and high value, and is performed at least once every six months. All these conditions are met with the calibration procedure of this assay, and therefore no additional calibration verification is required by CLIA.

Perform calibration verification whenever any of the follow occur:

- All of the reagents used for a test procedure are changed to new lot numbers, unless the laboratory can demonstrate that changing reagent lot numbers does not affect the range used to report patient test results, and control values are not adversely affected by reagent lot number changes.
- There is major preventative maintenance or replacement of critical parts that may influence the test/s performance. This includes when the laboratory sends a test system to the manufacturer for repairs. The laboratory must check the calibration of a repaired test system before resuming patients testing and reporting results.
- Control materials reflect an unusual trend or shift, or are outside of the laboratory's acceptable limits, and other means of assessing and correcting unacceptable control values fail to identify and correct the problem.
- The laboratory has determined that the test system's reportable range for patient test results should be check more frequently.

Calibration verification points are read from the conventional calibration curve. Values near the LOQ are expected to be within 30% of the target values; higher values are expected to be within 15% of the target values.

On a daily basis, the DLS 4030 method for measuring RBC fatty acids meets the requirement for calibration verification by using a minimum of 5 points to generate run-specific calibration curves. Curves are either linear or quadratic. Appropriate weighting of the curves has been determined for each fatty acid. The curves are designed to span the ranges of values for each fatty acid.

- **Calibration Expansion**

Based on results from the first 10 runs for NHANES 2019-2020, ALN, EPA, GLA, MR1, and PL1 had concentrations that were less than the lowest calibrator but greater than the LOD. In order to report these values, a calibration expansion is done every 6 months: 1) several dilutions of Std2 are analyzed to expand the calibration curve to be at or near the LOD for ALN, EPA, GLA, MR1, and PL1; and 2) diluted pools with concentrations at or near LOD are read off the expanded curve.

1. Expanded calibration curve
   a. Aliquot 40 µL of Std2 for MR1 calibrator concentration near LOD (Cal value: 4.048 uM)
   b. Aliquot 20 µL of Std2 for EPA calibrator concentration near LOD (Cal value: 1.43 uM)
   c. Prepare a diluted Std2 solution: 25 µL Std2 + 275 µL toluene
      i. Aliquot 100µL of solution for ALN and GLA concentration near LOD (Cal value: 0.544 and 0.244 uM)
      ii. Aliquot 20µL of solution for ALN, GLA, and PL1 concentrations near LOD (Cal values: 0.109, 0.0488 and 0.418 uM, respectively)

2. Add the following LOD levels in the specified volumes in duplicate to the run as follows:
   a. LOD_3: Aliquot 100 µL for MR1 concentration near LOD; target 1.156 mg/L
   b. LOD_6: Aliquot 100 µL for EPA and GLA concentrations near LOD; targets 0.549 and 0.194 mg/L, respectively
   c. LOD_6: Aliquot 25 µL for ALN and PL1 concentrations near LOD; targets 0.1257 and 0.299 mg/L, respectively

3. Calculations:
   a. percent diff = [(unknown*target)/unknown]*100
   b. Calculate SD and CV of the instrument result

4. Interpretation: The relative uncertainty (CV) near the LOD should be ≤ 100% (Taylor, John K (1987) Quality Assurance of Chemical Measurements Washington, D.C. Lewis Publishers).

2) JA-4030-G-02-Alternative In-House Proficiency Testing

❖ Background

An external proficiency testing program is not available for the analysis of RBC fatty acids. Because of this situation, an Audit-Sample Procedure alternative proficiency testing program, as described in the guideline of the **Clinical Laboratory Standards Institute** (CLSI) QMS24 “Using Proficiency Testing and Alternative Assessment to Improve Medical Laboratory Quality” was selected (1). Because of the lack of other laboratories performing the same type of testing, this procedure was considered the most appropriate among those described in this guideline.

❖ Principle

Aliquots of a pooled specimen are stored by the laboratory and analyzed periodically across time. Periodic analysis of aliquots of the audit samples assesses imprecision of the assay. The pooled specimens are blinded in a manner that the analysts do not recognize the type of the pool based on the pool ID. The Audit-Sample Procedure does not evaluate accuracy (i.e., bias), nor provide inter-laboratory comparison.

❖ Proficiency Testing Procedure

Generation, labeling/ aliquoting of pools
The set of proficiency testing pools were generated by Bioreclamation (Hicksville, NY), which are the same set used for our in-house blind QC program. Diluted washed red blood cells (50:50 saline:RBC) from nine donors was used to prepare nine pools that represent as wide a range of RBC fatty acid levels as possible without spiking. The resulting pools were aliquoted into vials with random IDs preventing decoding by the analyst.

Characterization of pools
To generate the target values, the analysts first characterized the pools by measuring 10 separate vials from each pool in duplicate across a minimum of 10 different runs to obtain the means and standard deviations.

Receipt of Samples
At least twice a year a proficiency testing challenge will be performed as requested by the project lead or supervisor. For that, the QA officer or supervisor will randomly select 5 vials for the analyst to run.

Analyzing PT Samples:
Allow samples to come to room temperature before testing and ensure proper mixing. If possible, scan in IDs with a barcode reader to avoid transcription errors. Test PT samples as part of regular patient workload following standard operating procedures. PT testing is performed by staff who routinely perform the testing. Rotate the performance of PT surveys among staff when there is more than one analyst performing the test regularly. Perform the same number of replicates for PT samples as we do for routinely tested patient samples unless the survey indicates otherwise. PT

results are reviewed and integrated in the same way as patient samples. Record results on a results sheet. Retain PT samples at least until results have been reviewed/approved by supervisor.

Documentation, Review, and Reporting:

The results will be summarized by the project lead and approved by the Lab Chief. The limits are determined using the characterization mean of the appropriate pool, plus or minus a factor times the appropriate pool standard deviation. If the results meet that criterion no further action is required. If less than 4 of the 5 proficiency testing samples are within the limits for a given analyte, the challenge is considered as failed and appropriate corrective action needs to be initiated. After identifying and correcting the problem, another set of 5 proficiency testing samples will be requested and the proficiency testing challenge will be repeated, only to prove that the problem was solved. We will not regrade the PT challenge. NB: Patient samples will be analyzed only after the proficiency testing challenge problem has been identified and solved, or it becomes clear that the failure(s) were random due to the large number of analytes per sample.

The analyst formally acknowledges on the results sheet that the PT samples have been handled the same way as patient samples.

The Lab Chief reviews the results and signs the results sheet to attest to the routine integration of the samples into the patient workload using the routine method.

The characterization data for the 9 pools can be found in:
\\cdc.gov\project\CCEHIP_NCEH_DLS_NBB_OC\QA\Blind QC\Open label blind QC\Blood\In house Blind QC\FA RBC_4030

The PT challenge data can be found in
\\cdc.gov\project\CCEHIP_NCEH_DLS_NBB_OC\QA\Data handling\In-house PT\4030 - RBC Fatty Acids

References

1) Clinical and Laboratory Standards Institute (CLSI). *Using Proficiency Testing and Alternative Assessment to Improve Medical Laboratory Quality*. CLSI guideline QMS24 (ISBN 1-56238-944-0 [Print]; ISBN 1-56238-945-9 [Electronic]). Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087 USA, 2016.

3) JA-4030-G-03-Sample Preparation

A. Preliminaries

1) SampleID numbers are scanned into the computer if they are barcoded.
2) Allow frozen RBCs (quality controls and unknowns) and standards to reach ambient (+15°C to +30°C) temperature, then vortex each sample individually or as a set prior to aliquoting.
3) Set up Excel run sheet containing sample IDs prior to starting sample preparation. This will be used later to build a sequence to run the samples and also used to keep track of any problems that may occur during the sample preparation.
4) A typical run consists of 9 calibrators (includes a blank), 3 QC samples (first set), 38-75 patient samples, 3 QC samples (second set).

B. Sample Preparation

1) Set-up and label one 16- x 100-mm screw cap culture tube and one 13- x 100-mm glass tube per sample. All subsequent steps are done in the fume hood except when items are in the oven.
2) Using an air displacement pipette, add 100 µL of standards and reagent blank to 16- x 100-mm screw cap culture tube.
3) Using a positive displacement pipette, add 100 µL of RBC to 16- x 100-mm screw cap culture tube.
4) Add 100 µL of internal standard mixture to each 16- x 100-mm culture tube.
5) Add 2 mL of acetonitrile: 6N hydrochloric acid (90:10, v:v).
6) Cap each tube tightly using Teflon-lined caps.
7) Heat samples in oven for 45 minutes at 104°C.
8) Remove samples from oven and allow samples to cool. If noticeable evaporation has taken place in any sample, then add additional acetonitrile (without hydrochloric acid) to bring back up to the approximate volume.
9) Add 2 mL of a solution of methanol:10N sodium hydroxide (90:10, v:v).
10) Recap tightly with the same teflon caps and heat in oven for 45 minutes at 104°C.
11) Remove samples from oven and allow samples to cool. If noticeable evaporation has taken place in any sample, then add equal proportions acetonitrile and methanol (without hydrochloric acid or sodium hydroxide) to bring back up to the approximate volume.
12) Re-acidify samples by addition of 350 µL of 6N HCl. If Hamilton Liquid-Liquid Extraction method will be used, then use a Pasteur pipette and tubing attached to the lab air line to blow off the vapors that are created after the addition of the acid to protect the Hamilton pipetting heads from corrosion.
13) Proceed with Hamilton Liquid-Liquid Extraction steps (step C).

C. Hamilton Liquid-Liquid Extraction

1) Turn on Hamilton Starlet
2) Open Microlab Star Run icon on the computer desktop
3) Open latest version of Fatty Acids extraction method.
4) Be sure all 13 x 100 mm culture tubes and hexane troughs are filled and placed in the appropriate carrier type and placed in the correct deck position according to the deck layout associated with the extraction method on the Hamilton Software. If extracting >40 samples, QC and calibrators, then you must refill the hexane trough after the first extraction is completed.
5) There are several questions to answer before the method can begin
   a. Tip count – be sure to correctly count where the tips begin and end

   b. How many calibrators, QC, samples, etc.? – this allows the method to be used no matter how many calibrators are being run. The method will do all the math and determine which carriers/positions are needed.
6) Using a 1-mL high volume tip, 1 mL of hexane is added to each 16- x 100- mm tube in triplicate to yield a 3mL total addition for the first extraction, then in duplicate to yield a 2mL total addition for the second and third extractions.
7) The two layers are mixed, using a 1-mL high volume tip, 1 mL of the organic layer is aspirated then dispensed at the bottom of the tube to go up through the aqueous layer. This mixing step is done ten times per tube.
8) Using a 1-mL high volume tip, 1 mL of hexane is transferred from each 16- x 100- mm tube to its respective 13- x 100- mm tube. This step is done twice per tube.
9) Steps 6) – 8) are repeated for a total of 3 hexane extractions. Proceed to sample preparation for GCMS (step D).

D. Sample Preparation for GCMS

1) Dry down samples in the SpeedVac (@ 45°C). This takes approximately 45 minutes to an hour and should not be stopped as long there is a liquid residue. Restart if liquid residue remains.
2) Prepare fresh daily a 7% pentafluorobenzyl bromide and 10% triethylamine in acetonitrile solution. Add 100 µL of the derivatizing solution to each tube.
3) Allow the solution to react for 15 minutes at room temperature.
4) Reconstitute residue with 1.0 mL of hexane.
5) Transfer the reconstituted sample (avoiding the bottom) using a clean Pasteur pipette to a labeled GCMS autosampler vial containing a glass insert, then immediately cap, and place on autosampler tray for injection.

See table below for general plan for splitting samples for GCMS analysis between two GCMS instruments (#1 & #2): fraction represents portion of sample volume (1-mL reconstituted).

Items	NBB #1	NBB #2
calibrator	1/2	1/2
QC	1/2	1/2
unknown #1-X	Half the unknowns	Half the unknowns
QC	1/2	1/2

E. GCMS Instrument Preparation

1) An Agilent GCMS system is used to quantitate dietary FA in extracted RBC.
2) GC preparation
   a. Septum should be changed prior to each run
   b. Typically, the liner should be changed every 2-3 runs
   c. Fill toluene and hexane wash vials; rinsing well with the respective solvent prior to filling
   d. Empty waste vials from autosampler
   e. Verify syringe is moving freely (remove from arm to gauge stickiness; replace with new syringe, if sticky)
   f. Load autosampler vials into appropriate positions according to sequence.
3) Typical Instrument Method (oven ramps, inlet temperatures, and split ratio are adjusted as needed)
   a. Oven: 230°C for 0 min, then 5°C/min to 234°C for 7 min; then 1°C/min to 250°C for 3 min
   b. Front inlet: injector temperature: 240°C; initial pressure: 47.377 psi; total flow: 105 mL/min; septum purge flow: 3 mL/min; gas saver: on at 2.0 min with a gas saver flow of 20.0 mL/min; gas type: Helium; typical split ratio: 50:1.

c. MSD transfer line: initial temperature: 250°C.
d. Injector: solvent A and B washes (pre and post injection): 5 each at 8 µL; sample washes: 3 at 8 µL; sample pumps: 2; injection volume: 1.0-µL; syringe size: 10.0-µL.
e. 3.5 min solvent delay
f. EMV mode relative
g. CI flow rate: 40
h. Capillary column: TG-POLAR; maximum temperature: 275°C; nominal length: 60.0 m; nominal diameter: 0.25 mm; nominal film thickness: 0.25 µm; mode: constant flow at 2 mL/min
i. Outlet pressure: vacuum

B. Reagents & Standards

1) JA-4030-R&S-01-Reagent Tracking

Tracking chemicals and other materials used in the preparation of reagents, calibrators, and quality control materials

For details on labeling requirements, see the Division of Laboratory Sciences Safety and Quality Joint Labeling Requirements.

❖ **Reagent Preparation**

Frequently Prepared Reagents
The following reagents specified in the APM are considered to be “frequently-prepared reagents”

- Acetonitrile:6N Hydrochloric acid, (90:10, v:v)
- Methanol:10N Sodium Hydroxide, (90:10, v:v)
- Derivatizing Solution: 7% pentafluorobenzyl bromide:10% triethylamine in acetonitrile
- GC sample syringe wash solution A: 100% toluene
- GC sample syringe wash solution B: 100% hexane

Frequently prepared reagent preparation is logged on the reagent bottles or a tag attached to the reagent bottle. The reagent bottle also has a label indicating for how long the reagent can be used (e.g., Expires 1 Month after Preparation). Chemicals (acetonitrile, methanol, sodium hydroxide, hydrochloric acid, pentafluorobenzyl bromide, triethylamine, toluene and hexane) used to prepare the “frequently-prepared reagents” are documented on tracking sheets in the laboratory. In each case the following information is included on the sheet:

- Name, supplier and catalog number
- Method and reagent(s) in which it is used
- Lot number
- CHaTS barcode (if available)
- Date received, started and stopped use
- Any important notes regarding these chemicals

❖ **Standards Preparation**

1) Stock Solutions

All calibrators and internal standards specified in the APM originate from single-analyte stock solutions. Chemicals used to prepare stock solutions are documented in individual records (either written or electronic) generated when the stock solution is prepared. For each preparation record the following information (when available) for each chemical:

- Name, supplier, catalog number, and lot number
- CHaTS barcode
- Date received
- A description of how it was used in the preparation of the stock solution

2) Working Standard Solutions

Stock solutions are combined in proportions specified in the APM to create mixed-analyte working standard solutions. Chemicals used to prepare working standard solutions are documented in individual records (either written or electronic) generated when the working standard is prepared. For each preparation record the following information (when available) for each chemical:

- Name, supplier, catalog number, and lot number
- CHaTS barcode

- Date received
- A description of how it was used in the preparation of the working standard solution

❖ Quality Control Materials

Human whole blood samples are used to prepare saline diluted washed red blood cells (RBC) quality control materials. In these cases, available information on the human whole blood samples used are tracked in the record. Quality control materials are aliquoted into cryovials for single use and kept frozen (-70°C). An antioxidant additive, BHT in methanol, is added to assist in preserving the fatty acids.

Chemicals may be used to amend (i.e., spike) analyte into a quality control material. The following information (when available) is included in the record:

- Name, supplier, catalog number, and lot number of material
- CHaTS barcode
- Date received
- A description of how it was used in the preparation of the material

For human blood samples, the following information (when available) is included in the record:

- Information regarding where and how the sample was obtained (e.g., commercial source, anonymous collection on-site)
- Sample ID (if applicable)
- A description of how these urine samples were used.

C. Instruments

1) JA-4030-I-01-GCMS Instrument Comparison and System Verification

❖ Instrument Comparison

When a method is analyzed on multiple instruments, an initial instrument comparison must be conducted to establish analytical comparability. All calibrators, quality control materials, and at least 30 samples that span the measurement range should be analyzed on each system. The same preparation should be analyzed on all systems on the same day. However, this may not be feasible. In this case, it is acceptable to prepare different aliquots of the samples and perform the analysis as close to the same time as possible. The results from each instrument should be plotted against the results from the original instrument. The parameters assessed are correlation (Pearson r >0.95), regression fit (r^2), and slope (m). The same data analysis and documentation procedure as described below under System Verification can be used.

❖ System Verification

Following the initial instrument comparison, semi-annual system verification is performed to ensure that the systems are maintaining comparability.

Requirement: According to DLS Policies and Procedures (section 12.2), if a DLS method is run on multiple instruments or at multiple sites, a set of at least five samples spanning the reportable range of the analytes must be run at least once every six months. The Pearson correlation coefficient of the compared results should be greater than 0.95, and if not, appropriate corrective action should be taken. In special situations, the Division Director may give written approval that the methods are sufficiently similar for the intended use of the data.

Procedure: No separate sample preparation is needed. A set of samples spanning approximately the reportable range that were analyzed on the primary instrument, are re-analyzed shortly on the secondary instrument. The time delay should be within the processed sample stability parameters determined during method validation (e.g., sample kept in autosampler or refrigerated/frozen for a certain time).

Data analysis:

- Identify a subset of results (n ≥5) from the two analyses and describe any inclusion or exclusion criteria applied (e.g., only include samples with analyte results ≥3x the LOD and ≤ highest calibrator).
- Determine the Pearson correlation coefficient. *Note:* Pearson correlation is a parametric test that requires normally distributed data. Most nutritional biomarkers show right-skewed analyte concentration distributions benefiting from a log-transformation to yield data that approximates a normal distribution. While Pearson analysis verifies correlation and not concordance, high concordance is expected for instrument comparisons because most critical variables are the same (measurement technique, sample preparation, operator, calibration, etc.) and only 1 variable changed (instrument).
- Optional: Assess Lin's rho coefficient for concordance. Perform regression and Bland-Altman bias analysis. Assess whether a similar proportion of samples is <LOD on both instruments. *Note:* Most nutritional biomarkers show non-constant variance (constant CV with increasing concentration); thus, weighted Deming regression and relative (%) Bland-Altman analysis are generally preferred.

Documentation, review, and approval:

• Summarize the results in a spreadsheet that contains the raw data, the data analyses, and the summary information and request review by the supervisor. For an example, see: Instrument comparison - Template with data 4063 caffeine.xlsx

• General supervisor reviews the data and, if acceptable, approves the data. Convert summary information tab to a PDF and electronically sign the PDF in the designated field. Add the signed PDF to your electronic QA Manual. For an example, see: Instrument comparison - Example signed PDF 4063 caffeine.pdf

2) JA-4030-I-02-GCMS Calibration, Startup and Shutdown Procedures

❖ **Mass Calibration**

A mass calibration for chemical ionization (CI) mode is performed using PFDTD in Positive CI (PCI) (masses 41, 266.9, and 599) and Negative CI (NCI) (masses 185, 351, and 448.9) mode. The software will set various parameters (such as repeller voltage, electron multiplier voltage and entrance lens offset) for maximum transmission while performing PCI and NCI tunes. The instrument will not complete a tune if it does not meet instrument specifications. Records of the tune reports are stored as PDF's on the local computer.

❖ **Agilent GCMS Startup and Tuning Procedure**

1. Power on the GC using the power button in the front on the bottom left
2. With the MSD side panel open and holding the door closed (if open), power on the MSD using the power button on the bottom. Once vacuum is obtained and the door holds by itself you can close the side panel.
3. The system will automatically start pumping down.
   a) If a manual pump down is necessary, open the instrument software and click on the tune icon
   b) Click ok to enter the CI tune panel.
   c) Select vacuum then MS vacuum control.
   d) Select Pump Down.
4. You might need to restart the software to reconnect with the instrument.
5. After the system has pumped down for at least 2 hours, a PCI tune must be performed followed by an NCI tune.
   a) From the instrument control window, select View then Tune and Vacuum Control (or select the tuning fork icon under Instrument).
   b) In the tune window, select File, then Load the parameters. Select the file pcich4.u (PCI tune file for methane gas) and click OK. Select apply to raise the MS temperatures of the MS Source (300°C) and MS Quad (150°C).
   c) **Methane pre-tune:** Once the MS temperatures reach the correct setpoint, select Setup then Methane Pre-Tune.
      (i) On the displayed profile scan verify:
         1. No peak at m/z 32 (mass indicates an air leak or system wasn't properly pumped down overnight)
         2. Peak at m/z 19 (protonated water) is less than 50% of the peak at m/z 17.
      (ii) Adjust the methane flow as needed to get the ratio of m/z 28/27 between 1.5 and 5.0.
   d) **PCI tune:** In the tune window, select Tune, then CI autotune. In the popup window leave the setting as is and click ok.

e) Once the tune is complete a pdf is created. Use the following parameters as a guideline for determining if the instrument is functioning properly:

   (i) EMV <2500 (due to EMV increases in the method the chromatography will max out if EMV>2500)

   (ii) Number of peaks are generally <500

   (iii) MS Source and MS Quad temperatures are close to setpoint (150°C)

   (iv) Turbo Speed = 100%

   (v) Hi Vac = very small near zero number

f) Be sure tune values are saved, then select File, then Load the parameters. Select the file ncich4.u (NCI tune file for methane gas) and click OK. Select apply to lower the MS temperatures of the MS Source (150°C) and MS Quad (150°C).

g) **NCI tune:** In the tune window, select Tune, then CI autotune. In the popup window leave the setting as is and click ok.

h) Once the tune is complete a pdf is created. Use the following parameters as a guideline for determining if the instrument is functioning properly:

   (i) Good peak shape for the three masses (185, 351, 449)

   (ii) PW50 = 0.6 for the three masses

   (iii) EMV <2500 (due to EMV increases in the method the chromatography will max out if EMV>2500)

   (iv) Number of peaks are generally <500

   (v) MS Source and MS Quad temperatures are close to setpoint (150°C)

   (vi) Turbo Speed = 100%

   (vii) Hi Vac = very small near zero number

   (viii) Relative abundances for peaks are approximately: 185 m/z: ~100%, 351 m/z: ~65%, 449 m/z: ~3%

   (ix) iAir/Water check: no appreciable % of H20, N2, O2, CO2, or N2/H2O

   (x) Column Flow = 2.00 mL/min

   (xi) Interface temp = 250°C

i) Save the tune values as ncich4.u and the tune report as a pdf in the CItunes folder. C:\ or D:\...\MassHunter\GCMS\1\5975\citunes

- **Agilent GCMS Shutdown Procedure:**

   1. From the instrument software select the tune icon
   2. Select ok to enter the CI panel
   3. Open the Vacuum menu in the software and select MS vacuum Control.
   4. Select Vent and follow any instructions presented.
   5. Set the GC/MSD interface heater and the GC oven temperatures to ambient (+15°C to +30°C) temperature.
   6. When prompted, turn off the GC and MSD power switch.

3) JA-4030-I-03-GCMS Time Segment Assignments

1. Open Chemstation software
2. Click File – Load data file
3. Click change path to find the main file folder for the most recent run, then choose the 3rd data file (highest calibrator) and click ok
4. Click on Chromatogram, then Extract Ion Chromatograms
5. Using **Table 1,** enter the masses associated with the 2 peaks of interest (last peak of previous segment and first peak of current segment) then click ok. Segment 1 always starts at 3.5 minutes (this is after the solvent front). The start time for the time segment should fall within the yellow highlighted space between the two peaks. The times will vary based on instrument/column.

Example for Segment 2:

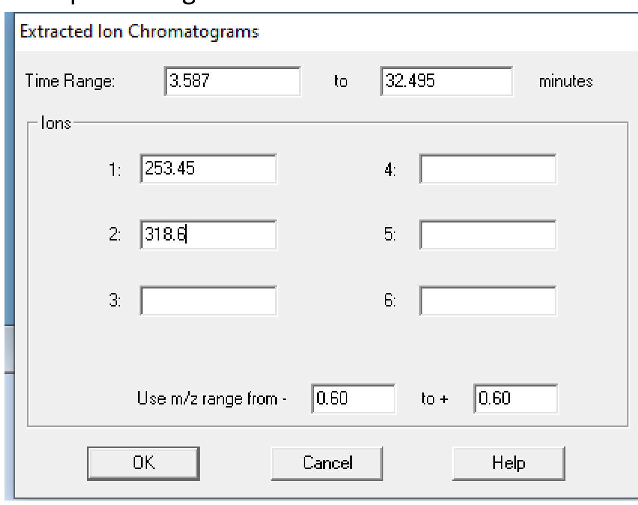
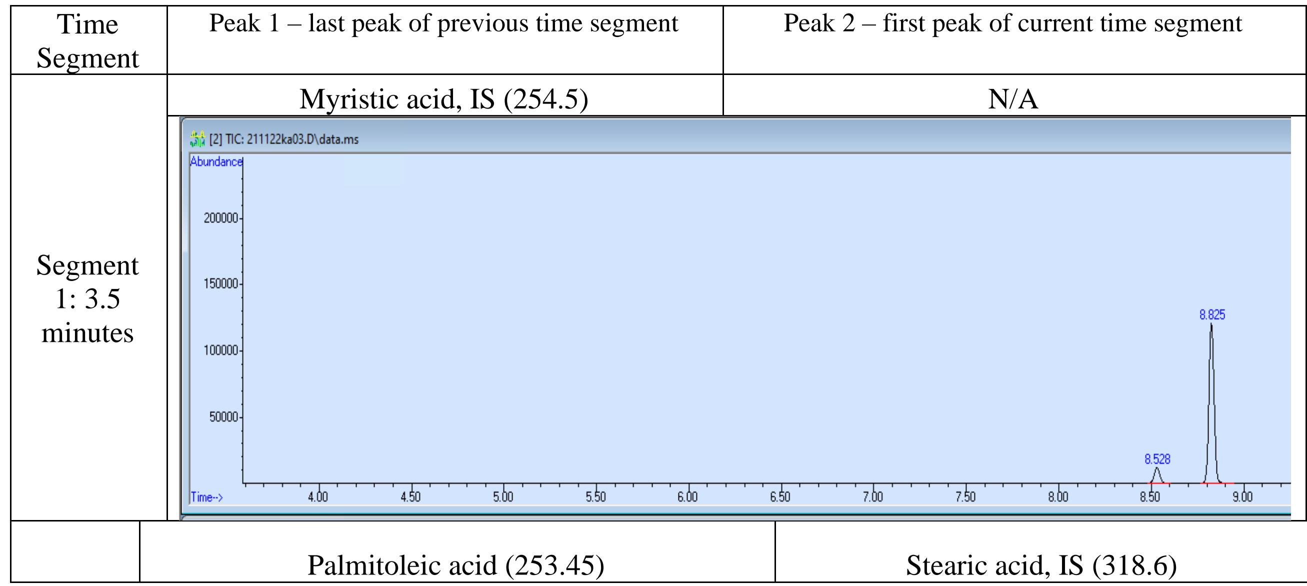
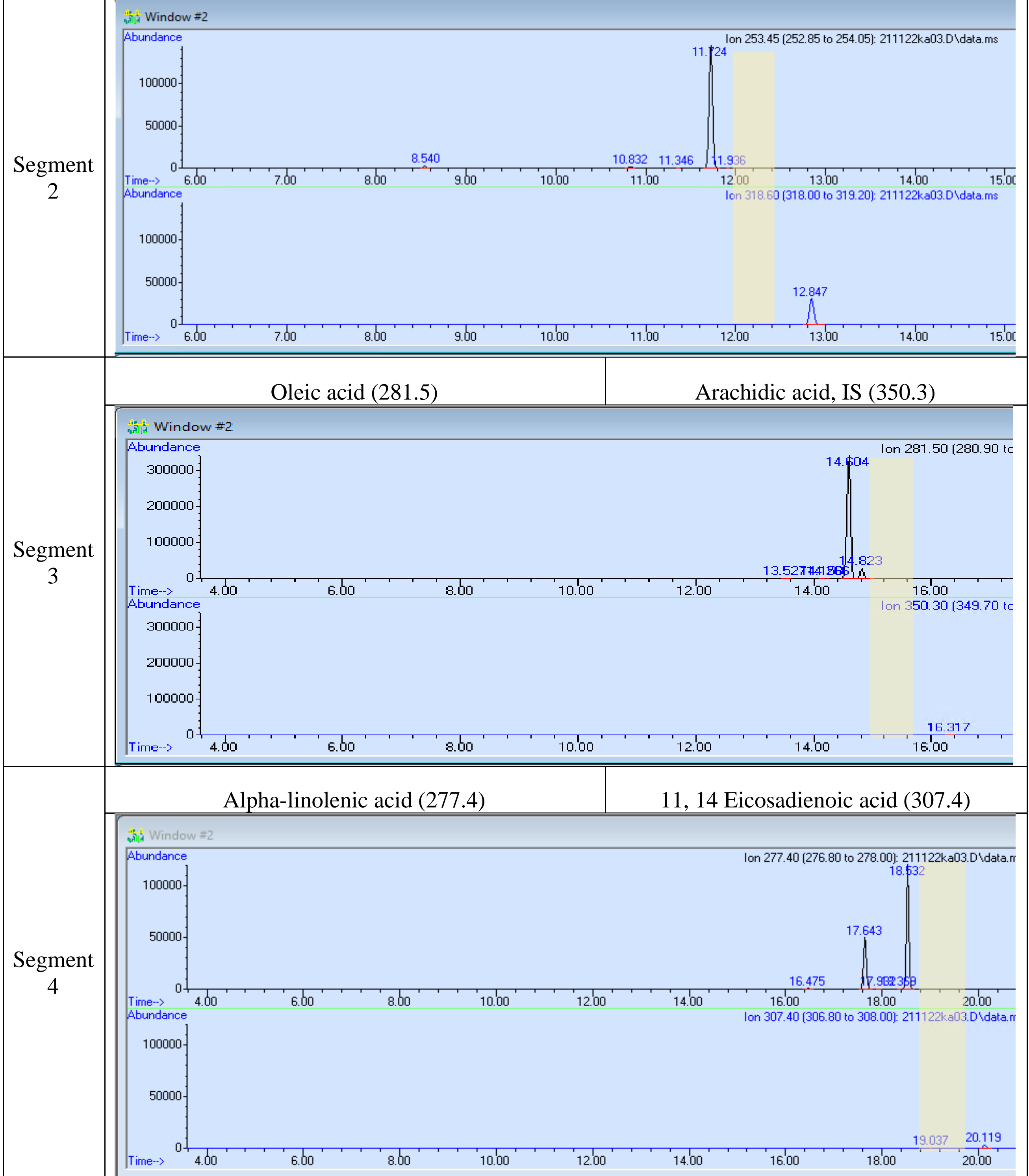


Table 1: Time segments



Time Segment	Peak 1 – last peak of previous time segment	Peak 2 – first peak of current time segment
Segment 1: 3.5 minutes	Myristic acid, IS (254.5)	N/A
	Palmitoleic acid (253.45)	Stearic acid, IS (318.6)


Segment 2
Window #2
Abundance
Ion 253.45 (252.85 to 254.05): 211122ka03.D\data.ms
Ion 318.60 (318.00 to 319.20): 211122ka03.D\data.ms
Time-->
Segment 3
Oleic acid (281.5)
Arachidic acid, IS (350.3)
Window #2
Abundance
Ion 281.50 (280.90 to
Ion 350.30 (349.70 to
Time-->
Segment 4
Alpha-linolenic acid (277.4)
11, 14 Eicosadienoic acid (307.4)
Window #2
Abundance
Ion 277.40 (276.80 to 278.00): 211122ka03.D\data.m
Ion 307.40 (306.80 to 308.00): 211122ka03.D\data.m
Time-->

Segment 5	Homo-gamma linolenic acid (305.4)	Arachidonic acid, IS (311.4)
Segment 6	Eicosapentaenoic acid (301.4)	Lignoceric acid, IS (371.5)

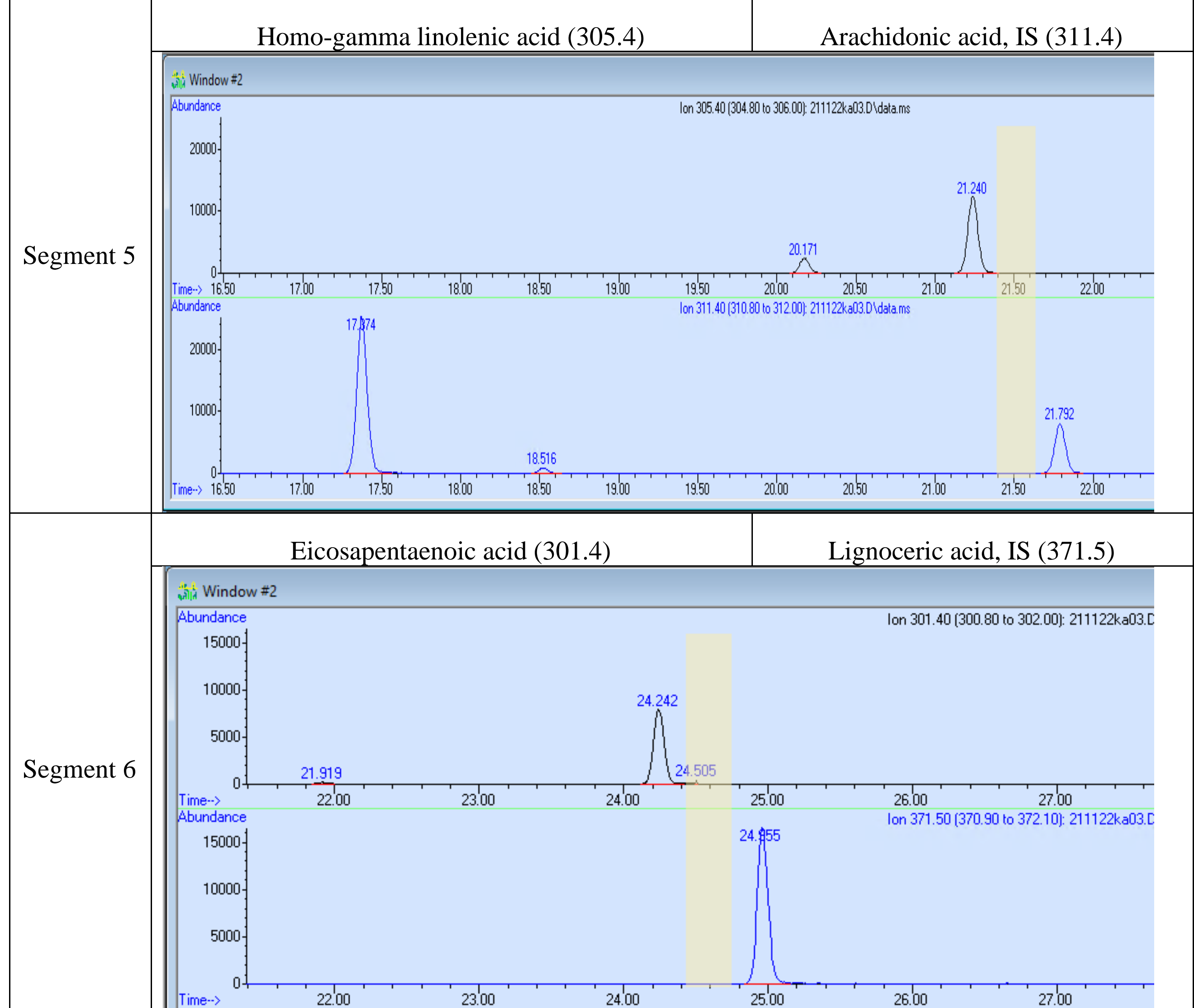
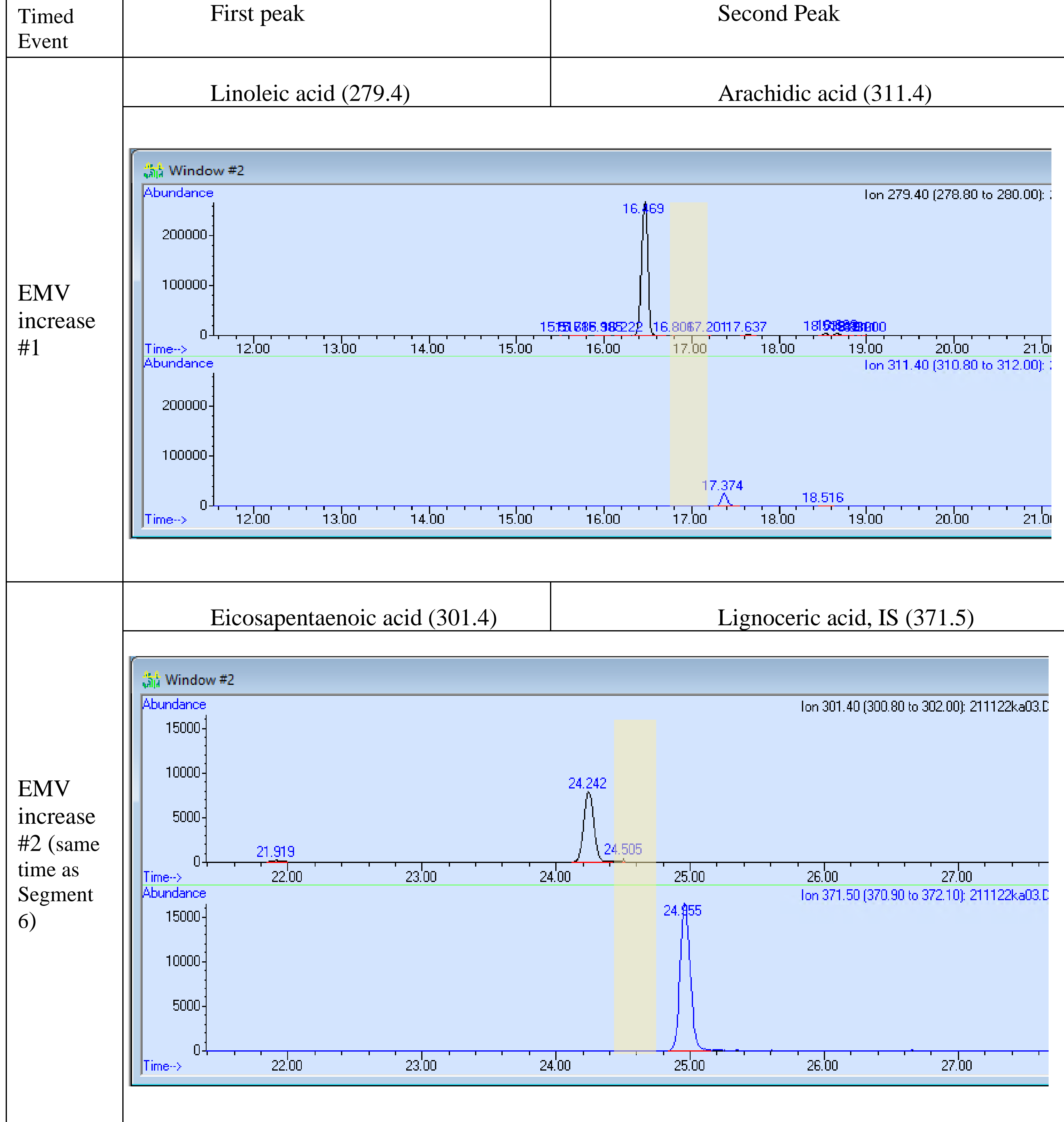
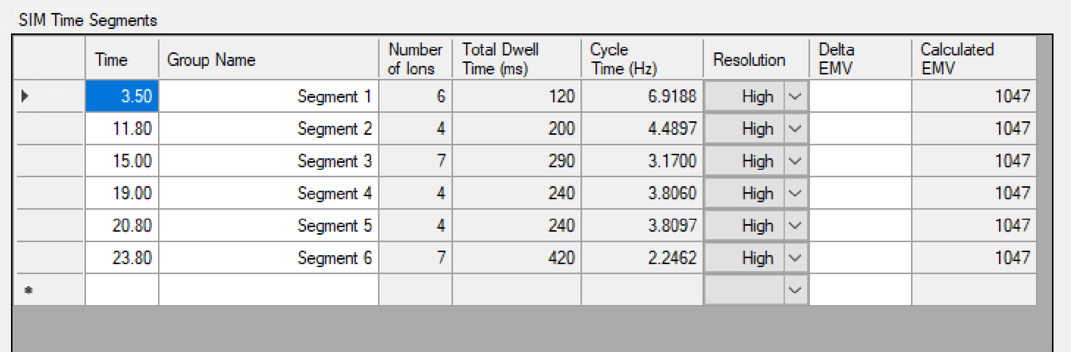


Table 2: Timed events



Timed Event	First peak	Second Peak
EMV increase #1	Linoleic acid (279.4)	Arachidic acid (311.4)
EMV increase #2 (same time as Segment 6)	Eicosapentaenoic acid (301.4)	Lignoceric acid, IS (371.5)

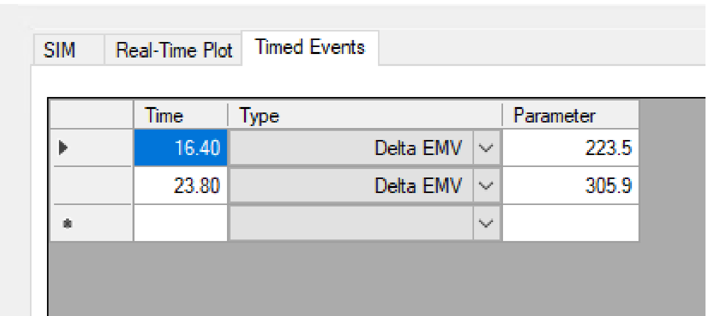
6. Update the RBC fatty acids instrument method loghseet with the new segment and EMV times.
7. Update instrument method with the new segment and EMV increase times.
   a. In Instrument control window, load the current instrument method. Click Instrument then Edit MS parameters (or click the gold quadrupole icon).
   b. Enter new time segments into the SIM Time Segments table:



SIM Time Segments

Time	Group Name	Number of Ions	Total Dwell Time (ms)	Cycle Time (Hz)	Resolution	Delta EMV	Calculated EMV
3.50	Segment 1	6	120	6.9188	High		1047
11.80	Segment 2	4	200	4.4897	High		1047
15.00	Segment 3	7	290	3.1700	High		1047
19.00	Segment 4	4	240	3.8060	High		1047
20.80	Segment 5	4	240	3.8097	High		1047
23.80	Segment 6	7	420	2.2462	High		1047

   c. Enter EMV increased in the Timed Events table:



SIM | Real-Time Plot | Timed Events

Time	Type	Parameter
16.40	Delta EMV	223.5
23.80	Delta EMV	305.9

D. Data review

1) JA-4030-DR-01-Computerization & Data System Management

- **Sample Identification**

  During sample preparation and analysis, samples are identified by sample IDs. A sample ID is a number that is unique to each sample that links the laboratory information to demographic data recorded by sample collectors.

- **Data Collection**

  Agilent GCMS raw data files are collected and stored using instrument software (MassHunter).

- **Data Back-up**
  - Raw data files and sequence files from instrument computers are generally transferred via ISLE, KVM-switch or encrypted thumb drive to NBB shared-drive:
    \\cdc.gov\project\CCEHIP_NCEH_DLS_NBB_Instr3\Fatty Acids 4 - 4030
  - Other instrument-related files from instrument computers (e.g., instrument method, processing method, etc.) are backed up to the CDC network periodically via an ISLE network. This is checked regularly by the analyst under the guidance of the project team leader.

- **Peak Integration and Chromatography Review**
  - This assay uses MassHunter software for peak-integration. Analysts perform peak integration using this software and produce a chromatography PDF file, a calibration curve PDF file, and an excel result file. The excel result file is importable to the STARLIMS database.

- **Data Import**
  - The final result files containing patient data as well as QC data are stored on the network. These files are further exported into STARLIMS Database for QC and statistical evaluation.

- **STARLIMS Data Review**
  - **Level I – Analyst**
    - Double click the STARLIMS icon on desktop
    - Under ‘Run-based Tasks’, select ‘Pending Runs Assigned to My Labs’
    - Choose ‘Show Pending Tests’ and select ‘4030 (Fatty Acids by GCMS (RBC))’
    - Click on ‘Add’ and select the Instrument
    - Run# and Equipment ID will be populated
    - [0] Run Instrument Macro – select the excel result file to run macro for STARLIMS import
    - [1] Upload Instrument File – import the post-macro result file to STARLIMS
    - [2] Mark Null Results – click this button which replaces the null with “|” (a pipetab)
    - [3] Evaluate Sample QC – check the sample QC flags according to the defined criteria
    - [4] Evaluate Run QC – evaluate bench QC via the DLS SAS Multi-Rule System QC program to determine QC pass/fail
    - [5] Set Run QC Statuses – *set analytes pass/fail based on SAS out-of-control assessment*
    - [6] Attach SAS QC file – upload both the SAS infile (.csv) and output file (.pdf)
    - Enter run bench QC (SAS) information in Run Comments column, prep date in user field 1, and IS lot in user field 2
    - Click on ‘Manage Attachments’ and upload the data review checklist for the run
    - *Click ‘Finish Results’ located under the test workflow steps and notify Project Lead*

- **Level II – Project Lead**
  - Double click the STARLIMS icon on desktop
  - Under 'Run-based Tasks', select 'Run Approval'
  - Choose 'Show Pending Tests' and select '4030 (Fatty Acids by GCMS (RBC))'
  - Review analyst run sheet, data review checklist, peak integration and Sample QC evaluation
  - Review and confirm Run QC evaluation
  - Assess blind QC results - click on 'Blind QC Results Only' tab, 'Assess Blind QC', 'Final Result' and 'Use Default Characterization Sets', 'Proceed to Next Step', 'OK'
  - Print blind QC report - click on 'BQC Reports', 'All data displayed in the datagrid', 'A paper-based report from template', 'NBB Blind QC Report v2', 'OK', 'Proceed to the SSRS Report', Save PDF
  - Enter bench QC (SAS) and blind QC evaluation status in the Run Comments column
  - Set results final - in 'All Results (S)' tab, click on 'Set Final' Wizard, select 'Process all samples displayed in the datagrid' and 'Run the Set Final Wizard' and click 'Proceed'
  - Choose set final criteria - check 'Required Sample QC Passed' and 'Required Run QC Passed'; check 'Pass' and 'Warn' for 'Allowable Results Statuses for Set Final'; choose date range to cover runs that may include the previous analysis of these samples; click 'Proceed'
  - Resolve samples with retest results and set final
  - Submit sample IDs and repeat instructions to the analyst to schedlue the repeats
  - Click on 'Manage Attachments' and upload the blind QC report and final data review checklist for the run
  - In Run Approval tab, and click Release Run and notify QA Officer

- **Level III – QA Officer**
  - Refer to \\cdc.gov\project\CCEHIP_NCEH_DLS_NBB_LABS\Databases\STARLIMS\Data review checklists\Starlims Data Review Checklist Level III.docx

- **Technical Supervisor (Lab Chief)**
  - Conduct random "spot checks" to verify proper handling of lab results for selected analytes, such as:
    - "Spot check" chromatography
    - Summarize data by comment code
    - Summarize retention times
    - Review blind QC results
    - Review reported value range and compare to LOD
    - Review bench QC for trending
    - Review calibration information
  - Discuss with Project Lead or QA Officer course of action on difficult questions

2) JA-4030-DR-02-Processing and Reporting a Run

Sample Scheduling

The Project Lead determines the priority of analysis for patient specimens based upon required reporting dates or other criteria determined by the Technical Supervisor.

In addition, the Project Lead schedules calibration verification at appropriate intervals.

In the event of analytical problems, the Project Lead will work with the Analyst to develop a troubleshooting procedure based upon the problem.

Agilent GC/MS data review

1. Double click on the MS Quantitative Analysis Icon to open the data review window.
2. Create new batch for the run you want to review
   a. Click ‘File’ then ‘New Batch’.
   b. Open folder with corresponding run date.
   c. Type run date (YYMMDDXa [X=instrument initial]). Ex: 140130Ea
   d. Click ‘Open’
   e. Click ‘Select All’ then click ‘OK’.
3. Apply method from previous batch and update method using a standard
   a. Click ‘Method’ then ‘Open’. Select ‘Open Method from Existing Batch’. (Note: do not choose a method from an existing batch with any calibration values removed from the calibration curve)
   b. Select method from the previous run by opening the run folder, then selecting the ‘YYMMDDXa.batch.bin’ file. Click ‘Open’.
   c. Click ‘Save As’ then open the current run folder. Open ‘Quant Results’ folder, then save method as “current method name”. Then click ‘Save’.
   d. Click ‘Exit’ then select ‘Analyze’ and click ‘Yes’ to apply method to batch.
   e. Highlight Std3 by clicking on it once.
   f. Click ‘Method’, then ‘Edit’.
   g. Click ‘Retention Time Setup’ under Method SetupTasks.
   h. Click ‘Update’ then ‘Update Retention Times’. Click ‘Select All’ then ‘OK’. Go through each analyte to ensure correct peaks are integrated.
   i. Click ‘Save As’, select current method, and click ‘Open’. Click ‘Yes’ to replace existing method.
   j. Click ‘Exit’ then select ‘Analyze’ and click ‘Yes’ to apply method to batch.
4. Review calibrator chromatography and update calibration curves.
   a. Click ‘View’ then ‘Compounds at a Glance’
   b. Click ‘Layout’ then ‘Load Layout’. Select ‘Calibrator_Review.quantcaglayout.xml’ located in ‘D:\MassHunter\Layouts\Quant\Compounds-at-a-Glance’. Then click ‘Open’.
   c. Verify integration for each analyte and internal standard for all calibrators. You can click the right arrow or Ctrl+N to view calibrator chromatography for each analyte.
   d. To make integration changes, double click on the chromatography, then click the ‘Integration’ icon. You can then right click on the mouse (and hold) on the left side of the peak and drag across the bottom of the peak (release the mouse button when touching the other side of the peak base).

e. Once all analytes/IS for the datafile are verified, exit out of the window by clicking the 'x' button on the top right corner or click 'File' then 'Close'.
f. Click 'Analyze Batch' or click 'Analyze' then 'Analyze Batch' to apply changes to the calibration curve.
g. To view calibration curve for each analyte, you can click the right arrow button in the Calibration Curve window. If any calibration curves are R2 < 0.985, then verify integration. If there is an integration issue, then correct it and repeat updating calibration curve steps. If there are no integration issues, then proceed with reviewing the run.
h. Once you have reviewed and updated calibration curve, Click 'Analyze' then 'Analyze Batch'. Click 'File' then 'Save Batch'.

5. Reviewing Calibrator, QC, and unknowns
   a. Click 'View' then 'Compounds at a Glance'
   b. Click 'Layout' then 'Load Layout'. Select 'Samp_Review' then click 'Open'. Maximize the Compounds at a Glance window.
   c. Verify integration for all calibrators, QC, and unknowns for each analyte and internal standard. You can review chromatography for all data files by analyte by clicking the right arrow button or Ctrl+N to view chromatography for each analyte.
   d. To make integration changes, double click on the chromatography, then click the 'Integration' icon. You can then right click on the mouse (and hold) on the left side of the peak and drag across the bottom of the peak (release the mouse button when touching the other side of the peak base).
   e. Exit out of the window by clicking the 'x' button on the top right corner or click 'File' then 'Close'.
   f. Click 'Analyze' then 'Analyze Batch'. Save batch by clicking 'File' then 'Save Batch'.

6. Generate Reports: 1) Calibration Curves (PDF), 2) Reviewed Chromatography (PDF), and 3) LIMS Import/Export file (Excel)
   a. Click 'Report' then 'Generate Report'.
   b. A Generate Report window will appear.
      i. Save the report in D:\\MassHunter\GCMS\1\data\"YYYY"\"coordinating run folder"\QuantReports\"coordinating run folder".
      ii. Report method is located in D:\\MassHunter\GCMS\1\methods\FA Report Method.m. FA report method contains 3 report templates for the 3 reports needed per file.
         1. Gen_Calibration.report.xml
         2. FA_QuantReport_ISTD_Compact_B_06_00.xltx
         3. FA_LIMS_Export_20130821.xltx
      iii. Make sure 'All samples' and 'All compounds' are checked under Samples/Compounds section.
      iv. Ensure 'Generate reports now' is selected under Generate section. Click 'OK'

7. Copying datafiles for import
   a. Go to D:\\MassHunter\GCMS\1\data\"YYYY ".
   b. Select and copy the main data folder for the run you just reviewed onto a McAfee encrypted flash drive.
   c. The datafiles are transferred to and stored at the appropriate network location: \\cdc.gov\project\CCEHIP_NCEH_DLS_NBB_Instr3\RBC Fatty Acids - 4030\1. Instrument Data\"coordinating instrument folder"\"YYYY"

3) JA-4030-DR-03-STARLIMS Data Review and Criteria

During sample preparation and analysis, samples are identified by their sample ID. The sample ID is a number that is unique to each sample that links the laboratory information to demographic data recorded by those who collected the sample.

Agilent GCMS result files are collected and stored using the instrument software (MassHunter). The results generated are mostly based on auto-integration by the MassHunter software, but do allow for manual peak selection and integration when necessary. The final processed result files containing patient data as well as QC data are exported via an encrypted thumb drive to the NBB Instrument network location: \\cdc.gov\project\CCEHIP_NCEH_DLS_NBB_Instr3\RBC Fatty Acids - 4030\1. Instrument Data (in their respective instrument folder). These files are further imported into STARLIMS database for statistical evaluation and QC check as described below.

Level I review:

1. In STARLIMS Pending Runs Assigned to My Labs under Run-based tasks, select the test 4030 (Fatty Acids by GCMS (RBC)), then click add. Choose the instrument used then click ok.
2. Prior to running instrument macro:
    a. Open instrument file at: \\cdc.gov\project\CCEHIP_NCEH_DLS_NBB_Instr3\RBC Fatty Acids - 4030\1. Instrument Data\”instrument name”\”year”\”datafile”\QuantReports\”datafile”\FA_LIMS_Export_20130821
    b. Add analyte/sample specific comment codes (1033, 1040 high, etc.) to “.xlsx” export file and save the file

NHANES Comments (Column AA on LIMS excel)	Description
0	OK
11	small clot present
12	clotted sample
18	empty vial/sample not received
21	quantity not sufficient for testing (QNS)
22	QNS for repeat testing
23	instrument error
24	lab error/contents spilled
Sample Info Comment Codes (column AB on LIMS excel)	**Description**
1014	Fatty Acids - ACN:HCl had evaporated after 1st heating (some or all); ACN (w/o HCl) was added (up to 2ml)
1015	Fatty Acids - MeOH:NaOH had evaporated after 2nd heating (some or all); MeOH (w/o NaOH) was added (up to 2ml)
1033	Fatty Acids - calibrator level for this analyte deleted from calibration curves used to calculate data
1040 high	result not valid: concentration was not calculated by software bc it does not extrapolate higher than the calibration curve

3. Select step 0 "Run Instrument Macro" and follow the prompts.
   a. Save instrument macro at: \\cdc.gov\project\CCEHIP_NCEH_DLS_NBB_Instr3\RBC Fatty Acids - 4030\1. Instrument Data\"instrument name"\"year"\"datafile"\QuantReports\"datafile"\FA_LIMS _Export_20130821_"Starlims Run #"
4. Select step 1 "Upload Instrument File" and follow the prompts.
5. Enter Run Information into the top table according to the table below

Run Information	STARLIMS Column
Run ID	External Run ID
Analyst	Assigned to
Prep date	User Field 1
IS lot	User Field 2

6. Select step 2 "Mark Null Results" – if the instrument file contained a null result this will replace the null with "|" (a pipetab)
7. Select step 3 "Evaluate Sample QC" – once this step completes verify the flagging was done properly
   a. Verifying calibrator flags
      i. R^2 <0.985 should be marked failed and code 1025 added (repeat for all analytes)
      ii. Code 1033 calibrator results should be ignored in pass/fail for accuracy
      iii. Accuracy <85% or >115% should be marked failed for the 3 highest calibrators per analyte (repeat for all analytes)
      iv. Filter for blank, [blank] > [lowest calibrator] should be marked failed and code 1039 added (repeat for all analytes). Exceptions: ALNP, EPAP, GLAP, MR1P, and PL1P are reportable because of bi-annual calibration expansion
   b. Internal standard recovery typically within mean ± 3SD; assess any results outside mean ± 3SD to determine if a repeat is necessary
   c. Verify patient results (mg/L) < LOD (mg/L) are marked code 37
   d. Verify patient results (mg/L) > highest calibrator (mg/L) are marked failed

8. Assess results for any samples with NHANES and Sample Info comment codes according to the table below

NHANES Comments (Column AA on LIMS excel)	Description	STARLIMS Status Message	How to handle
18, 21, and 22	Empty vial, QNS, or QNS for repeat	Fail: reported value changed to NR	Set final if no other data to report
23	instrument error	Fail: reported value changed to NR	Schedule for repeat
24	lab error/contents spilled	Fail: reported value changed to NR	Schedule for repeat
Sample Info Comment Codes (column AB on LIMS excel)	**Description**	**STARLIMS Status Message**	
1014	ACN:HCl had evaporated after 1st heating	warn	Evaluate IS recovery - if low, then schedule for repeat - if ok, then modify SQC assessment to pass
1015	MeOH:NaOH had evaporated after 2nd heating	warn	
1033	Fatty Acids - calibrator level for this analyte deleted from calibration curves used to calculate data	pass	Code applied to point removed; verify the point was removed and the accuracy evaluation does not include this point
1040 high	result not valid: conc. was not calculated by software bc it does not extrapolate higher than the calibration curve	if=0: warn if=#: fail	Schedule for a repeat with dilution; modify SQC assessment to fail if appropriate

9. Select step 4 “Evaluate Run QC”
   a. DLS QC evaluation criteria
      i. Choose - use default characterization sets
      ii. Choose – all QC results
      iii. Choose – the prior N* results within this date range; set N = 20
      iv. Click “Proceed to Next Step”
   b. QC Materials Results
      i. Click “Start the SASQC Wizard”
   c. QC Evaluation Wizard
      i. Save SAS Input File
         1. Location - \\cdc.gov\project\CCEHIP_NCEH_DLS_NBB_Instr3\RBC Fatty Acids - 4030\3. Bench QC review
         2. File name format – 4030_checkdata_vB_”start date”_”end date”.csv
         3. Click save, then ok for export complete
      ii. Send to SAS Server
      iii. Save DLS OOC QC Results PDF file
         1. Location - \\cdc.gov\project\CCEHIP_NCEH_DLS_NBB_Instr3\RBC Fatty Acids - 4030\3. Bench QC review

2. File name format – 4030_checkdata_vB_"start date"_"end date".pdf

iv. Click finished

10. Select step 5 "Set Run QC Statuses" - set analytes pass/fail according to SAS OOC printout
    a. Add code 61 to any failed reportable analytes using the modify SQC assessments and setting the comment code to 61 (Be sure to filter appropriately and only apply this code to the filtered datagrid)
11. Select step 6 "Attach SAS QC file" – attach SAS output csv and pdf files to the run
12. Evaluate any sample marked as "warn"; override SQC pass/fail as appropriate
13. Once Level I review is finalized select "finish results" located under the test workflow steps.
    a. Select yes to notify someone
        i. Chose "select one or more addressees…"
            1. Find NBB, Fat-Soluble Micronutrients Laboratory, then Team Lead and check the box next to the name
            2. If there are any comments you would like to add in the e-mail message then type that into the box on the bottom right
            3. Click continue

Level II review:

1. In STARLIMS the Run Approval under Run-based Tasks, select the test 4030 (Fatty Acids by GCMS (RBC)), then select the run and go to the All results (S) tab
2. Review Sample QC Evaluation
3. Review/Confirm QC Evaluation - Use job aid **JA-4030-DR-04-Out-of-Control Corrective Action (APM Appendix B, section D)** to investigate bench QC failures.
4. On the Blind QC Results only tab, assess the Blind QC results. Any flags outside 3SD?
5. Adjust sample QC for any Blind QC failures (add code 921 to analyst comment)
6. Run the 'Set Final' wizard (select appropriate batch processing and flagging task), then click proceed
7. Make sure the following boxes are checked
    a. Require sample QC passed
    b. Require Run QC passed
    c. Allowable Result Statuses for Set Final: pass and warn (UNCHECK these boxes if the set final wizard has already been run)
    d. Then click proceed
8. A window will pop-up stating # results records processed and if there are any replicates to process. If there are replicates, select yes otherwise select no.

9. Resolving replicates:
    a. Filter for analyte of interest
    b. Filter for resolved = n
    c. Review the duplicate results in the bottom datagrid, if the value selected as final is appropriate then choose the "check selected as Set Final" to resolve the duplicate otherwise adjust the final as appropriate.
10. Once review is finalized, Release the Run for Report and email Hui Ping and/or Supervisor

Final data reporting is done by the QA officer/Supervisor

1. From the main STARLIMS page select Final Release (Set Reportable)
2. Search for data pending final release/approval by:
    a. Type in Test number
    b. Run# or Run dates
    c. Study ID If it is a particular study
3. Review and finalize data: the critical elements that need to be looked at are Result Status, SQC, RQC
    a. Sort by Set Final column and make sure samples with set final checked both Sample QC & Run QC are checked as well
    b. Sort by DLS Comment Code column, check codes such as 37, 33…
    c. If necessary, modify SQC assessments by fill in the pop-up form accordingly
    d. View documents that are attached with the run, such as SAS QC data file and SAS output
4. Set reportable:
    a. If everything looks OK as a whole run, then use Set Reportable by Batch Process function
    b. Value will be marked as reportable when it meets following requirements:
       SQC passed; RQC passed; Set Final=yes; Results status = Pass & Warn
5. Resolve replicates
    a. Set reportable based on the first passing results, then save. Then you have done with Set Reportable for the run.

4) JA-4030-DR-04-Out-of-Control Corrective Action

❖ **Corrective Action Routine for SAS QC Failed Runs**

1. Verify there are no analyst remarks stating a problem in sample preparation of the QC in question.
   - If there is a sample preparation problem, apply appropriate code and set sample QC rejected for QC in question and re-run SAS not including the sample QC rejected QC pool values. Else go on to step 2.
2. Verify integration of the QC pool for the failed analyte(s). If there is an integration problem, then fix the integration (and verify the same integration problem is not seen in other QC pools, the calibrators or unknowns; if so, then correct problem), re-create the excel file, re-import into database and re-run SAS. If there is not an integration problem, then go to step 3.
3. Verify internal standard recovery for the failed analyte(s) in the QC pools. To do this look at the excel file and compare IS of QC pool with the run mean. If there is an IS recovery problem, apply code 1023 to the QC pool in question. On a case-by-case basis, evaluate whether it would be necessary to set sample QC rejected, therefore eliminating the QC values when SAS is re-run and also evaluate the IS recovery in the patient samples and calibrators. Else go to step 4.
4. Verify calibration of the failed analyte(s)
   - Verify calibration curve has an R2 >0.985; if there is a calibration curve problem, verify integration for all calibration levels. If there is an integration problem, fix integration and re-create the excel file, re-import into database, and re-run SAS. If there is no integration problem and R2 <0.985, apply code 1025.
   - Compare the slope and intercept with the previous 3 runs on the same instrument and/or historical information on regression parameters. If the current failed run shows a slope or intercept difference, investigate why (i.e., response problem, IS issue, etc.) apply appropriate code if necessary.
5. If QC failure is true, then repeat the run for all analyte failures. Write up QC failure corrective action write up in user field 3 as follows: QC failure corrective action: applied corrective action routine, “outcome.”

Appendix C: Ruggedness Testing

A. Derivatization - time

1) Principle: The extract is derivatized with pentafluorobenzyl bromide (PFBBr) in the presence of triethylamine (TEA) to form pentafluorobenzyl (PFB) esters. The current method specifies a reaction time of 15 minutes.

2) Proposal: To vary the reaction time during the derivatization phase to determine its effects on the 21 FA. A reaction time of 5 minutes and 25 minutes will be compared to the current methodology 15 minutes.

3) Findings: Method specifies 15 minutes; a shorter reaction time (5 minutes) and longer reaction time (25 minutes) were tested on 3 bench QC pools run in triplicate per reaction time. The following table shows the average percent difference from 15 minutes reaction time for each fatty acid (based on molar concentration).

Analyte Code	5 minutes (%)	25 minutes (%)
ALN	-3	-1
AR1	-4	-0.4
ARA	2	2
DA1	1	1
DHA	-3	-3
DP3	2	-3
DP6	1	-3
DTA	10	2
ED1	-8	-5
EN1	-7	-4
EPA	1	3
GLA	2	2
HGL	6	4
LG1	1	2
LNA	-0.2	0.2
MR1	13	20
NR1	1	-1
OL1	-1	-0.3
PL1	6	6
PM1	2	3
ST1	1	4

4) Derivatization – time summary: The avg (SD) percent difference for both derivatization times was 1% (7%) compared to the standard 15 minute reaction time. There does not appear to be a large difference between the three derivatization reaction times, therefore, to ensure a thorough reaction takes place 15 minutes will be used.

B. Derivatization – amount of PFBBr

1) Principle: The extract is derivatized with pentafluorobenzyl bromide (PFBBr) in the presence of triethylamine (TEA) to form pentafluorobenzyl (PFB) esters. The current method specifies a 7% PFBBr.

2) Proposal: To vary the amount of PFBBR used during the derivatization phase to determine its effects on the 21 FA. Two lower amounts (6% and 4%) will be compared to the current methodology of 7%.

3) Findings: Method specifies 7% PFBBr, 6% and 4% were tested on 3 bench QC pools run in triplicate per amount of PFBBr. The following table shows the average percent difference from 7% PFBBr for each fatty acid (based on molar concentration).

Analyte Code	4% PFBBr (%)	6% PFBBr (%)
ALN	-3	-2
AR1	-5	1
ARA	1	3
DA1	1	2
DHA	-1	-0.4
DP3	-2	-2
DP6	-2	-2
DTA	1	-1
ED1	-5	3
EN1	-4	3
EPA	1	5
GLA	0.4	-2
HGL	1	1
LG1	2	2
LNA	-0.2	1
MR1	12	5
NR1	2	4
OL1	-1	2
PL1	6	6
PM1	5	4
ST1	1	1

4) Derivatization – time summary: The avg (SD) percent difference for the varied PFBBr amounts compared to the standard 7% PFBBr were 0.4% (7%) and 2% (13%) for 4% PFBBr and 6% PFBBr respectively. Decreasing the amount of PFBBr yields similar results, however the method will continue to use 7% PFBBr to ensure a high enough concentration to complete the conversion.

C. Sonication - time

1) Principle: The calibrators, quality control pools, and unknowns are sonicated for 15 minutes prior to vortexing and aliquoting.

2) Proposal: To vary the sonication time (no sonication and 20 minutes) to see the effect in the 21 FA.

3) Findings: Method specifies 15 minutes sonication time; no sonication and 20 minutes were tested on 3 bench QC pools run in triplicate per time period. The following table shows the average percent difference from 15 minutes sonication for each fatty acid (based on molar concentration).

Analyte Code	No Sonication (%)	20 minutes (%)
ALN	-2	-5
AR1	1	-3
ARA	2	1
DA1	1	-1
DHA	1	-1
DP3	-1	-5
DP6	-0.4	-5
DTA	1	-4
ED1	-1	-1
EN1	-1	-1
EPA	1	2
GLA	-0.5	-2
HGL	1	-2
LG1	2	-0.4
LNA	1	-1
MR1	9	5
NR1	2	-1
OL1	2	-0.4
PL1	6	3
PM1	4	2
ST1	2	-2

4) Sonication – time summary: The avg (SD) percent difference for the varied sonication times compared to the standard 15-minute sonication was 1 (6) and -1 (5) for 0 minutes and 20 minutes respectively. Eliminating the sonication step showed increases for 15/21 analytes. The longer sonication time yielded losses for 16/21 analytes. The sonication step is not necessary for increasing fatty acid concentrations and therefore will be eliminated.

D. Hydrolysis - time

1) Principle: The calibrators, QC pools and unknowns are hydrolyzed for 45 minutes after addition of acid/base.

2) Proposal: To vary the hydrolysis time (30 minutes and 60 minutes) to see the effect on the 21 FA.

3) Findings: Method specifies 45 minutes hydrolysis time per acid/base addition, 30 minutes and 60 minutes were tested on 3 bench QC pools run in triplicate per time period. The following table shows the average percent difference from 45 minutes hydrolysis time for each fatty acid (based on molar concentration).

Analyte Code	30 minutes (%)	60 minutes (%)
ALN	-3	-4
AR1	3	11
ARA	-0.3	-1
DA1	-1	-0.2
DHA	-3	-3
DP3	-4	-6
DP6	-2	-3
DTA	-3	-6
ED1	2	4
EN1	4	6
EPA	7	3
GLA	4	1
HGL	0.5	-2
LG1	-1	0.4
LNA	-0.1	-1
MR1	10	16
NR1	-2	-0.2
OL1	1	-0.1
PL1	1	0.3
PM1	-0.3	3
ST1	-1	5

4) Hydrolysis – time summary: The avg (SD) percent difference for the varied hydrolysis times was 1% (8%) for both 30 minutes and 60 minutes compared to the standard 45-minute hydrolysis time. Overall, the shorter and longer hydrolysis times were similar to the current methodology, therefore the hydrolysis time will remain at 45 minutes.

E. Extraction efficiency

1) Principle: This method performs a triple extraction to extract the 21 FA from RBC.

2) Proposal: To test whether a double extraction is sufficient for extraction or whether quadruple extraction yields better results.

3) Findings: Method specifies a triple extraction; a double and quadruple extraction were tested on 3 bench QC pools run in triplicate per time period. The following table shows the average percent difference from triple extraction for each fatty acid (based on molar concentration).

Analyte Code	Double extraction (%)	Quadruple extraction (%)
ALN	-2	-3
AR1	-1	8
ARA	-0.4	-1
DA1	-1	-1
DHA	-3	-3
DP3	-3	-5
DP6	0.3	-6
DTA	2	-8
ED1	-6	-1
EN1	-1	0.2
EPA	0.1	1
GLA	3	3
HGL	4	-7
LG1	-1	-2
LNA	-1	-1
MR1	-8	29
NR1	-4	-3
OL1	-0.5	-1
PL1	-1	-3
PM1	-2	5
ST1	-7	8

4) Extraction efficiency summary: The avg (SD) percent difference for the varied extraction were -1% (5%) and 1% (12%) for double and quadruple extraction respectively. The double extraction yielded lower concentrations for 16/21 FA. The quadruple extraction yielded higher concentrations for only 7/21 FA. Although the quadruple extraction yielded an avg (SD) increase in the molar concentration for MR1 (myristic acid), this is a low-level analyte (10-20uM) with a high LOD (4 uM). This does not represent other low-level analytes ≤20 uM (ALN, AR1, ED1, EN1, EPA, GLA, and PL1) which are on avg (SD) 1% (6%) higher with the quadruple extraction. Additionally, the molar concentration increase for MR1 with the quadruple extraction only slightly increases the MR1 % of total from 0.28% to 0.34%. For these reasons, the number of extractions will remain at three.