Laboratory Procedure Manual

Analytes: ***Profile of 30 fatty acids - Arachidic, Arachidonic, Capric, Docosahexaenoic, Docosanoic, Docosapentaenoic (n3), Docosapentaenoic (n6), Docosatetraenoic, Eicosadienoic, Eicosapentaenoic, Eicosatrienoic, Eicosenoic, Lauric, Lignoceric, Linoleic, alpha-Linolenic, gamma-Linolenic, homo-gamma-Linolenic, Margaric, Myristic, Myristoleic, Nervonic, Oleic, Palmitic, Palmitoleic, Pentadecanoic, Stearic, Stearidonic, Tricosanoic, and cis-Vaccenic***

Matrix: **Plasma or Serum**

Method: **Gas Chromatography – Mass Spectrometry**

Method No: **4028.02**

Revised:

as performed by:

Fat-soluble Nutrients Laboratory
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.

Lab Number	Analyte	SAS Label (and SI units)
FAS_G	LBXCAP	Capric acid (C10:0) (μmol/L)
	LBXLAR	Lauric acid (C12:0) (μmol/L)
	LBXMR1	Myristic acid (14:0) (μmol/L)
	LBXPEN	Pentadecanoic acid (C15:0) (μmol/L)
	LBXPM1	Palmitic acid (16:0) (μmol/L)
	LBXMRG	Margaric acid (C17:0) (μmol/L)
	LBXST1	Stearic acid (18:0) (μmol/L)
	LBXAR1	Arachidic acid (20:0) (μmol/L)
	LBXDA1	Docosanoic acid (22:0) (μmol/L)
	LBXTSA	Tricosanoic acid (C23:0) (μmol/L)
	LBXLG1	Lignoceric acid (24:0) (μmol/L)
	LBXML1	Myristoleic acid (14:1n-5) (μmol/L)
	LBXPL1	Palmitoleic acid (16:1n-7) (μmol/L)
	LBXVC1	cis-Vaccenic acid (18:1n-7) (μmol/L)
	LBXOL1	Oleic acid (18:1n-9) (μmol/L)
	LBXEN1	Eicosenoic acid (20:1n-9) (μmol/L)
	LBXNR1	Nervonic acid (24:1n-9) (μmol/L)
	LBXLNA	Linoleic acid (18:2n-6) (μmol/L)
	LBXALN	alpha-Linolenic acid (18:3n-3) (μmol/L)
	LBXGLA	gamma-Linolenic acid (18:3n-6) (μmol/L)
	LBXSD1	Stearidonic acid (C18:4n-3) (μmol/L)
	LBXED1	Eicosadienoic acid (20:2n-6) (μmol/L)
	LBXHGL	homo-gamma-Linolenic acid (20:3n-6) (μmol/L)
	LBXET1	Eicosatrienoic acid (C20:3n-9) (μmol/L)
	LBXARA	Arachidonic acid (20:4n-6) (μmol/L)
	LBXEPA	Eicosapentaenoic acid (20:5n-3) (μmol/L)
	LBXDTA	Docosatetraenoic acid (22:4n-6) (μmol/L)
	LBXDP3	Docosapentaenoic acid (22:5n-3) (μmol/L)
	LBXDP6	Docosapentaenoic acid (22:5n-6) (μmol/L)
	LBXDHA	Docosahexaenoic acid (22:6n-3) (μmol/L)

1. Summary of Test Principle and Clinical Relevance

A. Clinical Relevance

The analysis of individual plasma or serum fatty acids is important in the recognition of essential fatty acid deficiency (2) and in the differential diagnosis of inborn errors of metabolism, such as mitochondrial fatty acid oxidation disorders (3,4,5). Long-chain polyunsaturated fatty acids are essential for normal development (6). The dietary content of saturated, monounsaturated, and polyunsaturated fatty acids influence the concentration of cholesterol in low-density and high-density lipoproteins, and consequently the development of atherosclerosis (7). Regular consumption of or supplementation with omega-3 polyunsaturated fatty acids can have beneficial effects on long-term cardiovascular health due to anti-inflammatory and possibly antiarrhythmic effects (8). The goal of this method is to obtain US reference ranges for most circulating fatty acids. Public health recommendations advise increasing or decreasing the intake of various classes of fatty acids (saturated, monounsaturated, polyunsaturated) but relatively little fatty acid biomarker data exist to support these recommendations and reference range data are scarce.

B. Test Principle

Esterified fatty acids are hydrolyzed primarily from triglycerides, phospholipids and cholesteryl esters using sequential treatment with mineral acid and base in the presence of heat. Using a modification of (1), total fatty acids are hexane-extracted from the matrix (100uL serum or plasma) along with an internal standard solution containing eighteen stable isotopically-labeled fatty acids 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 fatty acids of interest from other matrix constituents. Fatty acids are detected using electron capture negative-ion mass spectrometry within 34 minutes. Eleven saturated, six monounsaturated, and thirtenen polyunsaturated fatty acids (thirty fatty acids in total) are measured using selected ion monitoring. Quantitation is accomplished by comparing the peak area of the analyte in the unknown with the peak area of a known amount in a calibrator solution. Calculations are corrected based on the peak area of the internal standard in the unknown compared with the peak area of the internal standard in the calibrator solution.

Saturated	Fatty acids	Database Analyte Code	Carbon: Double bonds
1	Capric acid	CAP	C10:0
2	Lauric acid	LAR	C12:0
3	Myristic acid	MR1	C14:0
4	Pentadecanoic acid	PDE	C15:0
5	Palmitic acid	PM1	C16:0
6	Margaric acid	MRG	C17:0
7	Stearic acid	ST1	C18:0
8	Arachidic acid	AR1	C20:0
9	Docosanoic acid	DA1	C22:0
10	Tricosanoic acid	TSA	C23:0
11	Tetracosanoic acid	LG1	C24:0

Monounsaturated			
1	Myristoleic acid	ML1	C14:1n-5
2	Palmitoleic acid	PL1	C16:1n-7
3	*cis*-Vaccenic acid	VC1	C18:1n-7
4	Oleic acid	OL1	C18:1n-9
5	Eicosenoic acid	EN1	C20:1n-9
6	Nervonic acid	NR1	C24:1n-9
Polyunsaturated			
1	Linoleic acid	LNA	C18:2n-6
2	*alpha*-Linolenic acid	ALN	C18:3n-3
3	*gamma*-Linolenic acid	GLA	C18:3n-6
4	Stearidonic acid	SD1	C18:4n-3
5	Eicosadienoic acid	ED1	C20:2n-6
6	*homo-gamma*-Linolenic acid	HGL	C20:3n-6
7	Eicosatrienoic acid	ET1	C20:3n-9
8	Arachidonic acid	ARA	C20:4n-6
9	Eicosapentaenoic acid	EPA	C20:5n-3
10	Docosatetraenoic acid	DTA	C22:4n-6
11	Docosapentaenoic acid	DP3	C22:5n-3
12	Docosapentaenoic acid	DP6	C22:5n-6
13	Docosahexaenoic acid	DHA	C22:6n-3

2. Safety Precautions

Consider all plasma or serum 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 and/or 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 plasma 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 derivitizating 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. This step should be done in an oven designed for volatile organic solvents.

Reagents and solvents used in this study include those listed in Section 6. Material safety data sheets (MSDS) for these chemicals are readily accessible as hard copies in the lab. If needed, MSDS for other chemicals can be viewed at http:\\www.ilpi.com\msds\index.html

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 a LIMS database for review of the patient data, statistical evaluation of the QC data, and approval of the results. See "SOP Chromatography Review of 4028 Fatty Acids Results" for a step-by-step description of chromatography review and transfer to network locations.
C. Files stored on the CDC network are automatically backed up nightly by ITSO support staff.

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

A. For best results, a fasting sample should be obtained.
B. Specimens for fatty acids analysis may be fresh or frozen plasma or serum.
C. A 0.5-mL sample of plasma or serum is required to allow for repeat analyses; a volume of 100uL is required per analysis.
D. The appropriate amount of plasma or serum is dispensed into a Nalge 2.0-mL cryovial or other plastic screw-capped vial labeled with the specimen ID.
E. Specimens collected in the field are frozen and then shipped on dry ice by overnight carrier. Frozen samples are stored at -70^{o}C.
F. 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 analyte of interest in the specimen and it needs to be divided, the appropriate amount of blood, serum or plasma should be transferred into a sterile Nalge cryovial labeled with a new sample ID linked to the participant's ID.

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

A. Reagent Preparation

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

Add 100mL of 6N HCl to 900mL of acetonitrile and mix. Store at room temperature. Prepare fresh 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: 10N Sodium Hydroxide, (90:10, v:v)

Add 100mL of 10N sodium hydroxide to 900mL with methanol and mix. Store at room temperature. Prepare fresh 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) Derivitizing Solution – Prepare fresh daily a 7% pentafluorobenzyl bromide and 10% triethylamine in acetonitrile solution as shown in the table below.

# samples	Pentafluorobenzyl Bromide (PFBBr)	Acetonitrile (ACN)	Triethylamine (TEA)
< 20	140 uL	1.86 mL	200 uL
< 30	210 uL	2.79 mL	300 uL
< 40	280 uL	3.72 mL	400 uL
< 50	350 uL	4.65 mL	500 uL
< 60	420 uL	5.58 mL	600 uL
< 70	490 uL	6.51 mL	700 uL
< 80	560 uL	7.44 mL	800 uL
< 90	630 uL	8.37 mL	900 uL
< 100	700 uL	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: 5 fatty acids that were included in the calibration solutions were not reportable because none of the QC has concentrations that were above the limit of detection (LOD).

	Fatty Acid Name	Analyte Code	FW	Target Stock Conc (mM)	Target Amount in 5.0 mL Toluene (g)
1	*alpha*-Linolenic	ALN	278.48	50	0.07
2	Arachidic	AR1	312.54	25	0.039
3	Arachidonic	ARA	304.52	250	0.381
4	Capric	CAP	172.26	10	0.017*
5	Caprylic	CL1	144.21	50	0.036

6	Docosanoic	DA1	340.59	25	0.043
7	Docosadienoic	DD1	336.55	10	0.017
8	Docosenoic	DE1	338.59	25	0.042
9	Docosahexaenoic	DHA	328.57	75	0.123
10	Docosapentaenoic n-3	DP3	330.57	25	0.041
11	Docosapentaenoic n-6	DP6	330.57	25	0.041
12	Docosatrienoic	DT1	334.5	10	0.017
13	Docosatetraenoic	DTA	332.57	75	0.125
14	Eicosadienoic	ED1	308.53	25	0.039
15	Eicosenoic	EN1	310.54	25	0.039
16	Eicosapentaenoic	EPA	302.52	50	0.076
17	*gamma*-Linolenic	GLA	278.48	75	0.104
18	*homo-gamma*-Linolenic	HGL	306.53	125	0.192
19	Hexacosanoic	HSA	396.7	10	0.02
20	Lauric	LAR	200.32	50	0.05
21	Tetracosanoic	LG1	368.64	15	0.028
22	Linoleic	LNA	280.48	500	0.701
23	Myristoleic	ML1	226.38	75	0.085
24	Myristic	MR1	228.38	100	0.114
25	Margaric	MRG	270.45	10	0.014
26	Nervonic	NR1	366.63	50	0.092
27	Oleic	OL1	282.48	500	0.706
28	Pentadecanoic	PDE	242.4	10	0.012
29	Palmitoleic	PL1	254.43	125	0.159
30	Palmitic	PM1	256.43	500	0.641
31	Stearidonic	SD1	276.4	2	0.00276
32	Stearic	ST1	300.48	150	0.225
33	Tricosanoic	TSA	354.62	10	0.018
34	*cis*-Vaccenic	VC1	282.48	100	0.141

*this stock solution prepared in 10mL instead of 5mL

(a) Weigh all materials into labeled 16- 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 * 5mL). Cap and mix by gentle inversion. If necessary sonicate stock solutions until analyte is in solution.
(c) Note: Eicosatrienoic acid is added directly to Intermediate Stock 1

(2) Purity Check for Individual Stock Standard Solutions – in addition to obtaining manufacturer purity information
   (a) Aliquot 50uL of each individual stock standard solution to a 13 x 100mm culture tube
   (b) Add 100uL of Derivitizing solution (7% pentafluorobenzyl bromide and 10% triethylamine in acetonitrile solution; preparation shown in section 6.a.3 Reagent preparation)
   (c) Wait 15 minutes for derivitization to occur
   (d) Add 1mL hexane to each tube and mix
   (e) Transfer hexane to labeled GCMS vial and run in SIM and Scan modes on GCMS
   (f) After analysis and data review, for each stock standard solution sum peak areas of all known peaks. Divide peak area (of analyte of interest) by the sum of the peak areas (within analyte of interest chromatogram) to obtain percent purity. Percent purity should be taken to account for all analytes when assigning calibration values.

(3) Composition of Intermediate Stock 1 (prepared in 50-mL volumetric cylinder)

	Fatty Acid Name	Analyte Code	FW	Stock (mM) Calculated	mL to be added to prepare Intermediate Stock 1
1	*alpha*-Linolenic	ALN	278.48	49.57	1.51
2	Arachidic	AR1	312.54	25.14	0.5
3	Arachidonic	ARA	304.52	251.64	1.24
4	Capric	CAP	172.26	10.02	0.62
5	Caprylic	CL1	144.21	49.58	0.25
6	Docosanoic	DA1	340.59	15.66	1.6
7	Docosadienoic	DD1	336.55	9.97	0.63
8	Docosenoic	DE1	338.59	25.11	0.25
9	Docosahexaenoic	DHA	328.57	74.39	1.26
10	Docosapentaenoic n-3	DP3	330.57	24.79	1.26
11	Docosapentaenoic n-6	DP6	330.57	24.94	0.75
12	Docosatrienoic	DT1	334.5	9.94	0.63
13	Docosatetraenoic	DTA	332.57	75.52	0.25
14	Eicosadienoic	ED1	308.53	24.92	0.5
15	Eicosenoic	EN1	310.54	24.78	0.5
16	Eicosapentaenoic	EPA	302.52	50.3	0.99
17	*gamma*-Linolenic	GLA	278.48	75.59	0.5
18	*homo-gamma*-Linolenic	HGL	306.53	124.9	0.5
19	Hexacosanoic	HSA	396.7	5.55	1.13
20	Lauric	LAR	200.32	49.7	0.25
21	Tetracosanoic	LG1	368.64	9.41	1.99
22	Linoleic	LNA	280.48	500.53	2.5
23	Myristoleic	ML1	226.38	75.51	0.25
24	Myristic	MR1	228.38	100.15	1.25
25	Margaric	MRG	270.45	10.05	0.62
26	Nervonic	NR1	366.63	49.53	0.5
27	Oleic	OL1	282.48	500.71	2.5
28	Pentadecanoic	PDE	242.4	10	0.62
29	Palmitoleic	PL1	254.43	126.13	2.48
30	Palmitic	PM1	256.43	311.7	4.01
31	Stearidonic	SD1	276.4	2	3.13
32	Stearic	ST1	300.48	93.84	4
33	Tricosanoic	TSA	354.62	6.26	1
34	*cis*-Vaccenic	VC1	282.48	99.46	1.01
35	5Z, 8Z, 11Z-eicosatrienoic	ET1	306.5	10mg/mL	0.38mL of 10mg/mL solution

(4) Intermediate Stock 2 is prepared by adding 4.0 mL of Intermediate Stock 1 to 50-volumetric flask, then filling to the mark with Toluene. Mix by inversion.

(5) Standards 1-6 are prepared from the Intermediate Stock 1 or 2 solutions as follows:

a. Standard 6 (Std6): Add 20 mL of Intermediate Stock 1 to a 50-mL volumetric flask, then fill to the mark with Toluene. Mix by Inversion.

b. Standard 5 (Std5): Add 12 mL of Intermediate Stock 1 to a 50-mL volumetric flask, then fill to the mark with Toluene. Mix by Inversion.

c. Standard 4 (Std4): Add 6 mL of Intermediate Stock 1 to a 50-mL volumetric flask, then fill to the mark with Toluene. Mix by Inversion.

d. Standard 3 (Std3): Add 25 mL of Intermediate Stock 2 to a 50-mL volumetric flask, then fill to the mark with Toluene. Mix by Inversion.

e. Standard 2 (Std2): Add 2.5 mL of Intermediate Stock 2 to a 50-mL volumetric flask, then fill to the mark with Toluene. Mix by Inversion.

f. Standard 1 (Std1): Add 1 mL of Intermediate Stock 2 to a 50-mL volumetric flask, then fill to the mark with Toluene. Mix by Inversion. Standard 1 is not routinely used in the assay.

aliquot 225uL final standard solutions into appropriately labeled calibrator vials

Table of Target Concentrations for Calibrators

	Fatty Acid	Code	Std6 (uM)	Std5 (uM)	Std4 (uM)	Std3 (uM)	Std2 (uM)	Std1* (uM)
1	*alpha*-Linolenic	ALN	600	360	180	60	6	2.4
2	Arachidic	AR1	100	60	30	10	1	0.4
3	Arachidonic	ARA	2500	1500	750	250	25	10
4	Capric	CAP	50	30	15	5	0.5	0.2
5	Caprylic	CL1	100	60	30	10	1	N/A
6	Docosanoic	DA1	200	120	60	20	2	0.8
7	13,16- Docosadienoic	DD1	50	30	15	5	0.5	0.2
8	Docosenoic	DE1	50	30	15	5	0.5	0.2
9	Docosahexaenoic	DHA	750	450	225	75	7.5	3
10	Docosapentaenoic n-3	DP3	250	150	75	25	2.5	1
11	Docosapentaenoic n-6	DP6	150	90	45	15	1.5	0.6
12	13,16,19-Docosatrienoic	DT1	50	30	15	5	0.5	0.2
13	Docosatetraenoic	DTA	150	90	45	15	1.5	0.6
14	11,14-Eicosadienoic	ED1	100	60	30	10	1	0.4
15	11-Eicosenoic	EN1	100	60	30	10	1	0.4
16	Eicosapentaenoic	EPA	400	240	120	40	4	1.6
17	5Z, 8Z, 11Z-Eicosatrienoic acid	ET1	100	60	30	10	1	0.4
18	*gamma*-Linolenic	GLA	300	180	90	30	3	1.2
19	*homo-gamma*-Linolenic	HGL	500	300	150	50	5	2
20	Hexacosanoic	HSA	50	30	15	5	0.5	0.2
21	Lauric	LAR	100	60	30	10	1	0.4
22	Tetracosanoic	LG1	150	90	45	15	1.5	0.6
23	Linoleic	LNA	10000	6000	3000	1000	100	40
24	Myristoleic	ML1	150	90	45	15	1.5	0.6
25	Myristic	MR1	1000	600	300	100	10	4
26	Margaric	MRG	50	30	15	5	0.5	N/A
27	Nervonic	NR1	200	120	60	20	2	0.8
28	Oleic	OL1	10000	6000	3000	1000	100	40
29	Pentadecanoic	PDE	50	30	15	5	0.5	0.2
30	Palmitoleic	PL1	2500	1500	750	250	25	10
31	Palmitic	PM1	10000	6000	3000	1000	100	40
32	Stearidonic	SD1	50	30	15	5	0.5	N/A
33	Stearic	ST1	3000	1800	900	300	30	12
34	Tricosanoic	TSA	50	30	15	5	0.5	0.2
35	*cis*-Vaccenic	VC1	800	480	240	80	8	3.2

*Std1 is run every six months for expanded calibration for low level analytes.

(6) Internal standard solution – individual stock solutions are prepared so that each will yield the following final concentrations 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 FW 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).

	Internal Standard Acid Name	Label	Analyte Code	Target (uM)
1	Arachidic	d-39	AR1_IS	25
2	Capric	d-3	CAP_IS	15
3	Caprylic	d-3	CL1_IS	35
4	Docosanoic	d-4	DA1_IS	60
5	Docosahexaenoic	d-5	DHA_IS	125
6	Eicosapentaenoic	d-5	EPA_IS	40
7	Lauric	d-3	LAR_IS	50
8	Lignoceric	d-4	LG1_IS	50
9	Linoleic	13C	LNA_IS	3500
10	Myristic	d-27	MR1_IS	100
11	Margaric	d-3	MRG_IS	15
12	Oleic	13C	OL1_IS	2100
13	Pentadecanoic	d-3	PDE_IS	20
14	Palmitic	d-31	PM1_IS	2700
15	Stearic	d-35	ST1_IS	700
16	*alpha*-Linolenic	d-14	ALN _IS	65
17	Arachidonic	d-8	ARA_IS	800
18	Palmitoleic	d-14	PL1_IS	200

C. Preparation of Quality Control Materials

Normal serum or plasma (650uL) containing 3.33g BHT in methanol/L serum is aliquotted into 2.0-mL Nalgene cryovials, capped, and frozen. The QC pools are stored at -70°C.

Means plus range limits for all pools are established by analyzing duplicates for at least 20 runs. QC pools are prepared by analyzing numerous serum or plasma samples and selecting and blending the ones which best match the following criteria: 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 fatty acids (Palmitic, Oleic, Linoleic, Stearic and Arachidonic).

D. Other Materials

(1) 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., Corning, NY)
(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 uL) for Eppendorf pipette (Brinkmann Instruments Inc., Westbury, NY)
(i) Yellow tips (2-200 uL) 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, Rainin)
(p) Hamilton high volume (1mL) tips without filter (cat. no. 235904, Hamilton, Reno, NV)
(q) Hamilton standard volume (300uL) tips without filter (cat. no. 235902, Hamilton, Reno, NV)
(r) 20mL Disposable glass scintillation vials with caps
(s) GC vials and caps (C4000-2W and C4000-53B; Fisher Scientific, Fair Lawn, NJ)
(t) Various glass beakers, graduated cylinders and glass bottles, class A glassware

(2) GCMS supplies

(a) Helium, ultrapure (>99.99% purity) (Air Products, Inc., Atlanta, GA)
(b) Methane, ultrapure (>99.99% purity) (Air Products, Inc., Atlanta, GA)
(c) J&W DB-23 capillary GC column, 60.0m x 0.25mm x 0.25μm (Agilent, Wilmington, DE)
(d) Thermo Trace Gold TG-POLAR GC Column, 60.0m x 0.25mm x 0.25μm (Thermo, West Palm Beach, FL)
(e) Split/splitless liner (19251-60540, Agilent, Wilmington, DE)
(f) Liner o-ring (5188-5365, Agilent, Wilmington, DE)
(g) Split gold seal disk (5182-9652, Agilent, Wilmington, DE)
(h) Silver washer (5061-5869, Agilent, Wilmington, DE)
(i) Inlet Ferrule, 0.4mm 15%/85% graphite/vespel (5181-3323, Agilent, Wilmington, DE)
(j) Column nut for GC capillaries (5181-8830, Agilent, Wilmington, DE)
(k) Ferrule, 0.4mm 15%/85% graphite/vespel (5062-3508, Agilent, Wilmington, DE)
(l) Column nut for MS interface (05988-20066, Agilent, Wilmington, DE)
(m) Lens insulator, 597X MSD (G1370-20530, Agilent, Wilmington, DE)
(n) 73CI Repeller (G1999-20432, Agilent, Wilmington, DE)
(o) CI Filament for the 5973 and 5975 MSD (G1099-80053, Agilent, Wilmington, DE)
(p) ALS Syringe, 10ul tapered, fixed needle (5181-3360, Agilent, Wilmington, DE)
(q) Advanced Green 11mm septa (5183-4759, Agilent, Wilmington, DE)

(r) 4mL Wash vial with fill markings and cap (5182-0551, Agilent, Wilmington, DE)
(s) Diffusion caps for 4 mL wash vials (07673-40180, Agilent, Wilmington, DE)
(t) Various Swagelok fittings (Agilent or equivalent, Wilmington, DE)
(u) Copper tubing cutter (Agilent or equivalent, Wilmington, DE)

(3) Chemicals
(a) Methanol, HPLC grade, # AH230-4 (Burdick & Jackson, Muskegan, MI)
(b) Acetonitrile, HPLC grade, Acros # UN1648 (Fisher Scientific, Pittsburgh, PA)
(c) Hexanes, HPLC grade, # UN1208 (Tedia Company Inc, Fairfield, OH)
(d) Toluene, # T-323-4 (Fisher Scientific, Fair Lawn, NJ)
(e) 10N Sodium Hydroxide, #SS255-1 (Fisher Scientific, Fair Lawn, NJ)
(f) Pentafluorobenzyl bromide (Pierce, Rockford, IL)
(g) 6N Hydrochloric acid, # SA56-1 (Fisher Scientific, Suwanee, GA)
(h) Triethylamine, # W639-07 (J.T. Baker, Phillipsburg, NJ)

(4) Standards
(a) *alpha*-Linolenic (Nu-Chek Prep, U-62A)
(b) Arachidic (Nu-Chek Prep, N-20A)
(c) Arachidonic (Nu-Chek Prep, U-71A)
(d) Capric (decanoic) (Nu-Chek Prep, N-10A)
(e) Caprylic (octanoic) (Nu-Chek Prep, N-8A)
(f) Docosanoic (Nu-Chek Prep, N-22A)
(g) 13,16- Docosadienoic (Nu-Chek Prep, U-81A)
(h) Docosenoic (Nu-Chek Prep, U-79A)
(i) Docosahexaenoic (Nu-Chek Prep, U-84A)
(j) Docosapentaenoic n-3 (Nu-Chek Prep, U-101)
(k) Docosapentaenoic n-6 (Nu-Chek Prep, U-102)
(l) 13,16,19-Docosatrienoic (Nu-Chek Prep, U-82A)
(m) Docosatetraenoic (Nu-Chek Prep, U-83A)
(n) 11,14-Eicosadienoic (Nu-Chek Prep, U-68A)
(o) 11-Eicosenoic (Nu-Chek Prep, U-66A)
(p) Eicosapentaenoic (Nu-Chek Prep, U-99A)
(q) *gamma*-Linolenic (Nu-Chek Prep, U-63A)
(r) *homo-gamma*-Linolenic (Nu-Chek Prep, U-69A)
(s) Hexacosanoic (cerotic) (Cayman Chemical, 13354)
(t) Lauric (dodecanoic) (Nu-Chek Prep, N-12A)
(u) Tetracosanoic (Nu-Chek Prep, N-24A)
(v) Linoleic (Nu-Chek Prep, U-59A)
(w) Myristoleic (Nu-Chek Prep, U-36A)
(x) Myristic (Nu-Chek Prep, N-14A)
(y) Margaric (heptadecanoic) (Nu-Chek Prep, N-17A)
(z) Nervonic (Nu-Chek Prep, U-88A)
(aa) Oleic (Nu-Chek Prep, U-46A)

(bb) Pentadecanoic (Nu-Chek Prep, N-15A)
(cc) Palmitoleic (Nu-Chek Prep, U-40A)
(dd) Palmitic (Nu-Chek Prep, N-16A)
(ee) Stearidonic (Cayman Chemical, 90320)
(ff) Stearic (Nu-Chek Prep, N-18A)
(gg) Tricosanoic (Nu-Chek Prep, N-23A)
(hh) *cis*-Vaccenic (Nu-Chek Prep, U-48A)
(ii) 5Z, 8Z, 11Z-Eicosatrienoic (Cayman, 90190)

(5) Isotopically Labeled Standards
(a) Arachidic, d-39 (C/D/N Isotopes, D-1617)
(b) Capric, d-3 (C/D/N Isotopes, D-4021)
(c) Caprylic, d-3 (C/D/N Isotopes, D-3992)
(d) Docosanoic, d-4 (Isosciences , custom synthesis)
(e) Docosahexaenoic, d-5 (Isosciences , custom synthesis)
(f) Eicosapentaenoic, d-5 (Isosciences , custom synthesis)
(g) Lauric, d-3 (C/D/N Isotopes, D-4027)
(h) Lignoceric, d-4 (Isosciences , custom synthesis)
(i) Linoleic, 13C (Isosciences, custom synthesis)
(j) Myristic, d-27 (Cambridge Isotopes, I1-7220D)
(k) Margaric, d-3 (C/D/N Isotopes, D-5255)
(l) Oleic, 13C (Isosciences, custom synthesis)
(m) Pentadecanoic, d-3 (C/D/N Isotopes, D-5258)
(n) Palmitic, d-31 (Cambridge Isotopes, I1-10006B)
(o) Stearic, d-35 (Cambridge Isotopes, I1-7911A)
(p) *alpha*-Linolenic, d-14 (Cayman, custom synthesis)
(q) Arachidonic, d-8 (Cayman and/or Isosciences, custom synthesis)
(r) Palmitoleic, d-14 (Cayman, custom synthesis)

E. Instrumentation
(1) Agilent GC-MS (Agilent, Wilmington, DE)
(a) GC model: 7890A
(b) Autosampler model:7683A
(c) Mass Spectrometer model:5975C
(d) Software model: MSD Chemstation E.02.0049
(2) Thermo Scientific FREAS Mechanical Convention Oven model 625S (Thermo, Marietta, OH)
(3) Speedvac Plus SC110 A (Savant Instrument Co., Farmingdale, NY) or Genevac EZ-2 evaporator (Genevac, Inc., Valley Cottage, NY)
(4) Mixer Type 16700 Model # M16715 (Barnstead International, Dubuque, IA)
(5) Magnetic stirrer (Baxter Scientific Products, Stone Mountain, GA)
(6) Eppendorf micropipette (Brinkmann Instruments Inc., Westbury, NY)
(7) Eppendorf repeater pipette (Brinkmann Instruments Inc., Westbury, NY)
(8) Rainin Pos-D positive displacement pipettes various sizes (Rainin)

(9) Mettler Toledo AG 104 balance (Mettler Instrument Corp., Hightstown, NJ)

(10) Hamilton Starlet 8-channel with auto-load arm (Hamilton, Reno, NV)

(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 (highest calibrator from current run) is run to determine that the retention times and analyte responses are within expected limits.

Five calibration standards plus a reagent blank are prepared and extracted along with the samples. The calibration curve is injected at the beginning of the analytical run. Following the completion of the analytical run, calibration data are calculated using Chemstation 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 must fall within 15% of the target values, with the exception of low concentration calibrators that are approaching the LOD. All calibrators are typically prepared within a few percent (mean -5%; range -39 to 11%) of the targeted concentrations after correcting for purity, lot-to-lot correction, and calibration verification.

Calibration verification is conducted at least twice a year. For details, see **4028_SOP Calibration Verification Fatty Acids**.

This method uses toluene as the matrix for the calibrators. Matrix based calibration was tested by comparing the average slope and intercept parameters of three 10-point calibration curves prepared using stripped serum (Aalto Scientific, Ltd., Carlsbad, CA) with three 10-point toluene-based calibration curves. A ≤ 5% difference in the average calibration curve slope was observed between serum and toluene-based calibrations for 29/35 analytes. For the remaining analytes the percent difference ranged from 6-8%. Differences observed (toluene vs serum) were of a similar magnitude to slope variability observed within and between individual calibration curves of a particular matrix.

Serum- or plasma-based reference materials are not available for all fatty acids. NIST, NIH and CDC are working toward an inter-laboratory quality assurance program for various fatty acids in biological matrices. NIST SRM1950 has 8 certified and 19 reference fatty acid values in plasma.

Method figures of merit are presented in the **Appendix 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 B**.

B. Instrument Calibration

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 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 running the instrument in CI autotune. Please refer to the User's Manual and the **4028_SOP Agilent GCMS calibration** for additional details.

C. Hamilton Microlab Starlet Calibration Verification
Twice a year a Hamilton service engineer performs preventative maintenance including volume verification at 10uL and 1000uL.
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.

D. Pipettes (air displacement and positive displacement)
Pipettes are calibrated or calibration is verified on a semi-annual basis.

E. 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 plasma or serum, quality control plasma or serum, and standards to reach ambient temperature, then sonicate for 15 minutes and vortex each sample individually or as set prior to aliquotting. Visually check each sample for unusual specimen color or debris/precipitate.
(3) Set up Excel run sheet containing sample ID's 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 6 calibrators (includes a blank), 3 QC samples (first set), 38-68 patient samples, 3 QC samples (second set).

Sample Preparation Note: If necessary a combination of manual and Hamilton automated sample preparation and extraction could be done: i.e. manual sample preparation can be done then use the Hamilton Extraction method for the liquid-liquid extraction or Hamilton sample preparation can be used with the Manual Extraction method for the liquid-liquid extraction. For a detailed step-by-step description of the manual and Hamilton Microlab Starlet sample preparation see **4028_SOP Sample Preparation**.

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 steps are done in the fume hood except when items are in the oven.
(2) Add 100uL of serum or plasma to 16- x 100-mm screw cap culture tube.
(3) Add 100uL of internal standard mixture.
(4) Add 2mL of acetonitrile: 6N hydrochloric acid (90:10, v:v).
(5) Cap each tube tightly using Teflon-lined caps.
(6) Heat samples in oven for 45 minutes at 104°C.

(7) 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.
(8) Add 2mL of a solution of methanol:10N sodium hydroxide (90:10, v:v).
(9) Recap tightly with the same teflon caps and heat in oven for 45 minutes at 104°C.
(10) Remove samples from oven and allow samples to cool. If noticeable evaporation has taken place in any sample, then add additional methanol (without sodium hydroxide) to bring back up to the approximate volume.
(11) Re-acidify samples by addition of 350uL of 6N HCl. If Hamilton liquid-liquid extraction method will be used, then use a Pasteur pipette and tubing attached to the lab airline to blow off the vapors that are created after the addition of the acid to protect the Hamilton pipetting heads from corrosion.
(12) Proceed with either manual liquid-liquid extraction (step C) or Hamilton liquid-liquid extraction steps (step D).

C. Manual Double Liquid-Liquid Extraction
(1) Add 3mL of hexane to samples; cap each tube tightly; mix by inversion for 20 seconds.
(2) Allow time for layers to separate.
(3) Using a clean glass Pasteur pipette carefully draw up the top (organic) layer and place it into a 13 x 100mm tube. Avoid the bottom (aqueous) layer.
(4) Repeat steps 1) – 3) for double extraction. Proceed to sample preparation for GC-MS (step e.).

D. Hamilton Automated Triple Liquid-Liquid Extraction
(1) The Hamilton Microlab Starlet can be used for a triple liquid-liquid extraction of the samples.
(2) A brief description of the triple extraction is as follows:
(a) 3mL of hexane added to all samples
(b) after mixing 2mL of the hexane extract is transferred to 13 x 100mm culture tubes
(c) 2mL of hexane added to all samples
(d) after mixing 2mL of the hexane extract is transferred to 13 x 100mm culture tubes
(e) 2mL of hexane added to all samples
(f) after mixing 2mL of the hexane extract is transferred to 13 x 100mm culture tubes

E. Sample Preparation for GC-MS
(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 (as shown in the table in section 6.a section 3). Add 100uL of the derivitizing 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 GC-MS autosampler vial containing a glass insert, then immediately cap, and place on autosampler tray for injection.

F. GCMS Instrument Preparation
An Agilent GCMS system is used to quantitate dietary fatty acids in extracted serum or plasma.
(1) GC preparation
(a) Septum should be changed prior to each run
(b) Liner should be changed every other run
(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.

(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: 47.377 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; 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 8uL; sample washes: 3 at 8uL; sample pumps: 2; injection volume: 1.0-uL; syringe size: 10.0-uL.
   (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: 0.25mm; nominal film thickness: 0.25 μm; mode: constant flow at 2mL/min
   (i) Outlet pressure: vacuum

Table of Selected Ion Monitoring (SIM) masses

	No.	Fatty Acid	Analyte code	SIM (M/Z)	Internal Standard used for quantitation
Segment 1	1	Caprylic-d3	CL1_IS	146.2	
	2	Caprylic	CL1	143.2	CL1_IS
	3	Capric-d3	CAP_IS	174.2	
	4	Capric	CAP	171.2	CAP_IS
	5	Lauric-d3	LAR_IS	202.3	
	6	Lauric	LAR	199.3	LAR_IS
Segment 2	7	Myristic-d27	MR1_IS	254.5	
	8	Myristic	MR1	227.4	MR1_IS
	9	Myristoleic	ML1	225.3	MR1_IS
	10	Pentadecanoic-d3	PDE_IS	244.3	
	11	Pentadecanoic	PDE	241.3	PDE_IS
	12	Palmitic-d31	PM1_IS	286.6	
	13	Palmitic	PM1	255.45	PM1_IS
	14	Palmitoleic-d14	PL1_IS	267.4	
	15	Palmitoleic	PL1	253.45	PL1_IS
segment 3	16	Margaric-d3	MRG_IS	272.3	
	17	Margaric	MRG	269.3	MRG_IS
	18	Stearic-d35	ST1_IS	318.6	
	19	Stearic	ST1	283.4	ST1_IS
	20	13C-Oleic	OL1_IS	299.5	
	21	Oleic	OL1	281.5	OL1_IS
	22	*cis*-Vaccenic	VC1	281.5	OL1_IS
Segment 4	23	13C- Linoleic	LNA_IS	297.5	
	24	Linoleic	LNA	279.4	LNA_IS
	25	*alpha*-linolenic-d14	ALN_IS	291.4	
	26	*gamma*-Linolenic	GLA	277.4	ALN_IS
	27	*alpha*-Linolenic	ALN	277.4	ALN_IS
	28	Arachidic-d39	AR1_IS	350.3	
	29	Stearidonic	SD1	275.1	AR1_IS
	30	Arachidic	AR1	311.4	AR1_IS
Segment 5	31	11-Eicosenoic	EN1	309.4	AR1_IS
	32	5Z, 8Z, 11Z-Eicosatrienoic	ET1	305.3	AR1_IS
	33	11,14-Eicosadienoic	ED1	307.4	AR1_IS
	34	*homo-gamma*-Linolenic	HGL	305.4	ALN_IS
	35	Arachidonic-d8	ARA_IS	311.4	
	36	Arachidonic	ARA	303.4	ARA_IS
Segment 6	37	Docosanoic-d4	DA1_IS	343.5	
	38	Docosanoic	DA1	339.4	DA1_IS
	39	Docosenoic	DE1	337.4	DA1_IS
	40	Tricosanoic	TSA	353.4	DA1_IS
	41	13,16-Docosadienoic	DD1	335.3	DA1_IS
	42	Eicosapentaenoic-d5	EPA_IS	306.4	
	43	Eicosapentaenoic	EPA	301.4	EPA_IS
Segment 7	44	Docosahexaenoic-d5	DHA_IS	332.4	
	45	Docosatetraenoic	DTA	331.4	DHA_IS
	46	Docosapentaenoic n-6	DP6	329.4	DHA_IS
	47	13, 16, 19 Docosatrienoic	DT1	333.3	DHA_IS
	48	Docosapentaenoic n-3	DP3	329.4	DHA_IS
	49	Docosahexaenoic	DHA	327.4	DHA_IS
	50	Tetracosanoic-d4	LG1_IS	371.5	
	51	Tetracosanoic	LG1	367.4	LG1_IS
	52	Nervonic	NR1	365.4	LG1_IS
	53	Hexacosanoic	HSA	395.4	LG1_IS

G. Processing and reporting a run

1) The Agilent Chemstation 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.
2) For a detailed step-by-step description, see **4028_SOP Chromatography Review of Fatty Acids Results** for instructions to review chromatography. See **4028_SOP Analyst (or Project Lead) Review of Fatty Acids Results** for review of the data in the LIMS database.
   (a) Reviewing the chromatography
      – When the batch run is finished acquiring the data, the data is reviewed in Chemstation. Chromatograms for each fatty acid and stable isotope labeled standard are checked for retention times, peak shapes, peak separation, intensity and/or potential interferences.
   (b) Uploading the data file into LIMS
      – After review of the chromatography, the instrument data files is uploaded into a LIMS database for further review
   (c) Reviewing in LIMS database and reporting data
      – In the LIMS database the following is verified: good stable isotope labeled standards recovery, excellent calibration curves, low background in the blank, quality control materials are within characterized mean and SD, and any sample repeats are confirmed

Calculations

The Chemstation 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 concentrations in unknown samples are calculated using the regression parameters. Calculations are based on the single analysis of five (Std2 – Std6) standard concentrations according to the following formula: Concentration = Response factor (amount/area ratio) x peak area ratio x multiplier (dilution factor). Note: Twice a year calculations are based on the single analysis of six (Std1 – Std6) standard concentrations to fulfill CDC requirements for expanded calibration. The following table lists calibration curve type and weighting associated with each analyte:

	Fatty Acid Name	Analyte Code	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	Capric	CAP	quadratic	$1/x^2$
5	Caprylic	CL1	linear	$1/x$
6	Docosanoic	DA1	linear	$1/x^2$
7	13, 16 Docosadienoic	DD1	linear	$1/x^2$
8	Docosenoic	DE1	quadratic	$1/x$
9	Docosahexaenoic	DHA	linear	$1/x^2$
10	Docosapentaenoic n-3	DP3	linear	$1/x^2$
11	Docosapentaenoic n-6	DP6	linear	$1/x^2$
12	13, 16, 19 Docosatrienoic	DT1	quadratic	$1/x^2$
13	Docosatetraenoic	DTA	quadratic	$1/x^2$
14	11-14, Eicosadienoic	ED1	quadratic	$1/x^2$
15	11-Eicosenoic	EN1	quadratic	$1/x^2$
16	Eicosapentaenoic	EPA	quadratic	$1/x^2$
17	5Z, 8Z, 11Z-Eicosatrienoic	ET1	quadratic	$1/x^2$
18	*gamma*-Linolenic	GLA	linear	$1/x^2$
19	*homo-gamma*-Linolenic	HGL	quadratic	$1/x^2$
20	Hexacosanoic	HSA	linear	$1/x^2$
21	Lauric	LAR	quadratic	$1/x^2$

22	Tetracosanoic	LG1	linear	$1/x^2$
23	Linoleic	LNA	linear	$1/x^2$
24	Myristoleic	ML1	quadratic	$1/x^2$
25	Myristic	MR1	quadratic	$1/x^2$
26	Margaric	MRG	quadratic	1/x
27	Nervonic	NR1	quadratic	$1/x^2$
28	Oleic	OL1	linear	$1/x^2$
29	Pentadecanoic	PDE	linear	$1/x^2$
30	Palmitoleic	PL1	quadratic	$1/x^2$
31	Palmitic	PM1	quadratic	$1/x^2$
32	Stearidonic	SD1	quadratic	1/x
33	Stearic	ST1	quadratic	$1/x^2$
34	Tricosanoic	TSA	linear	$1/x^2$
35	*cis*-Vaccenic	VC1	quadratic	$1/x^2$

H. System Maintenance (other than daily maintenance)

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

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

(3) 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.

I. CDC Modifications

This document represents the second official method for the CDC lab for measuring 35 Fatty Acids.

9. **Reportable Range of Results**

The reportable range of results for each fatty acid is between the lowest and highest standards whose approximate values are shown in the table below:

	Fatty Acid	Code	Lowest Standard (uM)	Highest Standard (uM)
1	*alpha*-Linolenic	ALN	2.4	600
2	Arachidic	AR1	0.4	100
3	Arachidonic	ARA	10	2500
4	Capric	CAP	0.2	50
5	Caprylic	CL1	1	100
6	Docosanoic	DA1	0.8	200
7	13,16- Docosadienoic	DD1	0.2	50
8	Docosenoic	DE1	0.2	50
9	Docosahexaenoic	DHA	3	750
10	Docosapentaenoic n-3	DP3	1	250
11	Docosapentaenoic n-6	DP6	0.6	150
12	13,16,19-Docosatrienoic	DT1	0.2	50
13	Docosatetraenoic	DTA	0.6	150
14	11,14-Eicosadienoic	ED1	0.4	100
15	11-Eicosenoic	EN1	0.4	100
16	Eicosapentaenoic	EPA	1.6	400
17	5Z, 8Z, 11Z-Eicosatrienoic acid	ET1	0.4	100
18	*gamma*-Linolenic	GLA	1.2	300
19	*homo-gamma*-Linolenic	HGL	2	500
20	Hexacosanoic	HSA	0.2	50

21	Lauric	LAR	0.4	100
22	Tetracosanoic	LG1	0.6	150
23	Linoleic	LNA	40	10000
24	Myristoleic	ML1	0.6	150
25	Myristic	MR1	4	1000
26	Margaric	MRG	0.5	50
27	Nervonic	NR1	0.8	200
28	Oleic	OL1	40	10000
29	Pentadecanoic	PDE	0.2	50
30	Palmitoleic	PL1	10	2500
31	Palmitic	PM1	40	10000
32	Stearidonic	SD1	0.5	50
33	Stearic	ST1	12	3000
34	Tricosanoic	TSA	0.2	50
35	*cis*-Vaccenic	VC1	3.2	800

Fatty acids in grey text (CL1, DD1, DE1, DT1, and HSA) are being monitored but not reported.

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 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.

B. Bench Quality Controls

Three bench QC pools are prepared by blending units of serum or plasma to achieve low, intermediate, and high levels of key fatty acids (Palmitic, Oleic, Linoleic, Stearic and Arachidonic). QC pools are aliquoted into 200-300 2-mL vials and stored at -70C. 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 (pre- and post- unknowns).

The results from the pools are checked after each run. The system is declared “in control” if the results pass the following tests:
Multi-rule quality control system: quality control rules for three QC pools per run

1) If all three QC run means are within $2S_m$ limits and individual results are within $2S_i$ limits, accept the run
2) If 1 of the 3 QC run means is outside a $2S_m$ limit – reject run if:
   a. 1_{3s}: Any of the three QC results are outside the 3S limit
   b. 2_{2s}: Two of the three QC results in the run are outside the 2S limit (same side of mean)
   c. 10_x: Ten sequential QC results (across pools and across runs) are on the same side of the mean.
3) If one of the six QC individual results is outside a 2 S_i limit – reject run if:

a. Outlier – One individual result is beyond the characterization mean $\pm$ 4 S_i or

b. R_{4s}: Sequential QC results (either within the run or across runs) are outside the 2S limit on the opposite sides of the mean

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 (9). 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 using bench QC. The initial limits are established by analyzing QC pool material in 20 consecutive runs and then are reevaluated as needed. When necessary, limits are updated to include more runs. QC results are stored in the LIMS database.

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

A. 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.

B. Look for sample preparation errors, e.g., analyst forgot to add isotope-labeled standard or derivitizing agent.

C. Check the calibration of the pipettes.

D. If the steps outlined above do not result in correction of the "out of control" values for QC materials, consult the supervisor for other appropriate corrective actions.

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

12. **Limitations of Method; Interfering Substances and Conditions**

A. Due to the complexity of this assay, not all analytes being monitored are found in the quality control pools. At this time 5 fatty acids (Caprylic, Docosadienoic, Docosenoic, Docosatrienoic, and Hexacosanoic) are non-reportable because we lack QC materials with concentrations greater than the LOD for them. We continue to collect data for non-reportable fatty acids in order to assess whether some individuals have measurable concentrations, but will not report any analyte that is not in the quality control pools.
B. The most common causes of imprecision are intermittently inaccurate micropipettors and pipetting errors.
C. Stock standards, stable isotope labeled standards and specimens should be mixed thoroughly by vortexing before pipetting.
D. Handling stocks and internal standards in step-wise sequential manner will minimize the chances of cross-contaminations.
E. Also changing of gloves after preparations of stock and working standards and internal standards is recommended to avoid any contamination.

F. 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 B.** Please refer to Chapter 21 of the 2012 *DLS Policies and Procedures Manual* for further information on ruggedness testing.

13. **Reference Ranges (Normal Values)**

The reference ranges in fasted, noninstitutionalized civilians aged ≥20y from the cross-sectional 2003–2004 National Health and Nutrition Examination Survey for 24 of the 35 fatty acids analyzed in this method (data for new fatty acids are not yet available).

Analyte Code	Geometric mean (uM)	5th (uM)	50th (uM)	95th (uM)	Sample Size
ALN	63.1	30	61.4	137	1,801
AR1	23.4	16.2	23.2	33.6	1,757
ARA	776	484	789	1,180	1,807
DA1	69.3	45.2	69.5	102	1,739
DE1	3.44	0.712	3.62	10.3	1,604
DHA	125	61.1	121	277	1,808
DP3	41.6	24.5	41.4	72.7	1,808
DP6	19.6	9.8	19.3	39	1,808
DTA	25	14.4	24.7	43	1,808
ED1	21.2	12.4	20.9	36.9	1,805
EN1	13.6	7.56	13.3	25.9	1,805
EPA	42.1	17.1	40.9	113	1,806
GLA	46.9	20.2	49	100	1,795
HGL	151	87.5	151	262	1,806
LG1	54	35.3	53.8	80.9	1,743
LNA	3,450	2,370	3,430	4,980	1,806
ML1	6.57	1.79	6.5	23.9	1,808
MR1	119	48.1	116	308	1,796
NR1	74.9	49.8	75	116	1,696
OL1	2,100	1,220	2,070	3,850	1,798
PL1	217	84	213	563	1,805
PM1	2,710	1,690	2,630	4,710	1,805
ST1	692	471	684	1,040	1,806
VC1	146	83.6	143	262	1,762

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 the -70°C freezer 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 at -20°C or -70°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 fatty acids 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 at -70°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 that 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.

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

A LIMS 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 serum or plasma from these analyses for non-NHANES studies may be discarded at the request of the principal investigator, or may be transferred to the CDC CASPIR facility for use by other investigators with the permission of the principal investigator. Very little residual material will be available after NHANES analyses are completed, and these vials may be routinely autoclaved.

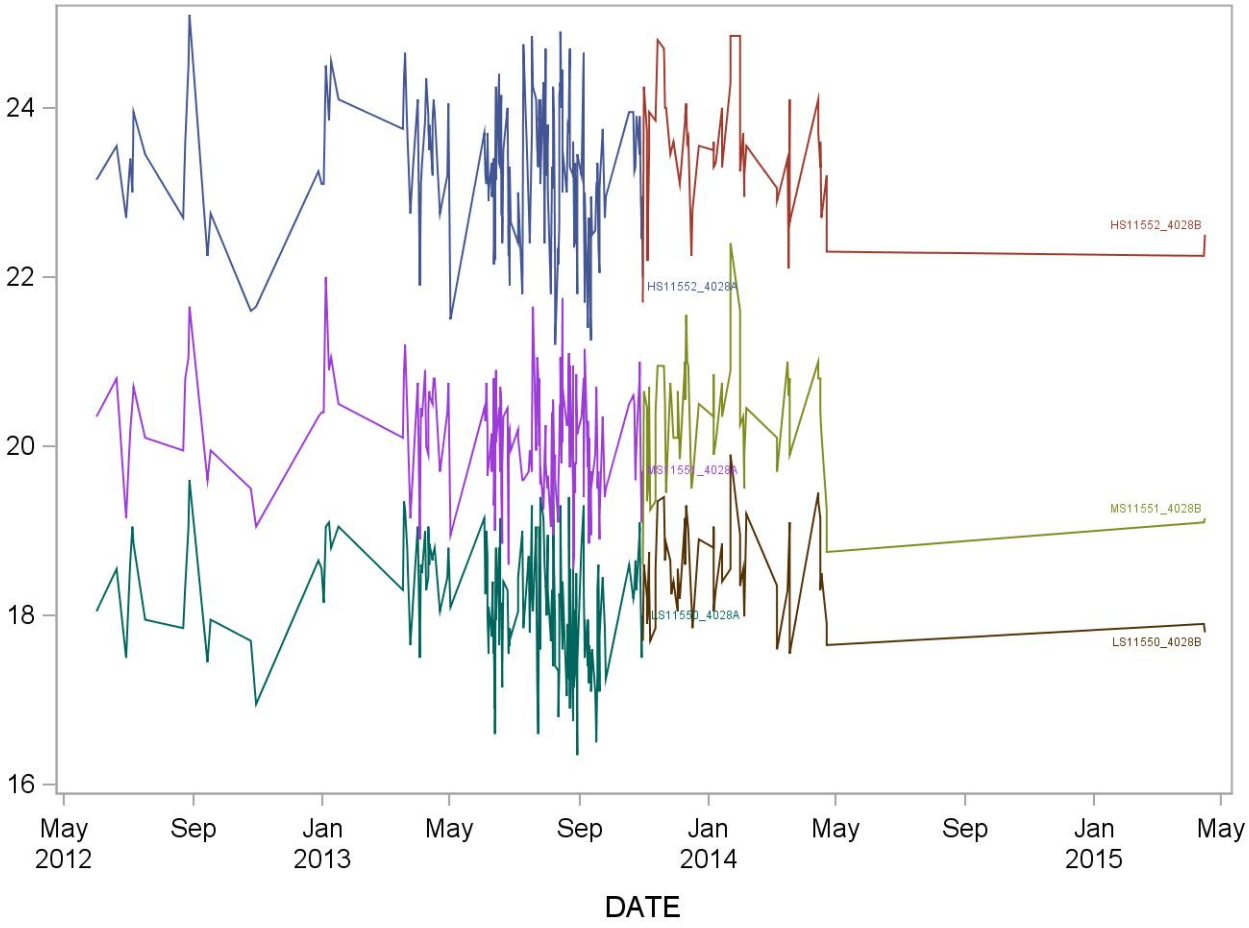
The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the specimens are stored in a freezer at -70°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. **Summary Statistics and QC Graphs**

Please see following pages

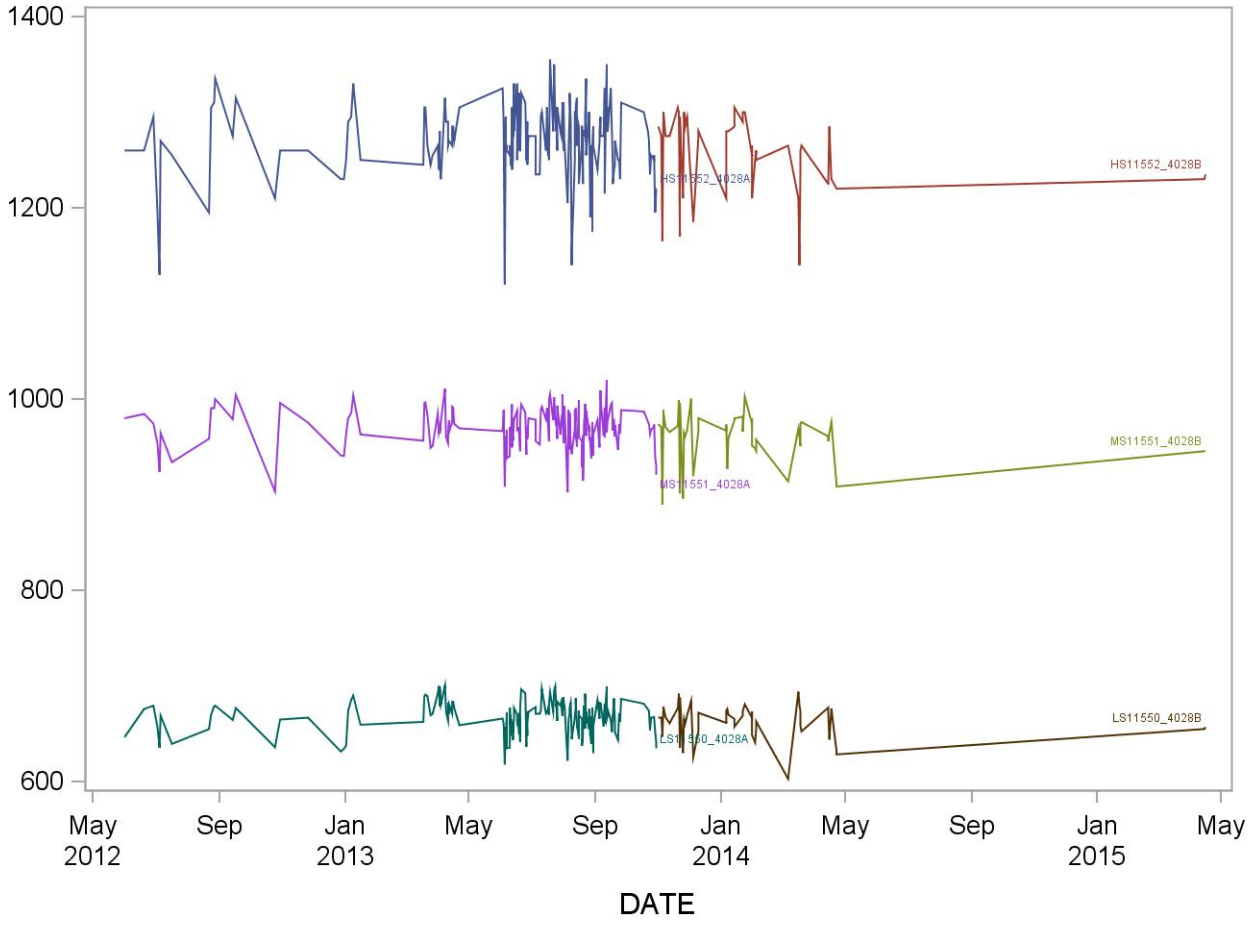
2011-2012 Summary Statistics and QC Chart for Arachidic acid (20:0)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	173	01JUN12	31OCT13	23.244	0.806	3.5
LS11550_4028A	173	01JUN12	31OCT13	18.150	0.699	3.9
MS11551_4028A	173	01JUN12	31OCT13	20.105	0.651	3.2
HS11552_4028B	54	31OCT13	16APR15	23.416	0.729	3.1
LS11550_4028B	58	31OCT13	16APR15	18.501	0.548	3.0
MS11551_4028B	58	31OCT13	16APR15	20.278	0.729	3.6



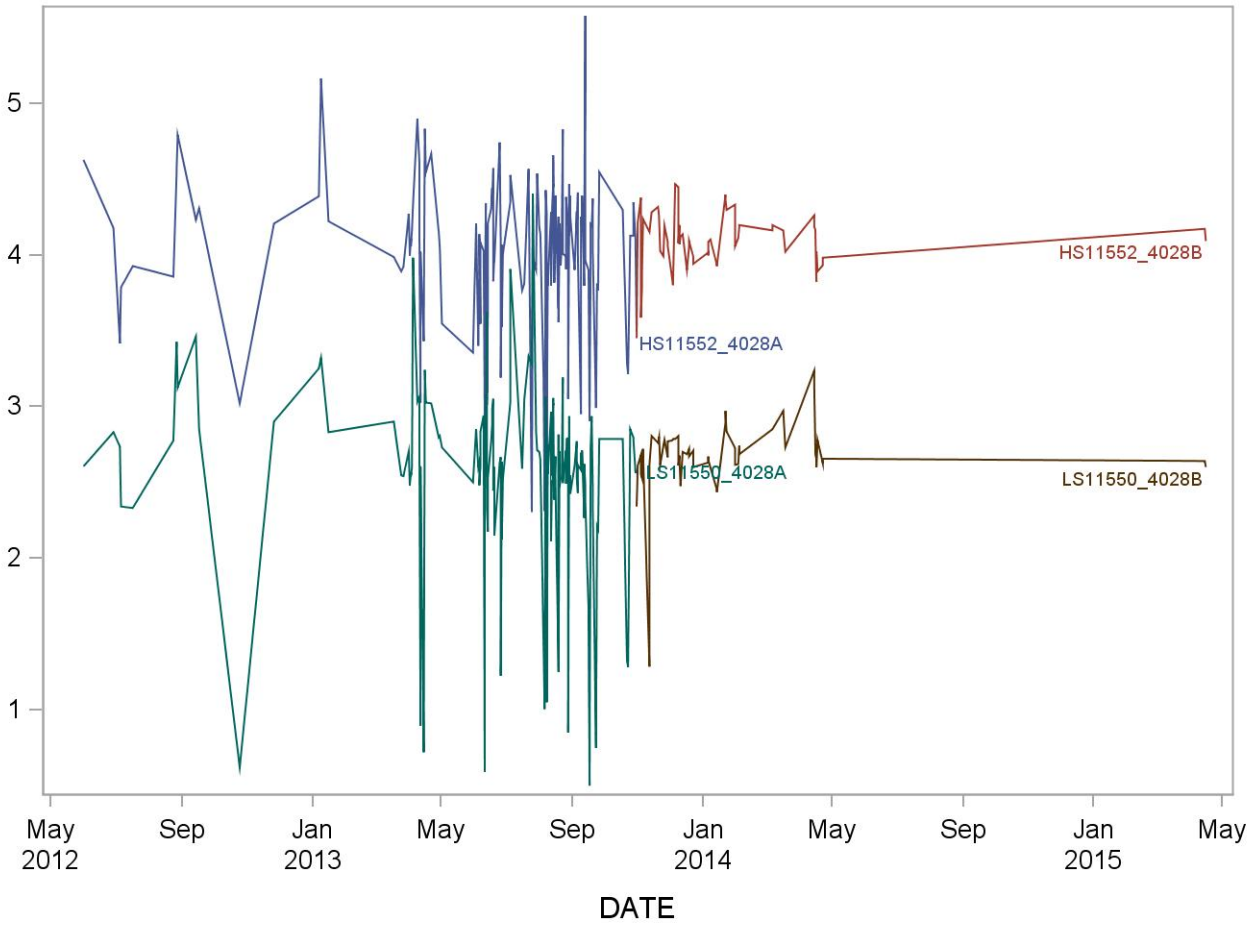
2011-2012 Summary Statistics and QC Chart for Arachidonic acid (20:4n-6)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	181	01JUN12	30OCT13	1268.4	39.0	3.1
LS11550_4028A	181	01JUN12	30OCT13	667.1	16.7	2.5
MS11551_4028A	181	01JUN12	30OCT13	968.9	20.7	2.1
HS11552_4028B	49	01NOV13	16APR15	1253.8	42.1	3.4
LS11550_4028B	51	01NOV13	16APR15	662.6	18.3	2.8
MS11551_4028B	51	01NOV13	16APR15	959.4	28.2	2.9



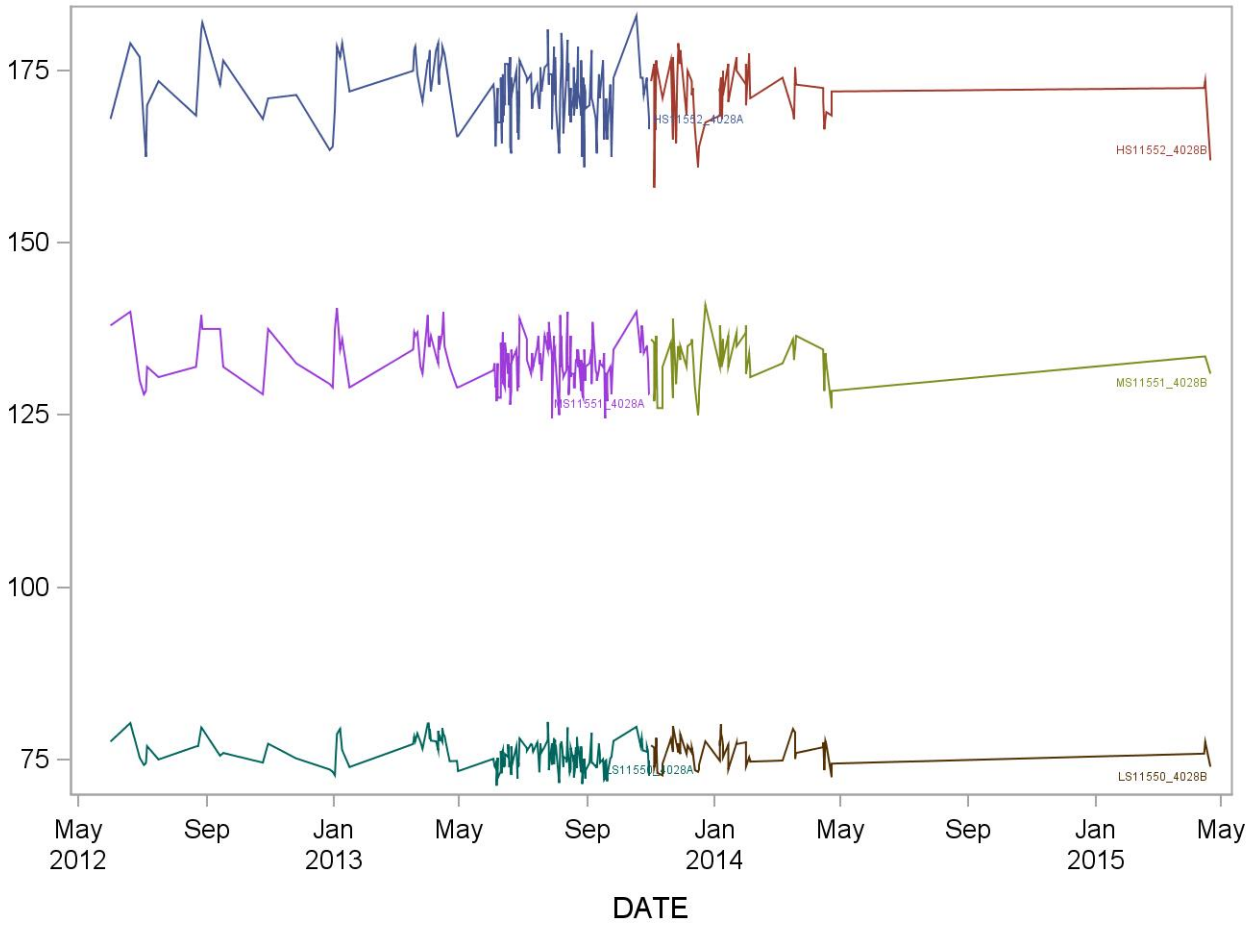
2011-2012 Summary Statistics and QC Chart for Capric acid (C10:0)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	151	01JUN12	31OCT13	4.018	0.504	12.5
LS11550_4028A	150	01JUN12	31OCT13	2.558	0.628	24.6
HS11552_4028B	58	31OCT13	16APR15	4.093	0.192	4.7
LS11550_4028B	60	31OCT13	16APR15	2.680	0.236	8.8



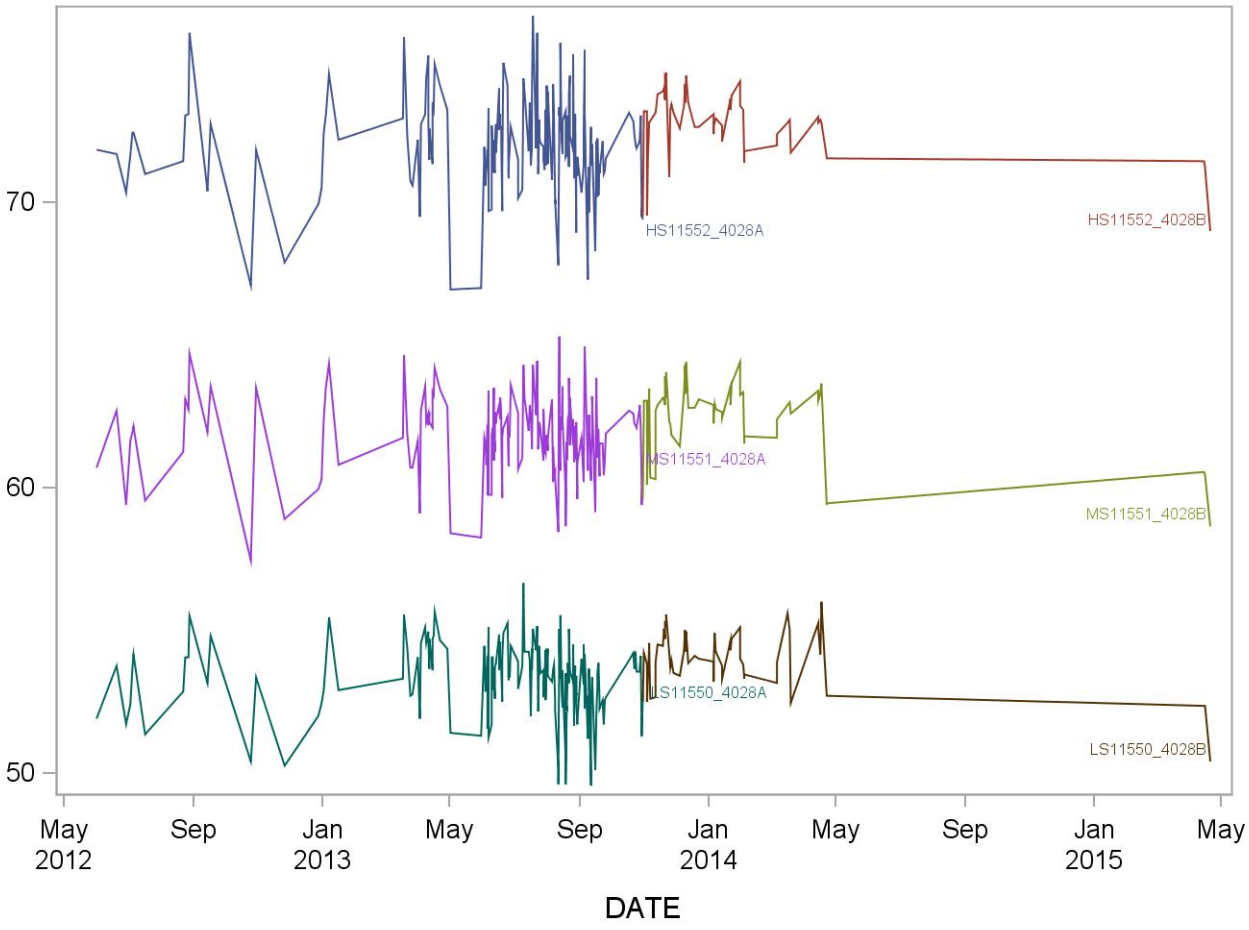
2011-2012 Summary Statistics and QC Chart for Docosahexaenoic acid (22:6n-3)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	175	01JUN12	30OCT13	171.934	4.583	2.7
LS11550_4028A	175	01JUN12	30OCT13	75.670	2.058	2.7
MS11551_4028A	175	01JUN12	30OCT13	132.723	3.432	2.6
HS11552_4028B	61	01NOV13	21APR15	171.418	4.580	2.7
LS11550_4028B	63	01NOV13	21APR15	76.178	1.881	2.5
MS11551_4028B	63	01NOV13	21APR15	133.024	3.527	2.7



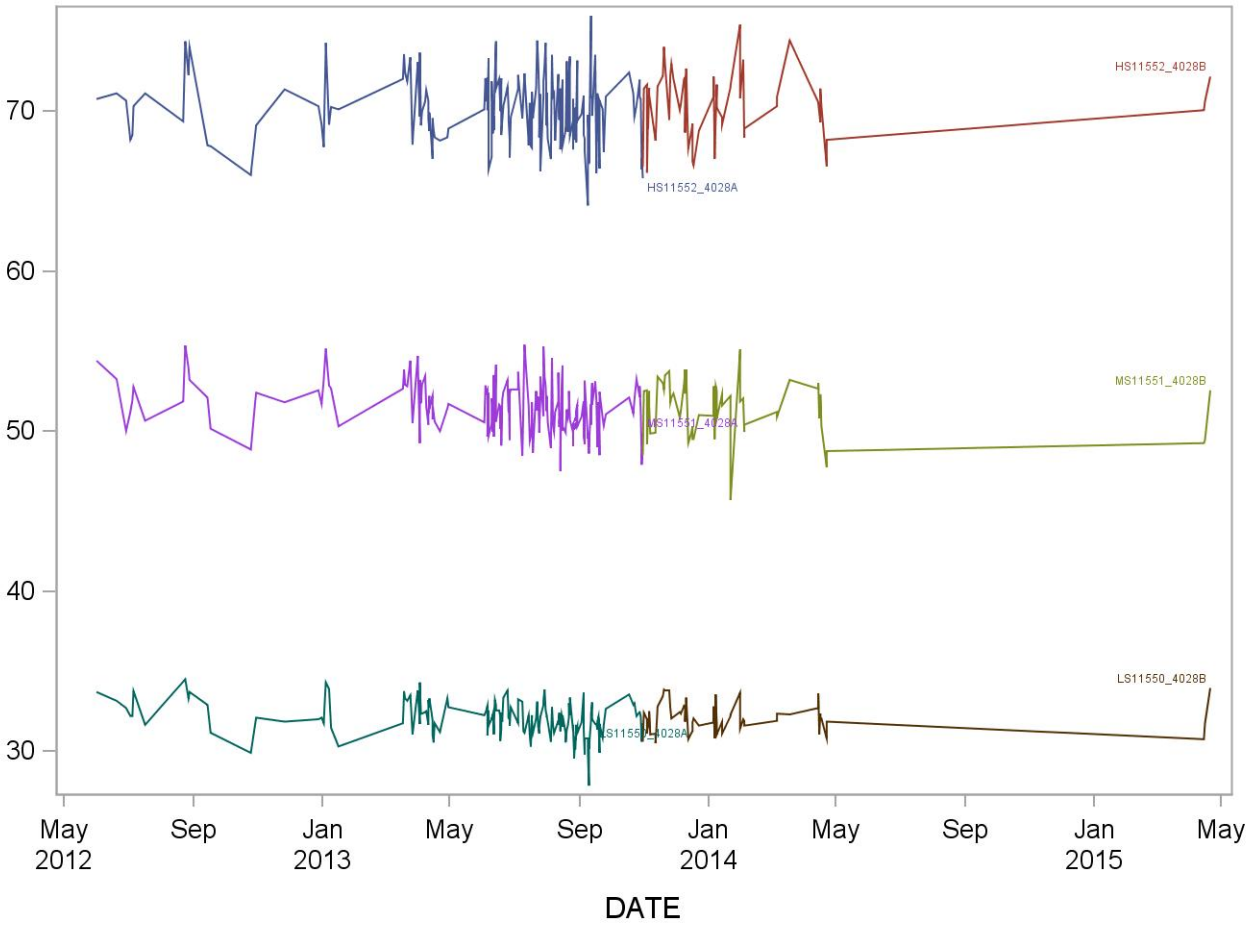
2011-2012 Summary Statistics and QC Chart for Docosanoic acid (22:0)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	179	01JUN12	31OCT13	71.95	1.73	2.4
LS11550_4028A	179	01JUN12	31OCT13	53.34	1.27	2.4
MS11551_4028A	179	01JUN12	31OCT13	61.80	1.41	2.3
HS11552_4028B	57	31OCT13	21APR15	72.74	1.18	1.6
LS11550_4028B	59	31OCT13	21APR15	53.96	1.02	1.9
MS11551_4028B	59	31OCT13	21APR15	62.44	1.31	2.1



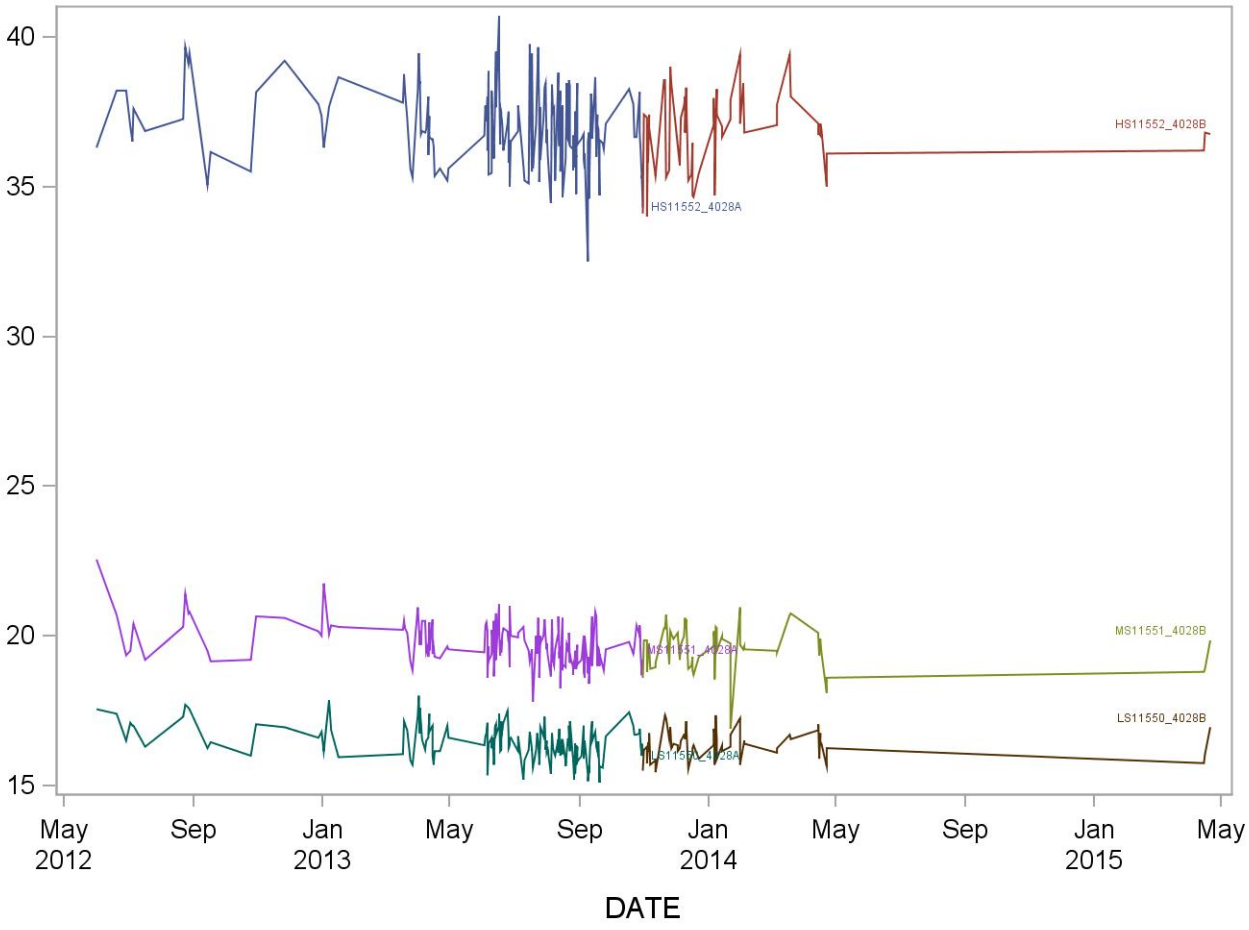
2011-2012 Summary Statistics and QC Chart for Docosapentaenoic acid (22:5n-3)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	173	01JUN12	31OCT13	70.044	2.069	3.0
LS11550_4028A	173	01JUN12	31OCT13	31.993	1.050	3.3
MS11551_4028A	173	01JUN12	31OCT13	51.417	1.593	3.1
HS11552_4028B	58	31OCT13	21APR15	70.372	2.052	2.9
LS11550_4028B	60	31OCT13	21APR15	32.077	0.900	2.8
MS11551_4028B	60	31OCT13	21APR15	51.333	1.719	3.3



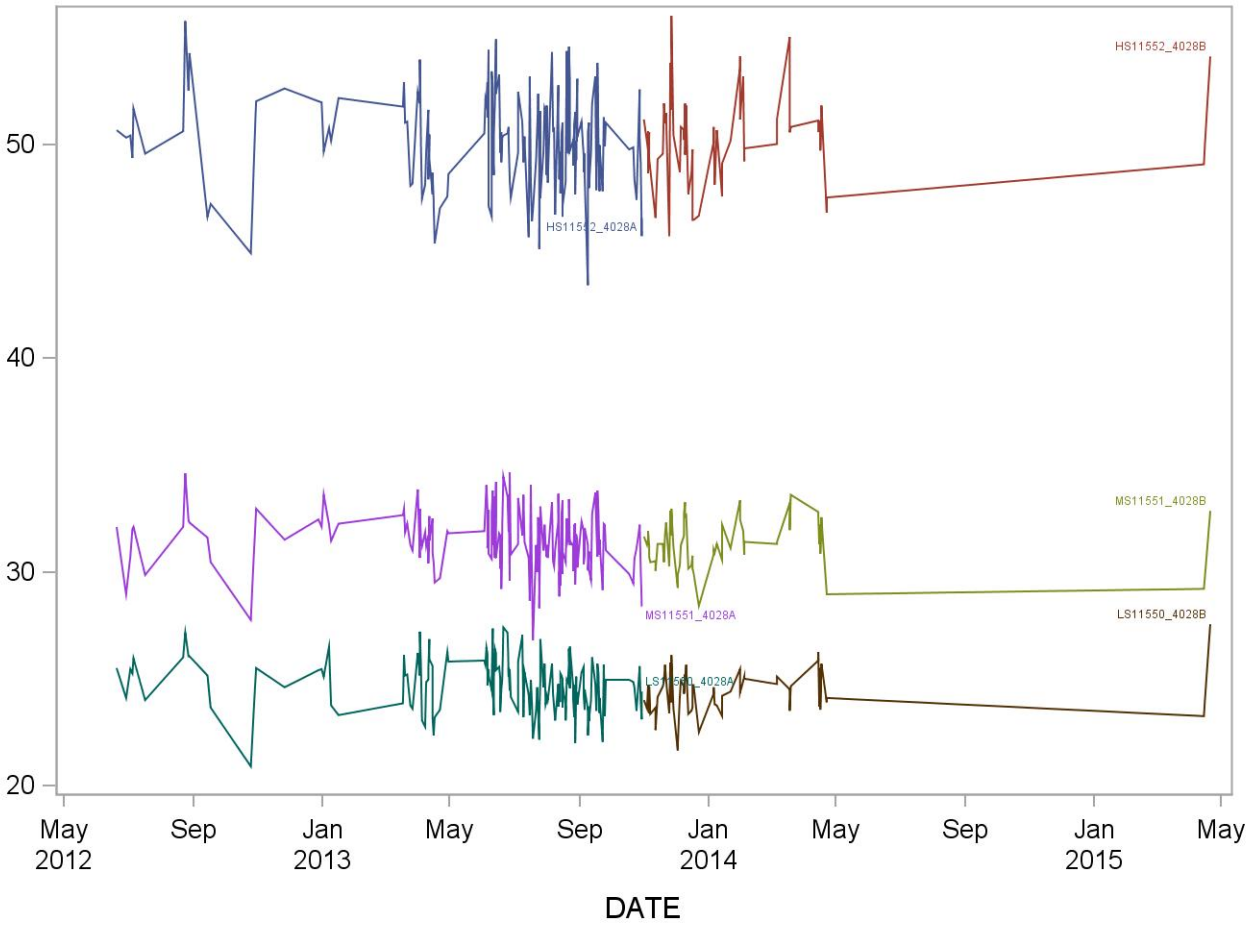
2011-2012 Summary Statistics and QC Chart for Docosapentaenoic acid (22:5n-6)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	170	01JUN12	31OCT13	36.907	1.303	3.5
LS11550_4028A	170	01JUN12	31OCT13	16.435	0.588	3.6
MS11551_4028A	170	01JUN12	31OCT13	19.696	0.701	3.6
HS11552_4028B	59	31OCT13	21APR15	36.865	1.256	3.4
LS11550_4028B	63	31OCT13	21APR15	16.375	0.473	2.9
MS11551_4028B	63	31OCT13	21APR15	19.598	0.706	3.6



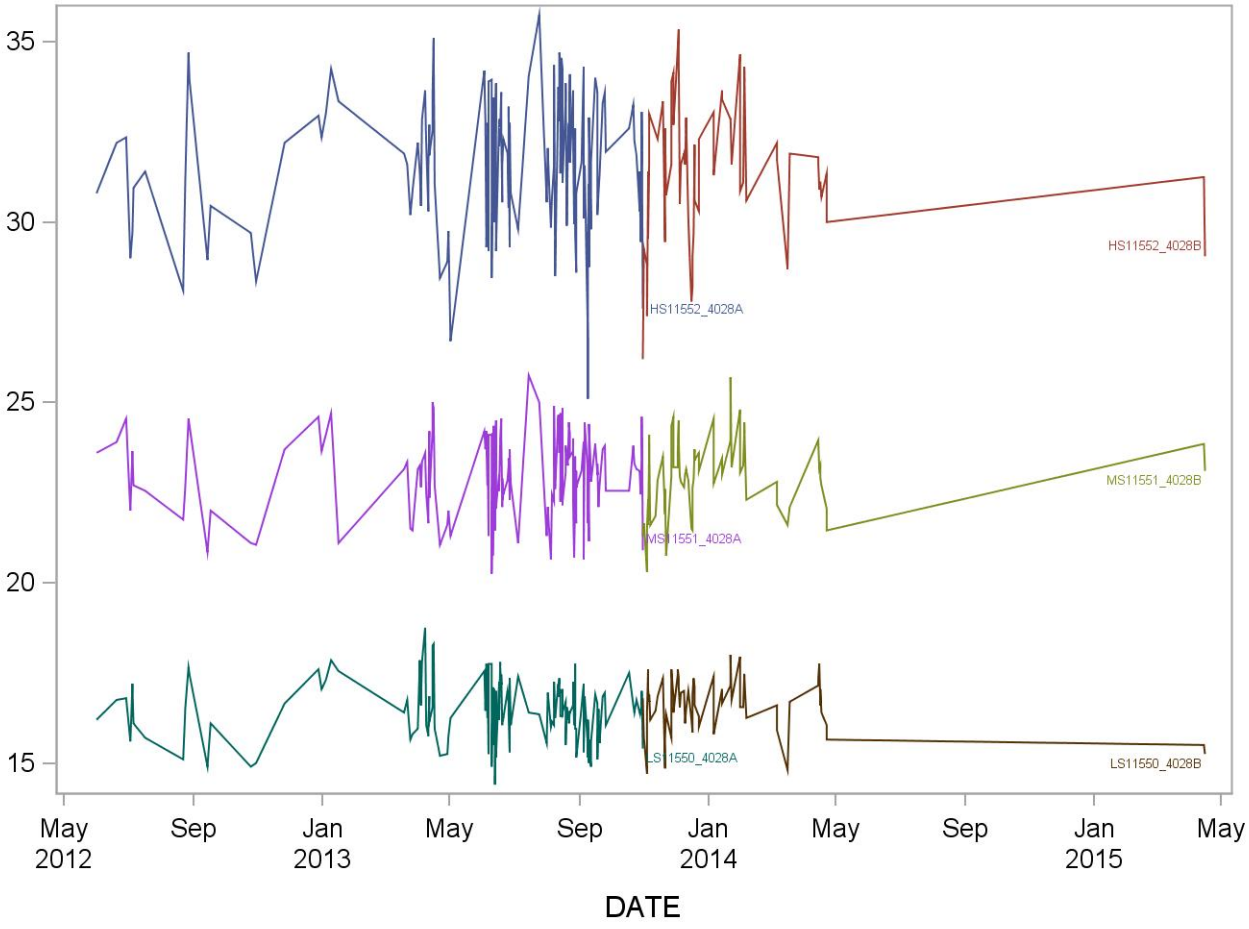
2011-2012 Summary Statistics and QC Chart for Docosatetraenoic acid (22:4n-6)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	167	20JUN12	30OCT13	49.976	2.228	4.5
LS11550_4028A	167	20JUN12	30OCT13	24.652	1.193	4.8
MS11551_4028A	167	20JUN12	30OCT13	31.450	1.428	4.5
HS11552_4028B	59	01NOV13	21APR15	50.239	2.145	4.3
LS11550_4028B	63	01NOV13	21APR15	24.371	1.039	4.3
MS11551_4028B	63	01NOV13	21APR15	31.317	1.206	3.9



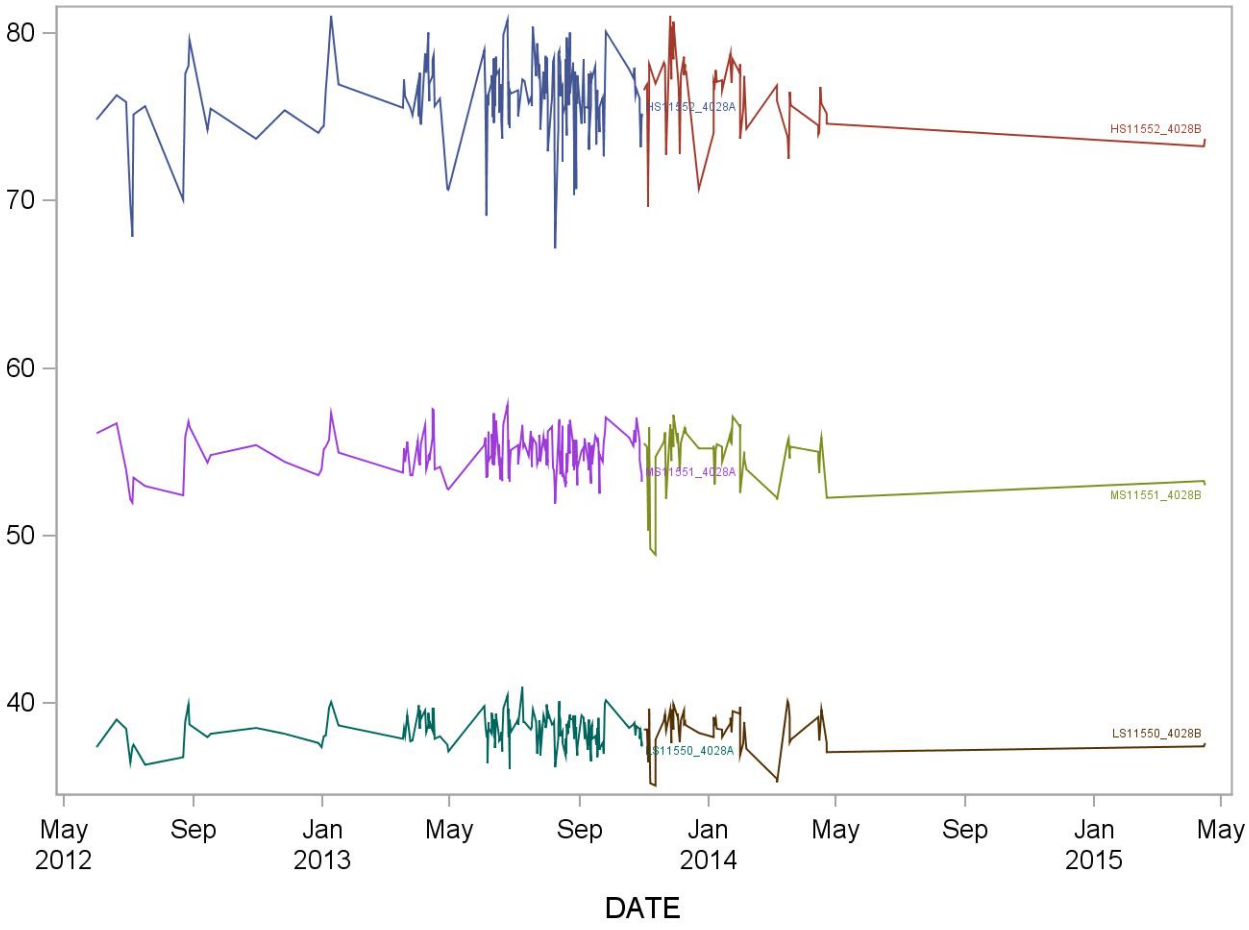
2011-2012 Summary Statistics and QC Chart for Eicosadienoic acid (20:2n-6)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	147	01JUN12	31OCT13	31.619	1.897	6.0
LS11550_4028A	147	01JUN12	31OCT13	16.442	0.831	5.1
MS11551_4028A	147	01JUN12	31OCT13	23.037	1.157	5.0
HS11552_4028B	60	31OCT13	16APR15	31.403	1.890	6.0
LS11550_4028B	64	31OCT13	16APR15	16.622	0.749	4.5
MS11551_4028B	64	31OCT13	16APR15	22.955	1.054	4.6



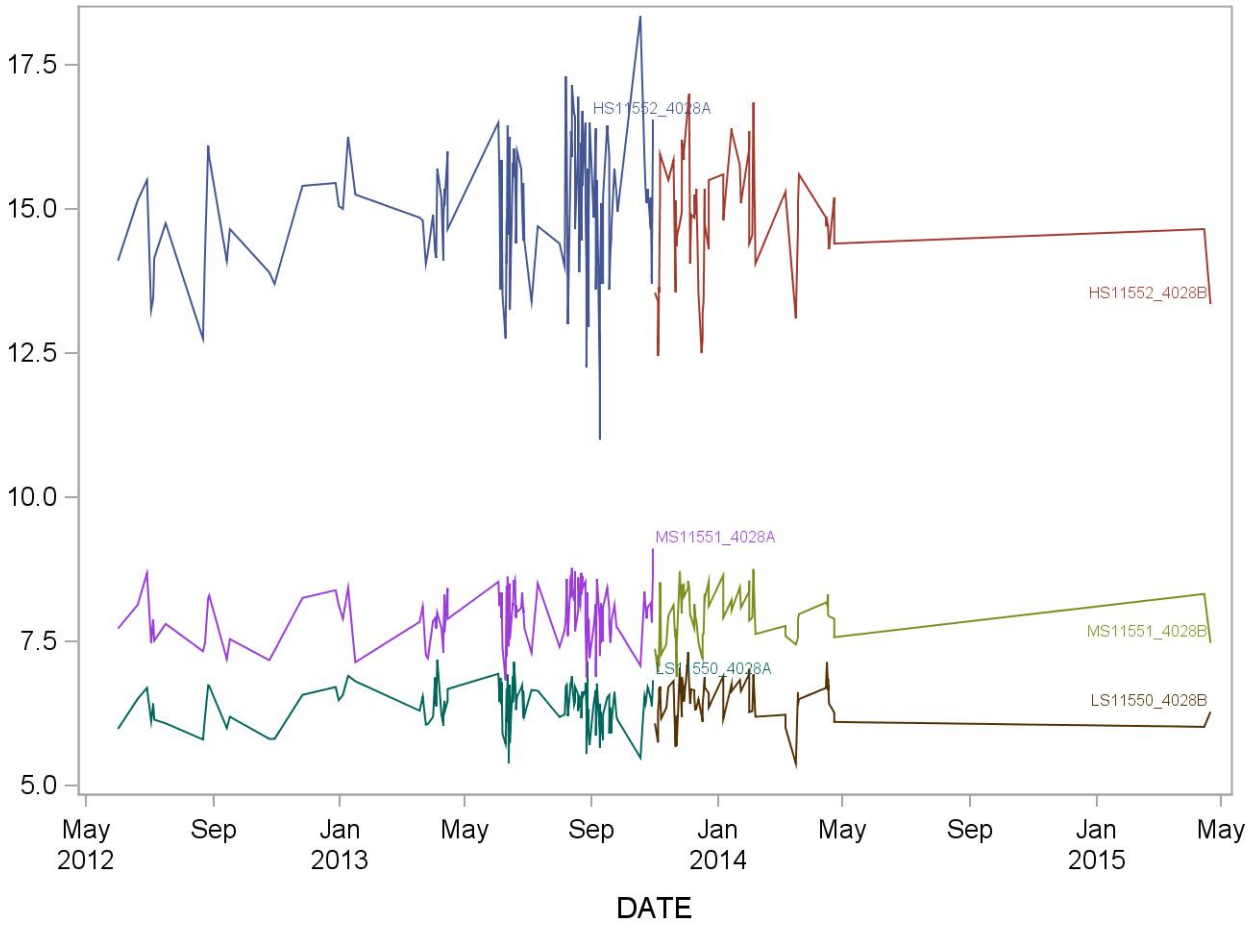
2011-2012 Summary Statistics and QC Chart for Eicosapentaenoic acid (20:5n-3)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	178	01JUN12	30OCT13	76.120	2.393	3.1
LS11550_4028A	178	01JUN12	30OCT13	38.388	0.897	2.3
MS11551_4028A	178	01JUN12	30OCT13	54.889	1.209	2.2
HS11552_4028B	59	01NOV13	16APR15	76.206	2.378	3.1
LS11550_4028B	63	01NOV13	16APR15	38.347	1.151	3.0
MS11551_4028B	63	01NOV13	16APR15	54.692	1.832	3.3



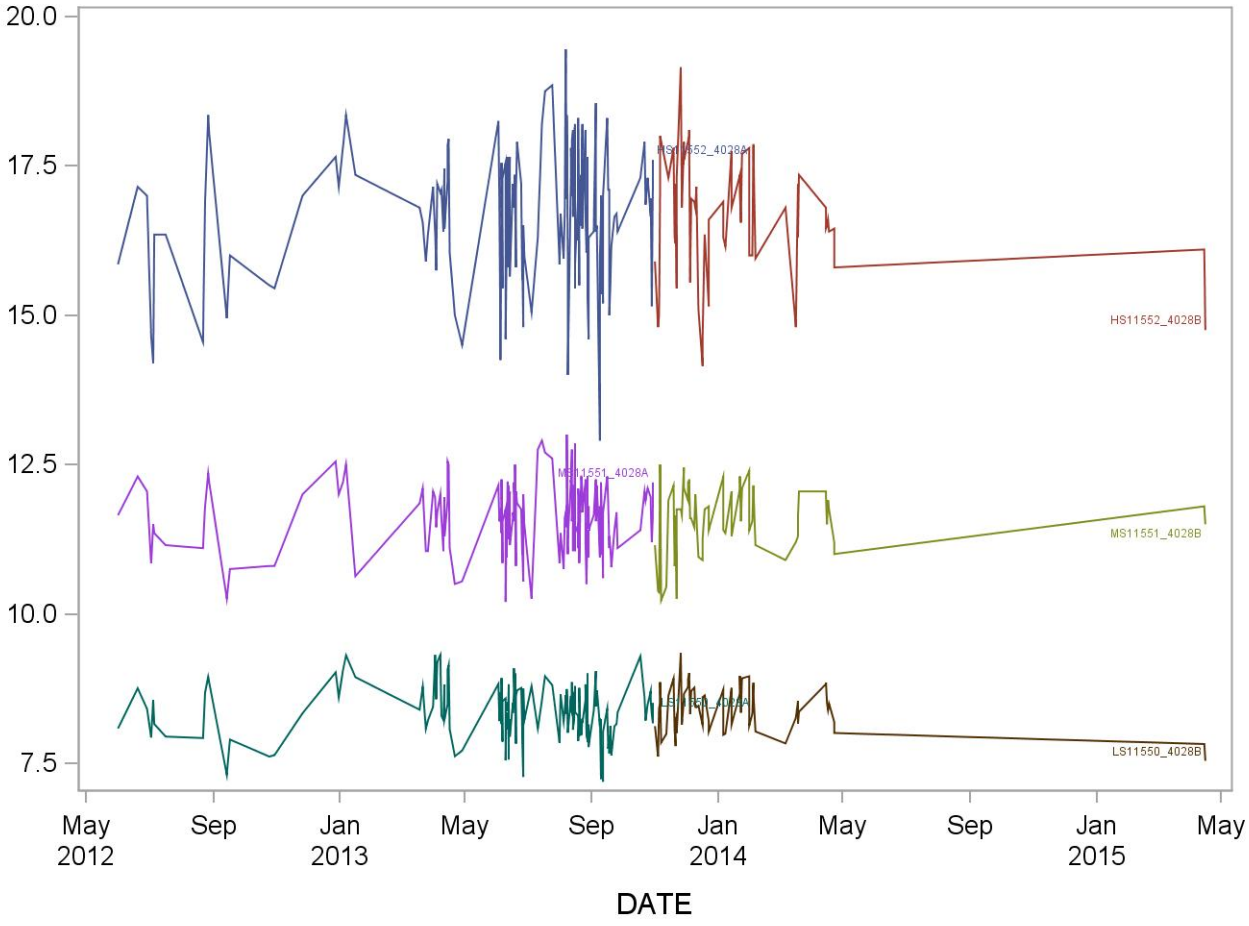
2011-2012 Summary Statistics and QC Chart for Eicosatrienoic acid (C20:3n-9)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	132	01JUN12	30OCT13	14.972	1.166	7.8
LS11550_4028A	131	01JUN12	30OCT13	6.401	0.377	5.9
MS11551_4028A	132	01JUN12	30OCT13	7.963	0.468	5.9
HS11552_4028B	64	01NOV13	21APR15	14.791	1.028	6.9
LS11550_4028B	68	01NOV13	21APR15	6.478	0.375	5.8
MS11551_4028B	68	01NOV13	21APR15	7.923	0.451	5.7



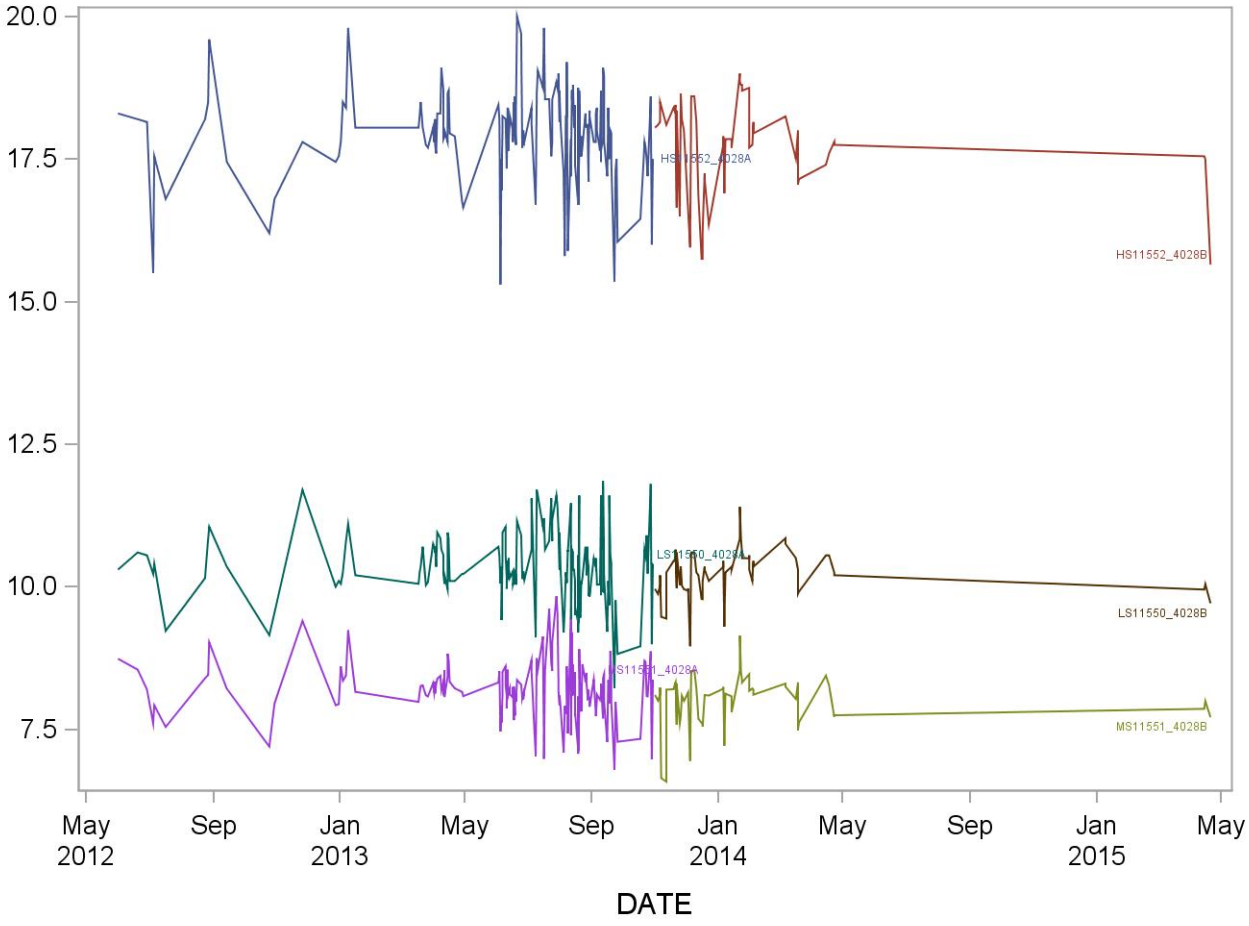
2011-2012 Summary Statistics and QC Chart for Eicosenoic acid (20:1n-9)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	151	01JUN12	30OCT13	16.654	1.103	6.6
LS11550_4028A	151	01JUN12	30OCT13	8.354	0.445	5.3
MS11551_4028A	151	01JUN12	30OCT13	11.623	0.597	5.1
HS11552_4028B	59	01NOV13	16APR15	16.614	1.020	6.1
LS11550_4028B	63	01NOV13	16APR15	8.418	0.391	4.6
MS11551_4028B	63	01NOV13	16APR15	11.602	0.571	4.9



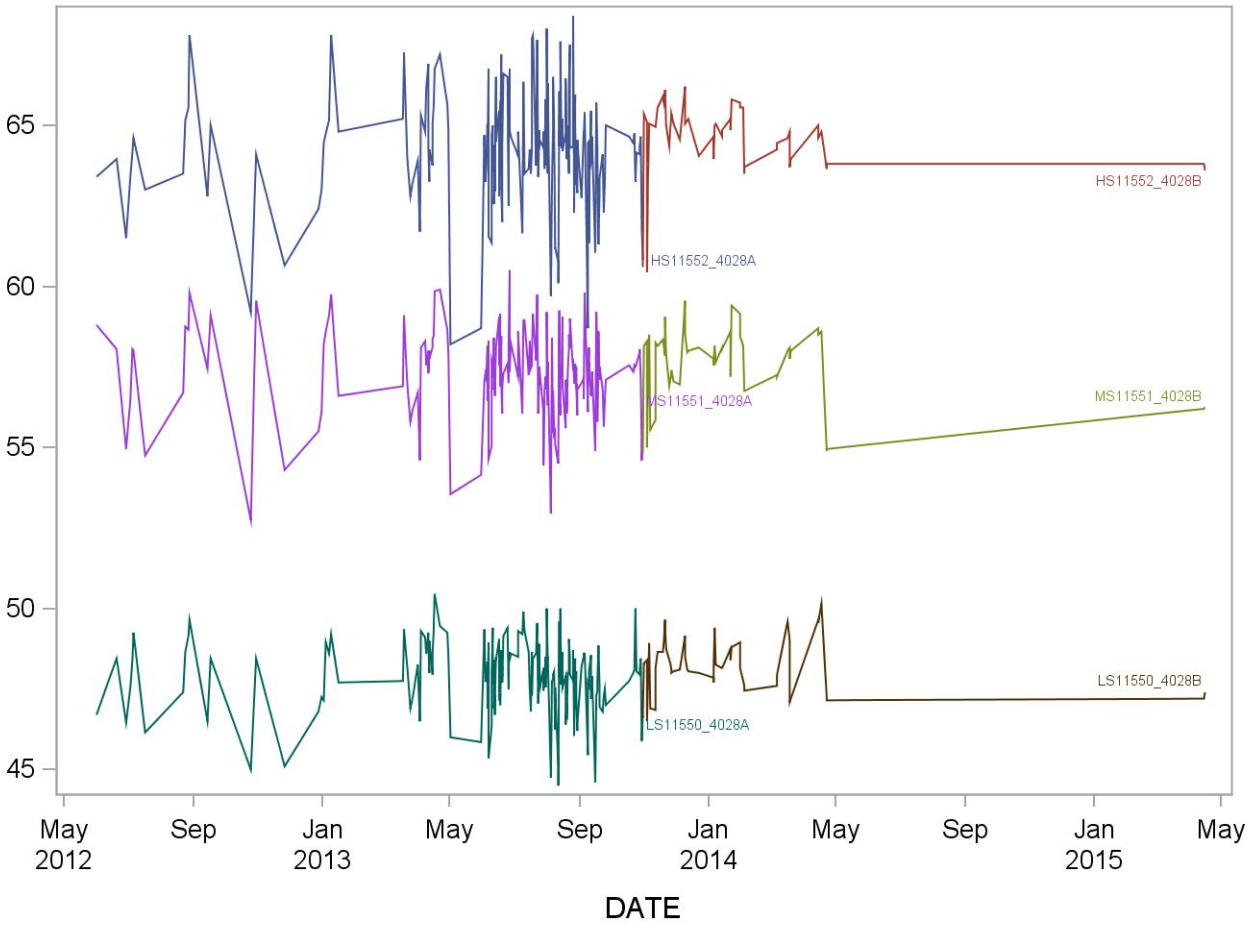
2011-2012 Summary Statistics and QC Chart for Lauric acid (C12:0)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	171	01JUN12	30OCT13	17.929	0.799	4.5
LS11550_4028A	171	01JUN12	30OCT13	10.362	0.616	5.9
MS11551_4028A	171	01JUN12	30OCT13	8.191	0.496	6.1
HS11552_4028B	56	01NOV13	21APR15	17.739	0.854	4.8
LS11550_4028B	60	01NOV13	21APR15	10.232	0.406	4.0
MS11551_4028B	60	01NOV13	21APR15	8.021	0.445	5.6



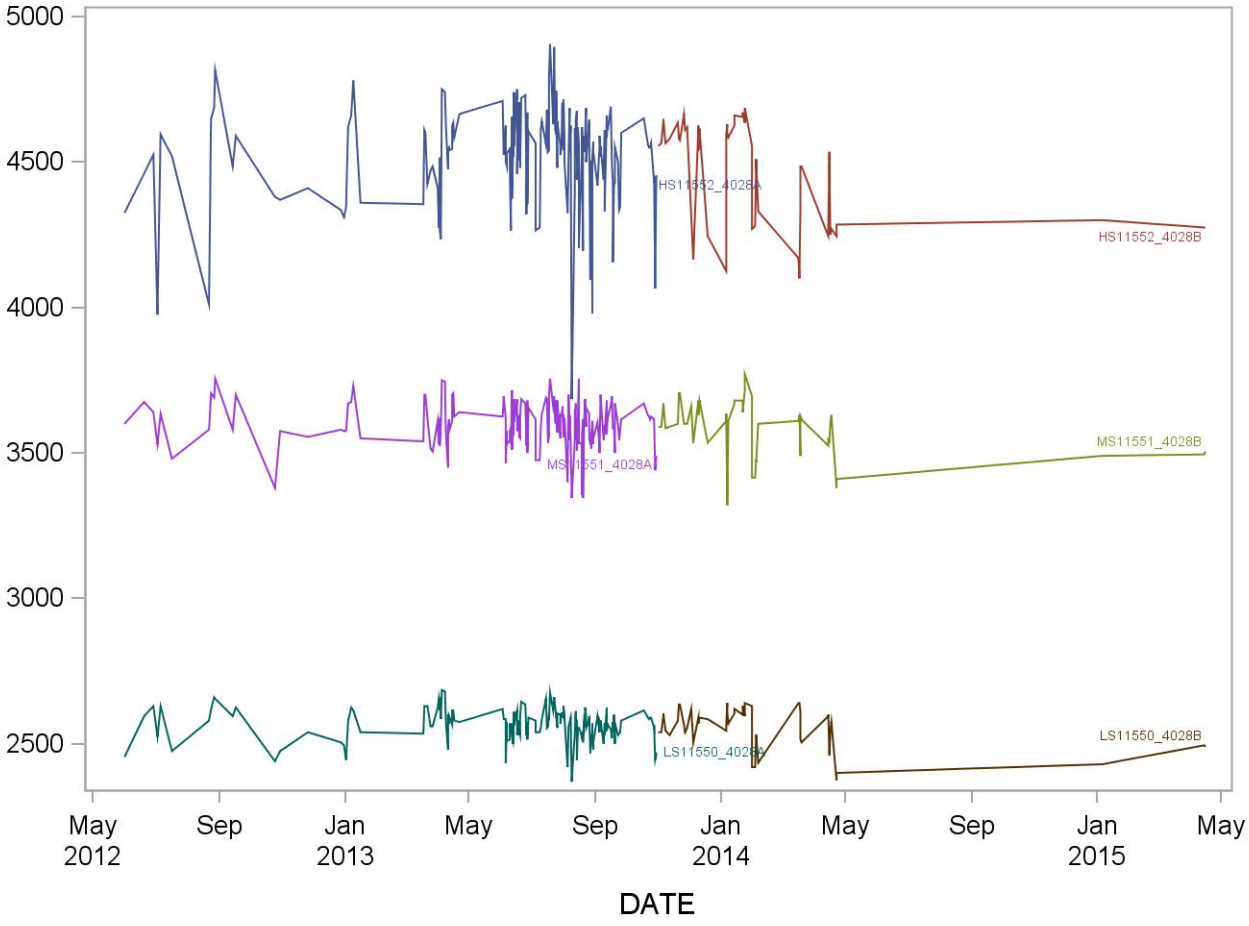
2011-2012 Summary Statistics and QC Chart for Lignoceric acid (24:0)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	181	01JUN12	31OCT13	64.176	1.848	2.9
LS11550_4028A	181	01JUN12	31OCT13	47.874	1.134	2.4
MS11551_4028A	181	01JUN12	31OCT13	57.254	1.369	2.4
HS11552_4028B	54	31OCT13	16APR15	64.637	1.087	1.7
LS11550_4028B	56	31OCT13	16APR15	48.225	0.822	1.7
MS11551_4028B	56	31OCT13	16APR15	57.588	1.103	1.9



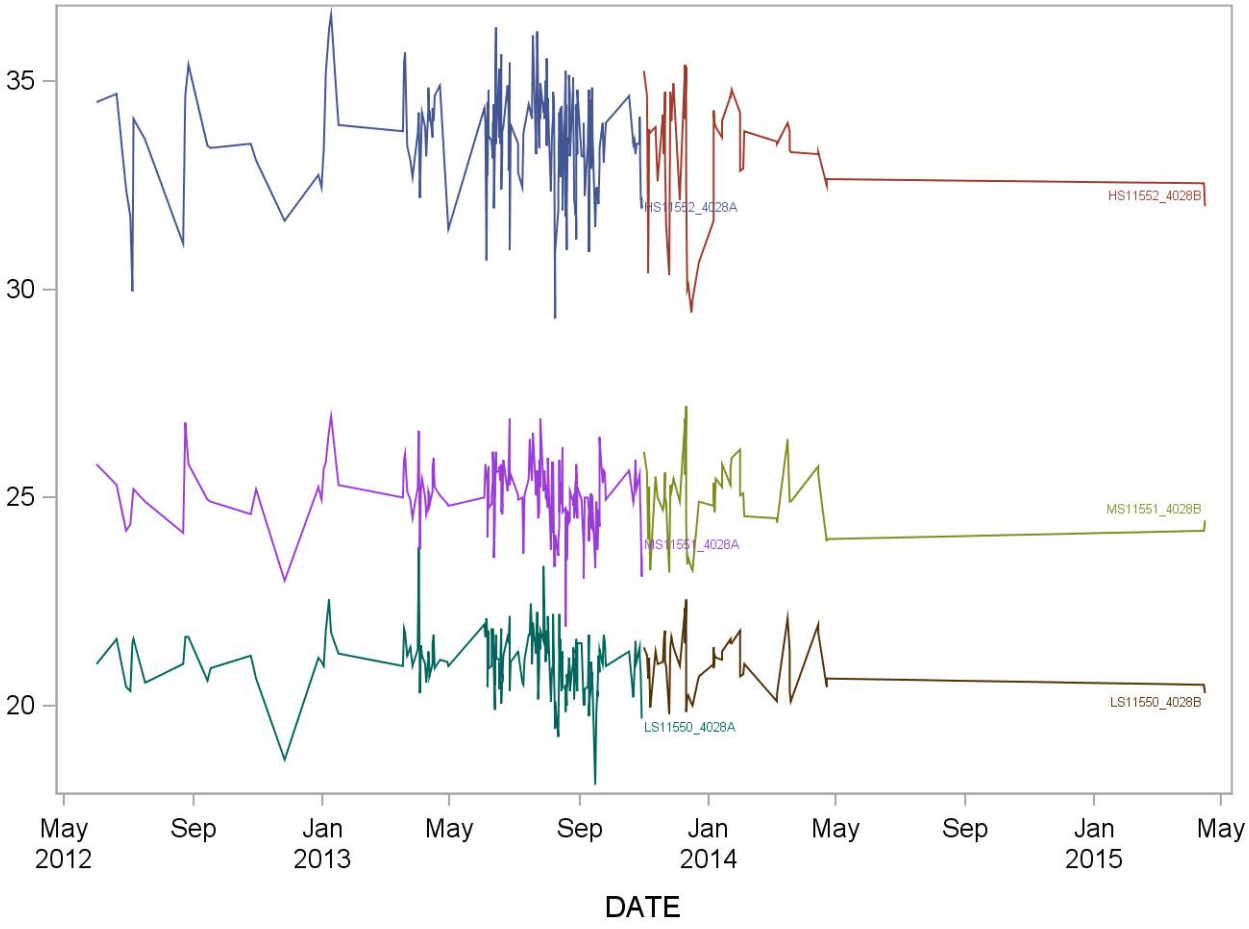
2011-2012 Summary Statistics and QC Chart for Linoleic acid (18:2n-6)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	181	01JUN12	30OCT13	4505.7	185.9	4.1
LS11550_4028A	181	01JUN12	30OCT13	2562.3	55.3	2.2
MS11551_4028A	181	01JUN12	30OCT13	3595.4	79.7	2.2
HS11552_4028B	51	01NOV13	16APR15	4461.3	181.5	4.1
LS11550_4028B	52	01NOV13	16APR15	2556.7	68.3	2.7
MS11551_4028B	52	01NOV13	16APR15	3583.1	99.3	2.8



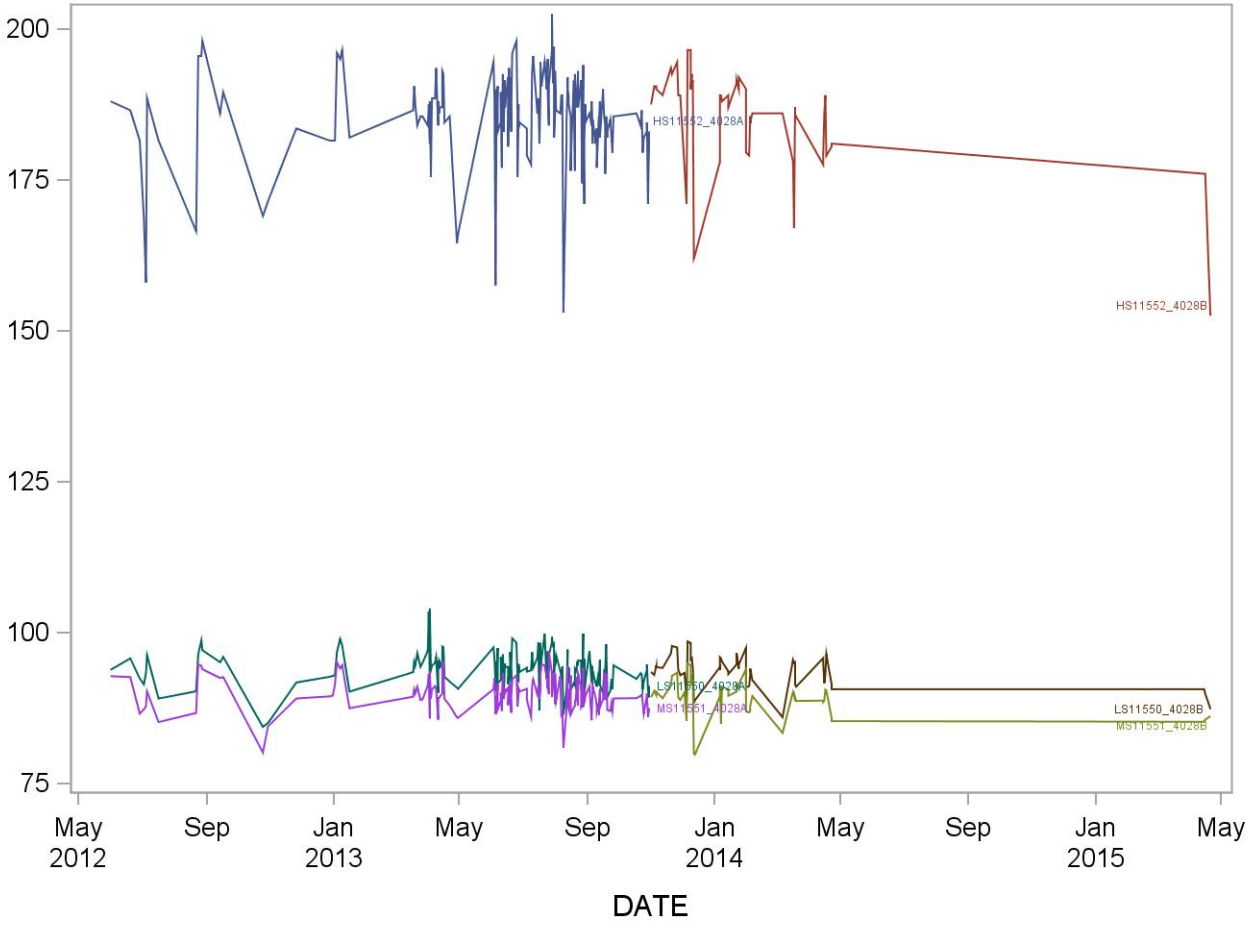
2011-2012 Summary Statistics and QC Chart for Margaric acid (C17:0)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	182	01JUN12	30OCT13	33.571	1.263	3.8
LS11550_4028A	182	01JUN12	30OCT13	21.044	0.736	3.5
MS11551_4028A	182	01JUN12	30OCT13	25.035	0.792	3.2
HS11552_4028B	54	01NOV13	16APR15	33.157	1.525	4.6
LS11550_4028B	55	01NOV13	16APR15	21.016	0.648	3.1
MS11551_4028B	55	01NOV13	16APR15	24.968	0.883	3.5



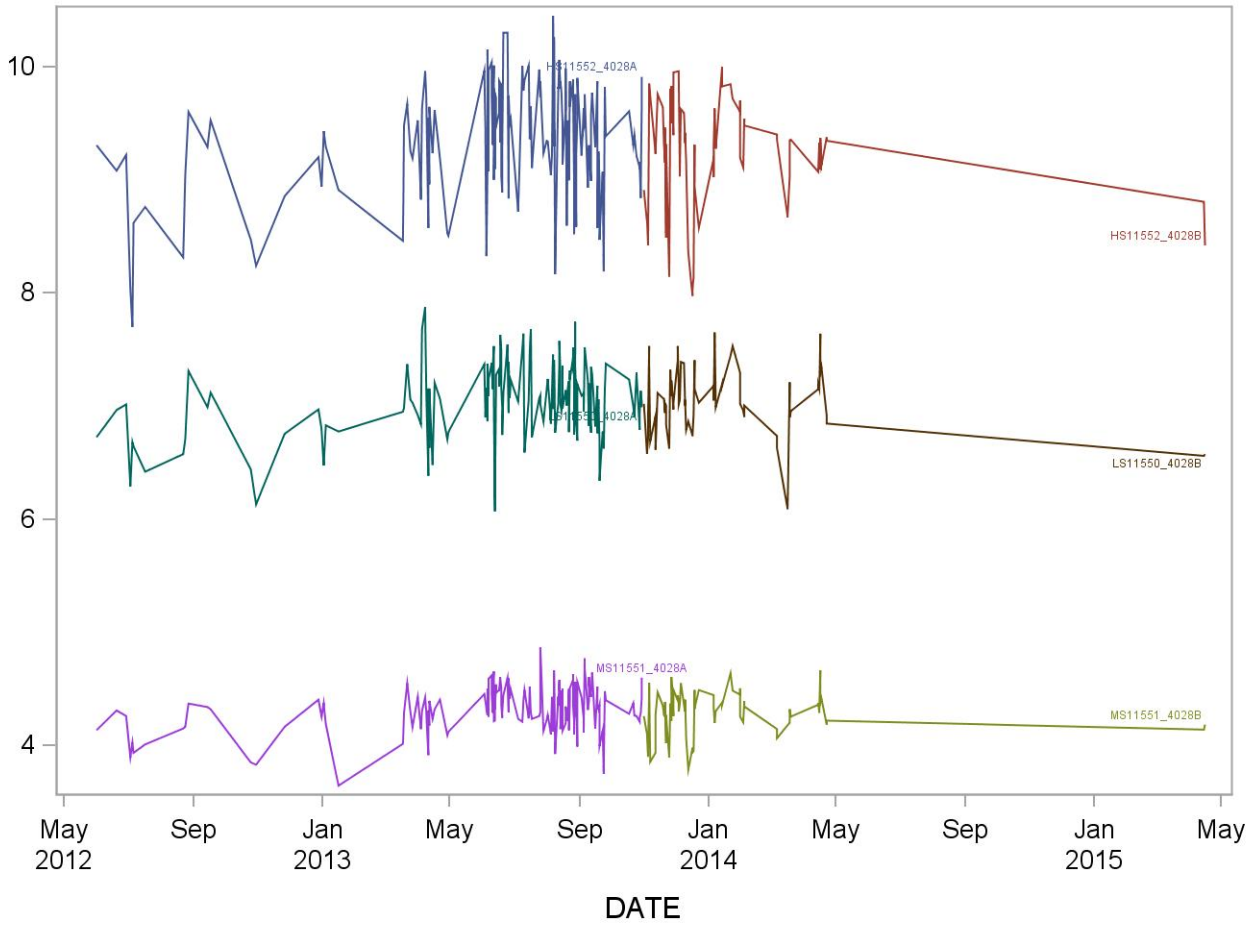
2011-2012 Summary Statistics and QC Chart for Myristic acid (14:0)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	178	01JUN12	30OCT13	185.298	7.526	4.1
LS11550_4028A	178	01JUN12	30OCT13	93.849	2.941	3.1
MS11551_4028A	178	01JUN12	30OCT13	89.740	2.664	3.0
HS11552_4028B	53	01NOV13	21APR15	184.547	8.980	4.9
LS11550_4028B	54	01NOV13	21APR15	93.683	2.770	3.0
MS11551_4028B	54	01NOV13	21APR15	89.215	3.116	3.5



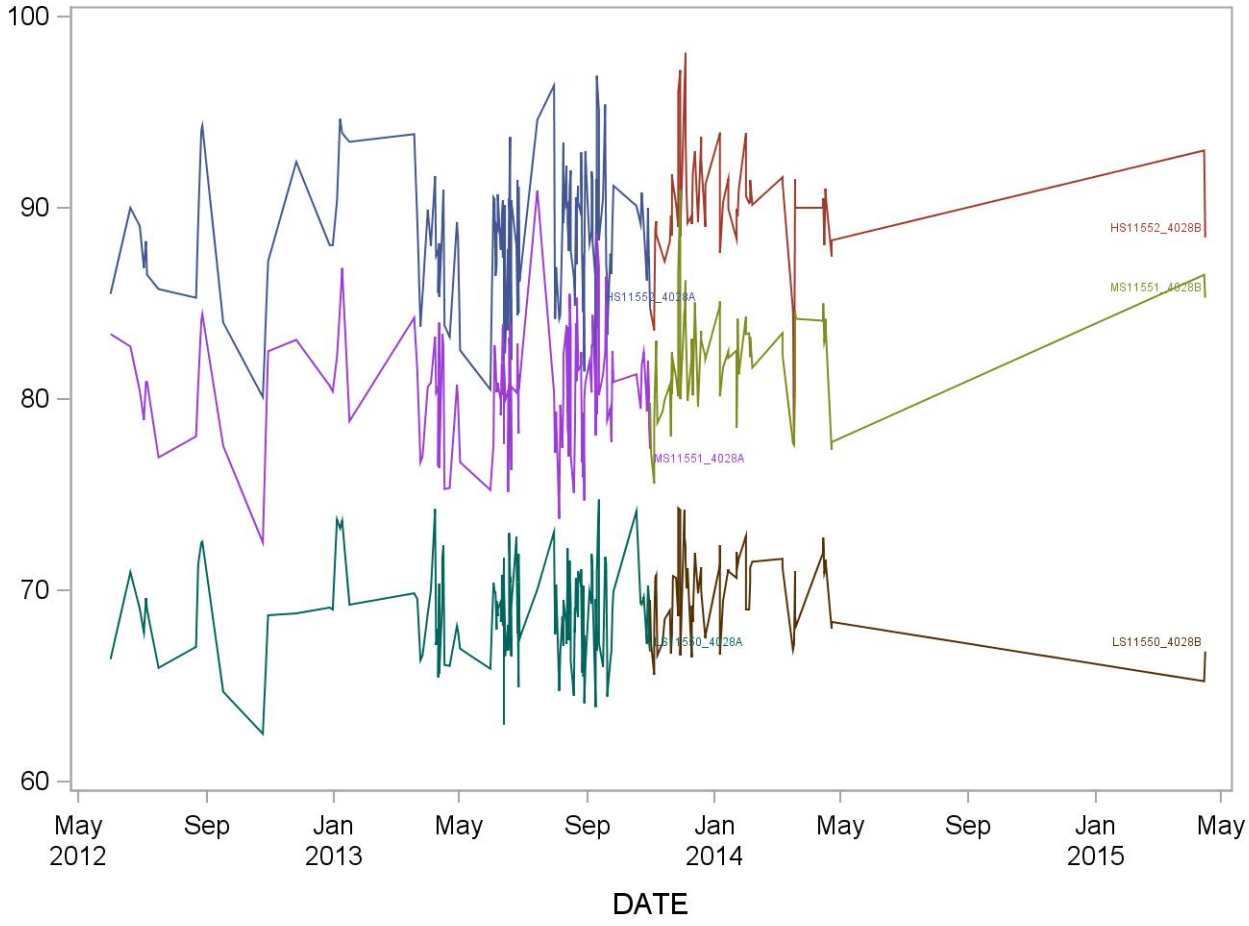
2011-2012 Summary Statistics and QC Chart for Myristoleic acid (14:1n-5)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	156	01JUN12	30OCT13	9.370	0.512	5.5
LS11550_4028A	156	01JUN12	30OCT13	7.058	0.327	4.6
MS11551_4028A	156	01JUN12	30OCT13	4.329	0.201	4.7
HS11552_4028B	66	01NOV13	16APR15	9.228	0.505	5.5
LS11550_4028B	70	01NOV13	16APR15	7.047	0.288	4.1
MS11551_4028B	70	01NOV13	16APR15	4.283	0.195	4.6



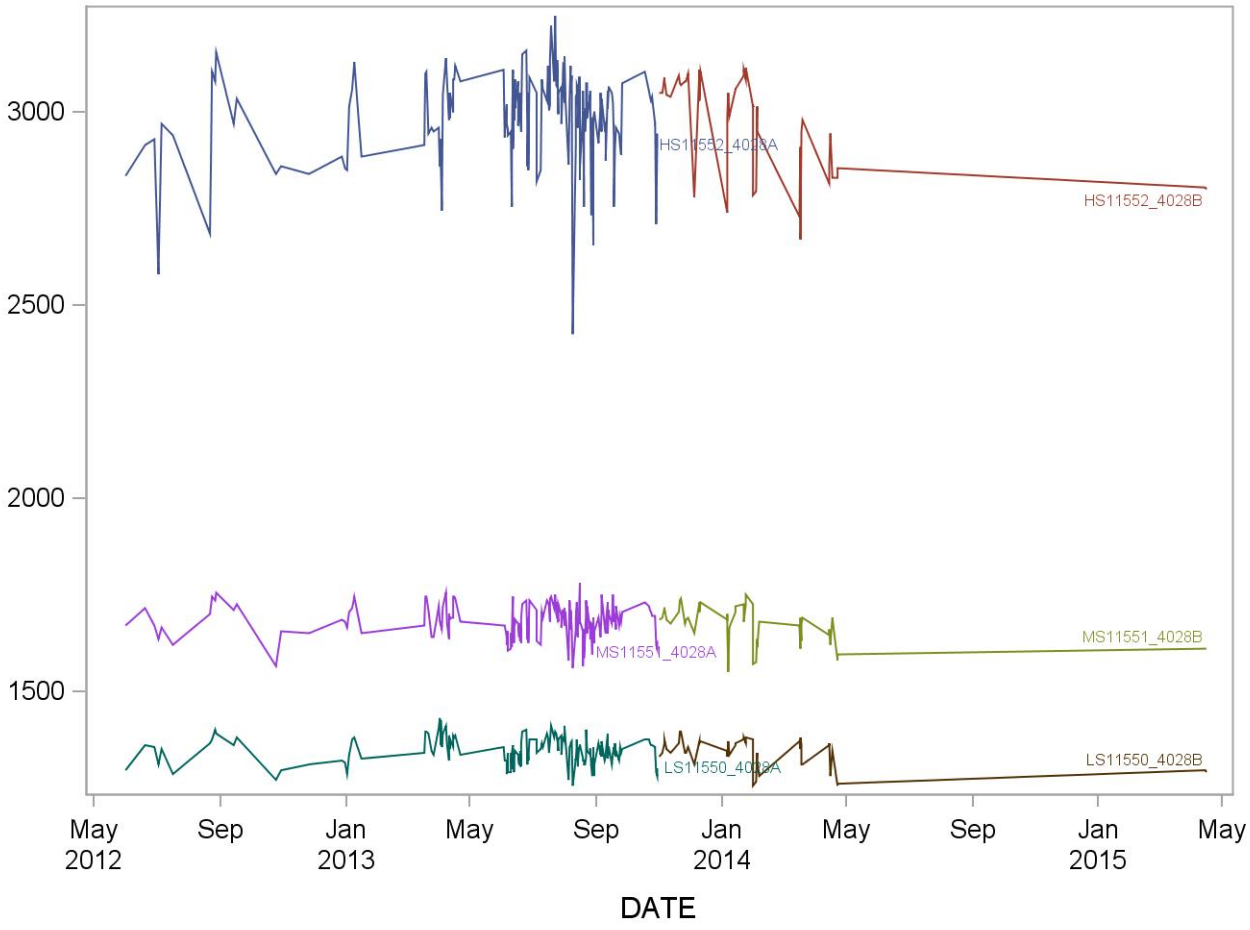
2011-2012 Summary Statistics and QC Chart for Nervonic acid (24:1n-9)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	148	01JUN12	31OCT13	88.418	3.295	3.7
LS11550_4028A	148	01JUN12	31OCT13	68.777	2.415	3.5
MS11551_4028A	148	01JUN12	31OCT13	80.546	2.892	3.6
HS11552_4028B	64	31OCT13	16APR15	89.994	3.181	3.5
LS11550_4028B	68	31OCT13	16APR15	69.837	2.190	3.1
MS11551_4028B	68	31OCT13	16APR15	82.097	2.706	3.3



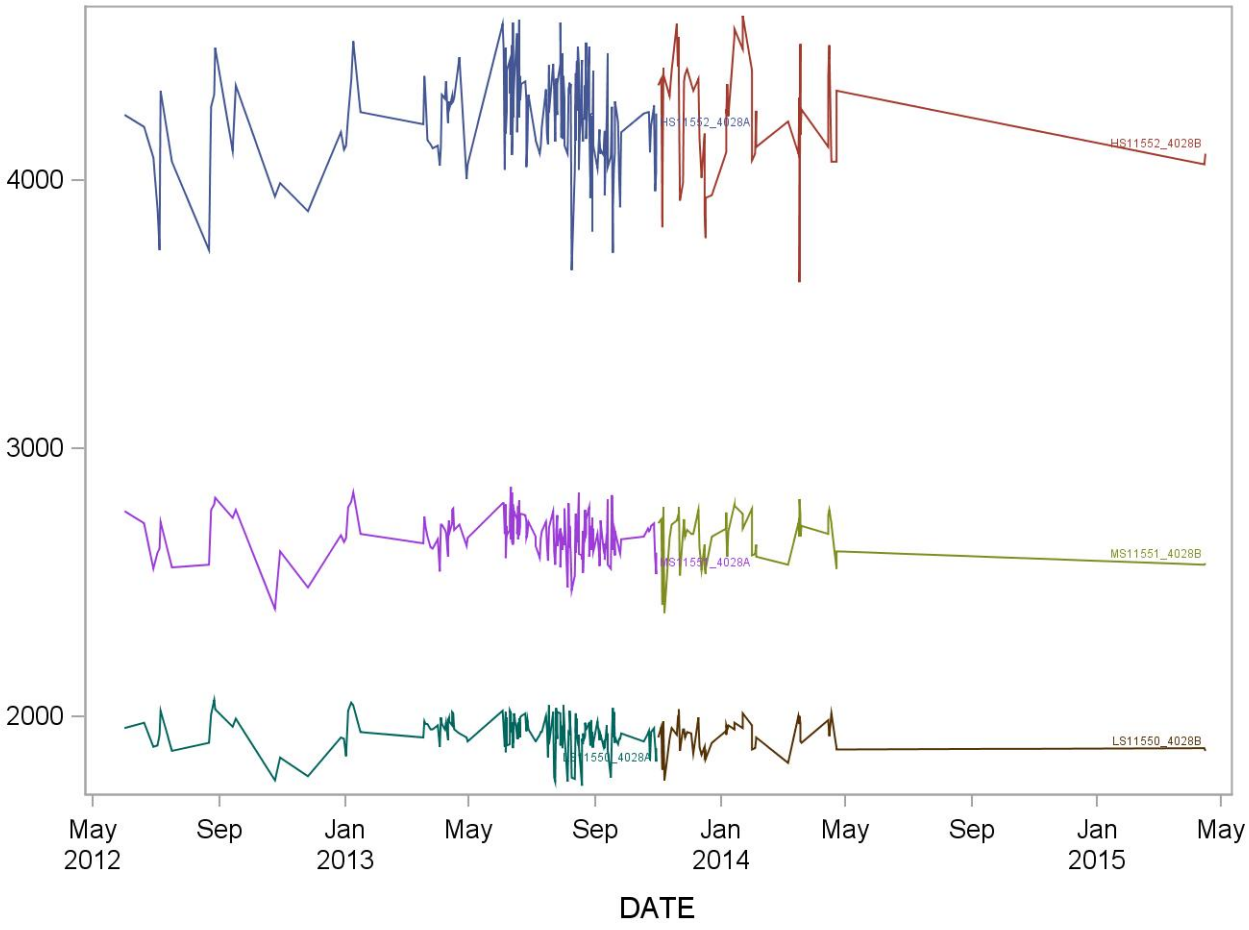
2011-2012 Summary Statistics and QC Chart for Oleic acid (18:1n-9)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	180	01JUN12	30OCT13	2982.9	117.7	3.9
LS11550_4028A	180	01JUN12	30OCT13	1345.9	33.3	2.5
MS11551_4028A	180	01JUN12	30OCT13	1680.0	41.8	2.5
HS11552_4028B	48	01NOV13	16APR15	2966.7	127.0	4.3
LS11550_4028B	48	01NOV13	16APR15	1341.5	37.5	2.8
MS11551_4028B	48	01NOV13	16APR15	1672.3	49.7	3.0



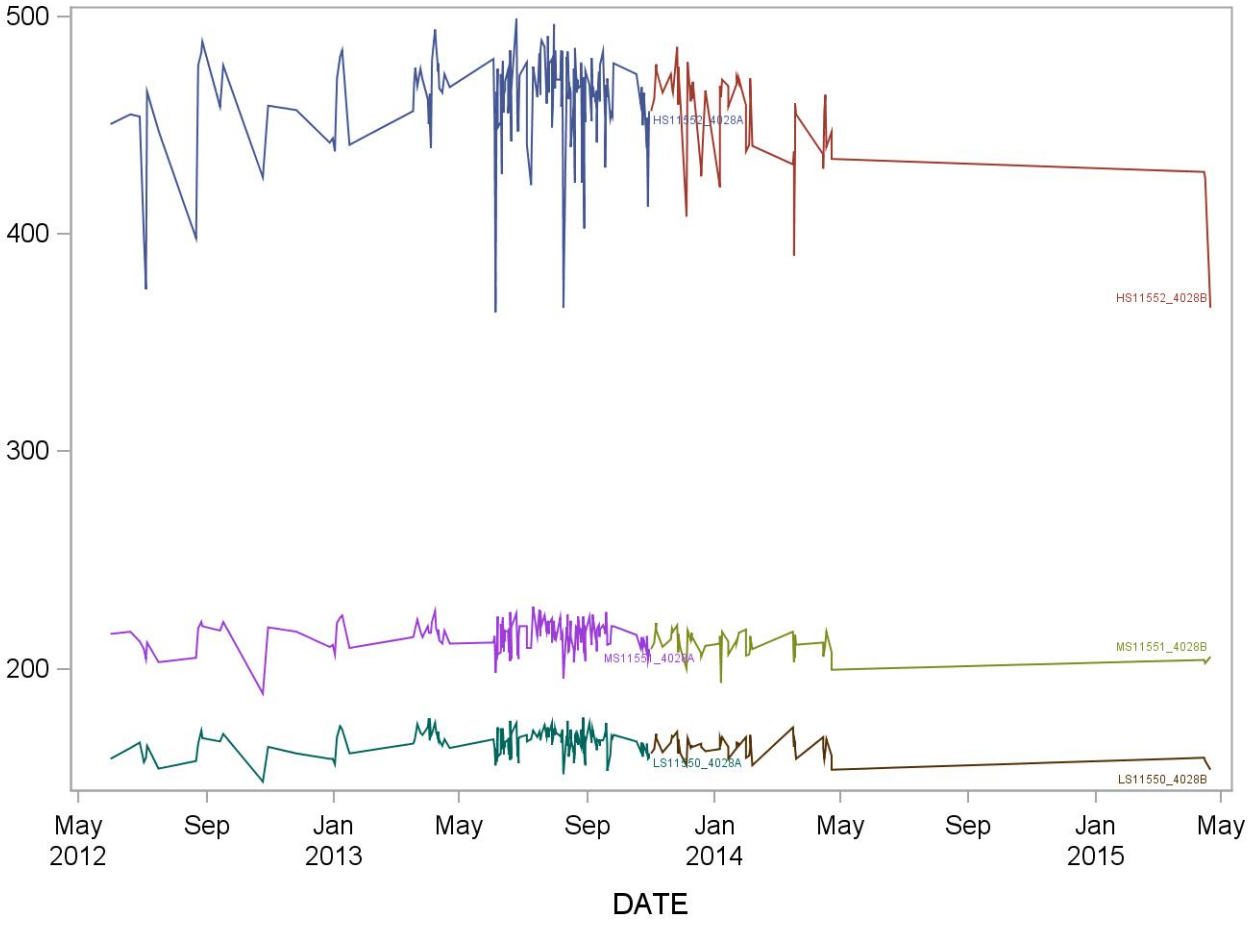
2011-2012 Summary Statistics and QC Chart for Palmitic acid (16:0)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	177	01JUN12	30OCT13	4225.9	177.7	4.2
LS11550_4028A	177	01JUN12	30OCT13	1935.2	61.8	3.2
MS11551_4028A	177	01JUN12	30OCT13	2676.7	78.0	2.9
HS11552_4028B	55	01NOV13	16APR15	4233.7	225.8	5.3
LS11550_4028B	57	01NOV13	16APR15	1925.2	56.9	3.0
MS11551_4028B	57	01NOV13	16APR15	2666.4	96.1	3.6



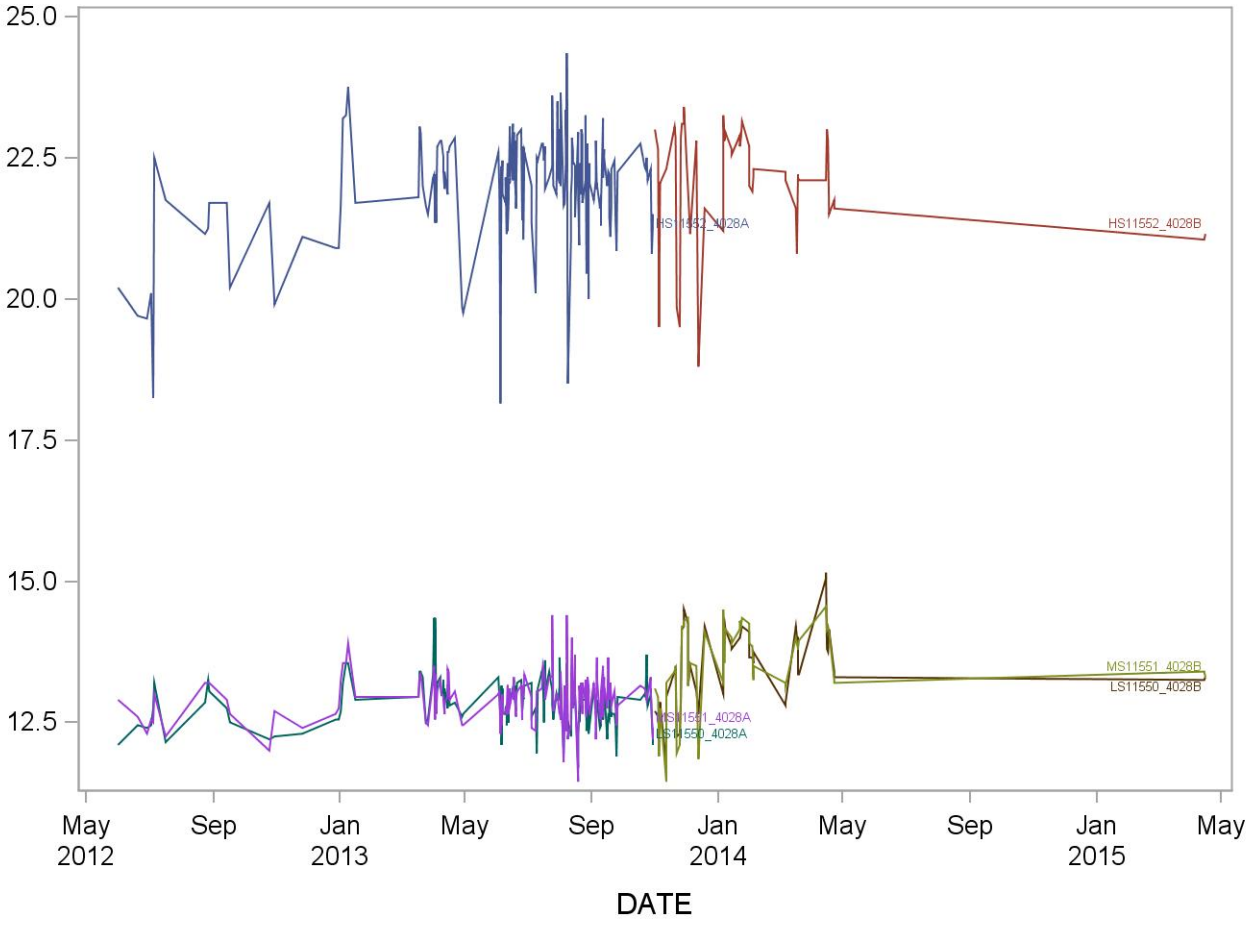
2011-2012 Summary Statistics and QC Chart for Palmitoleic acid (16:1n-7)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	173	01JUN12	30OCT13	461.379	21.794	4.7
LS11550_4028A	173	01JUN12	30OCT13	166.283	5.394	3.2
MS11551_4028A	173	01JUN12	30OCT13	215.029	6.409	3.0
HS11552_4028B	55	01NOV13	21APR15	452.091	23.101	5.1
LS11550_4028B	55	01NOV13	21APR15	163.818	4.402	2.7
MS11551_4028B	55	01NOV13	21APR15	211.100	5.675	2.7



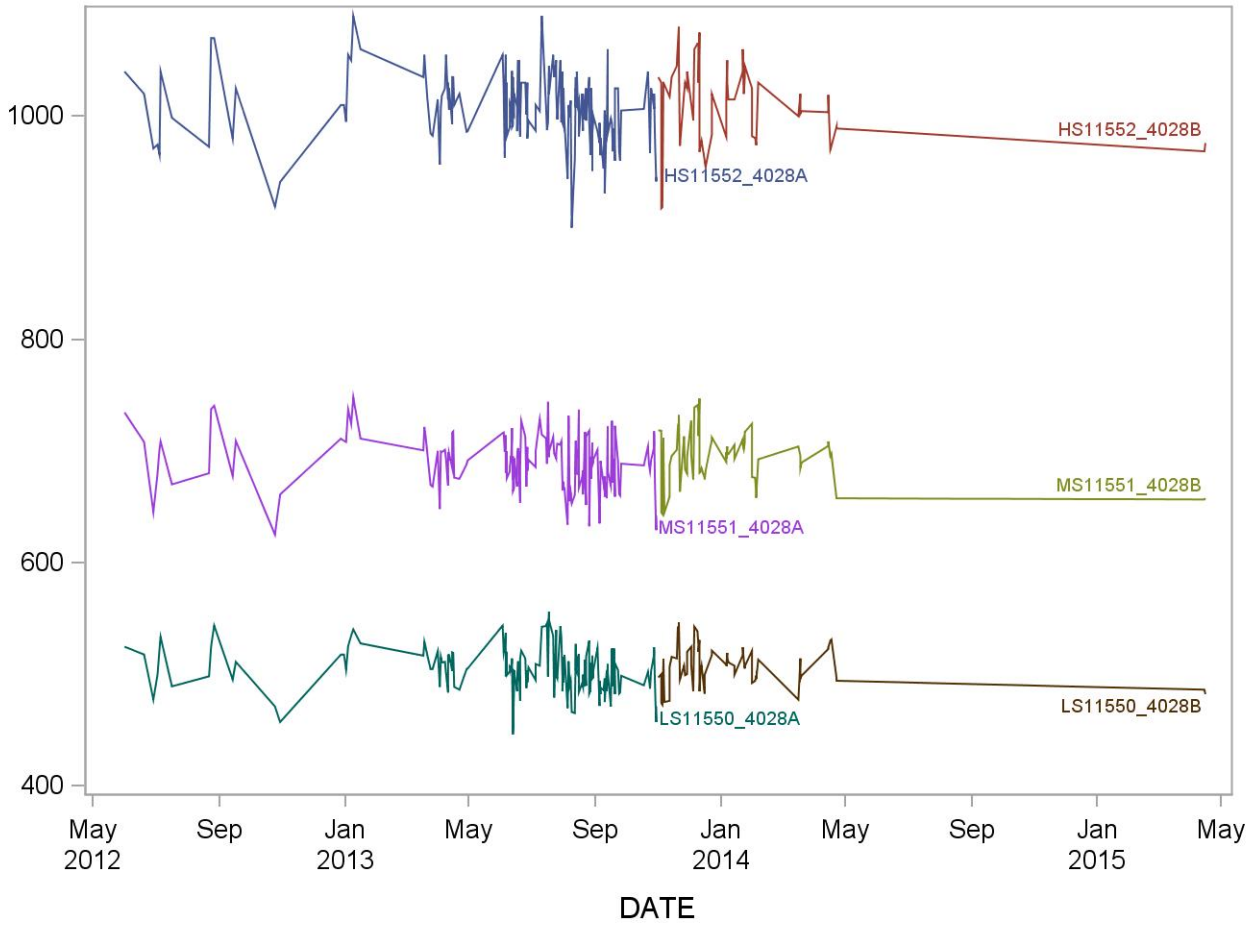
2011-2012 Summary Statistics and QC Chart for Pentadecanoic acid (C15:0)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	178	01JUN12	30OCT13	21.928	0.985	4.5
LS11550_4028A	178	01JUN12	30OCT13	12.845	0.396	3.1
MS11551_4028A	178	01JUN12	30OCT13	12.913	0.407	3.2
HS11552_4028B	51	01NOV13	16APR15	22.019	1.072	4.9
LS11550_4028B	54	01NOV13	16APR15	13.527	0.717	5.3
MS11551_4028B	54	01NOV13	16APR15	13.533	0.765	5.7



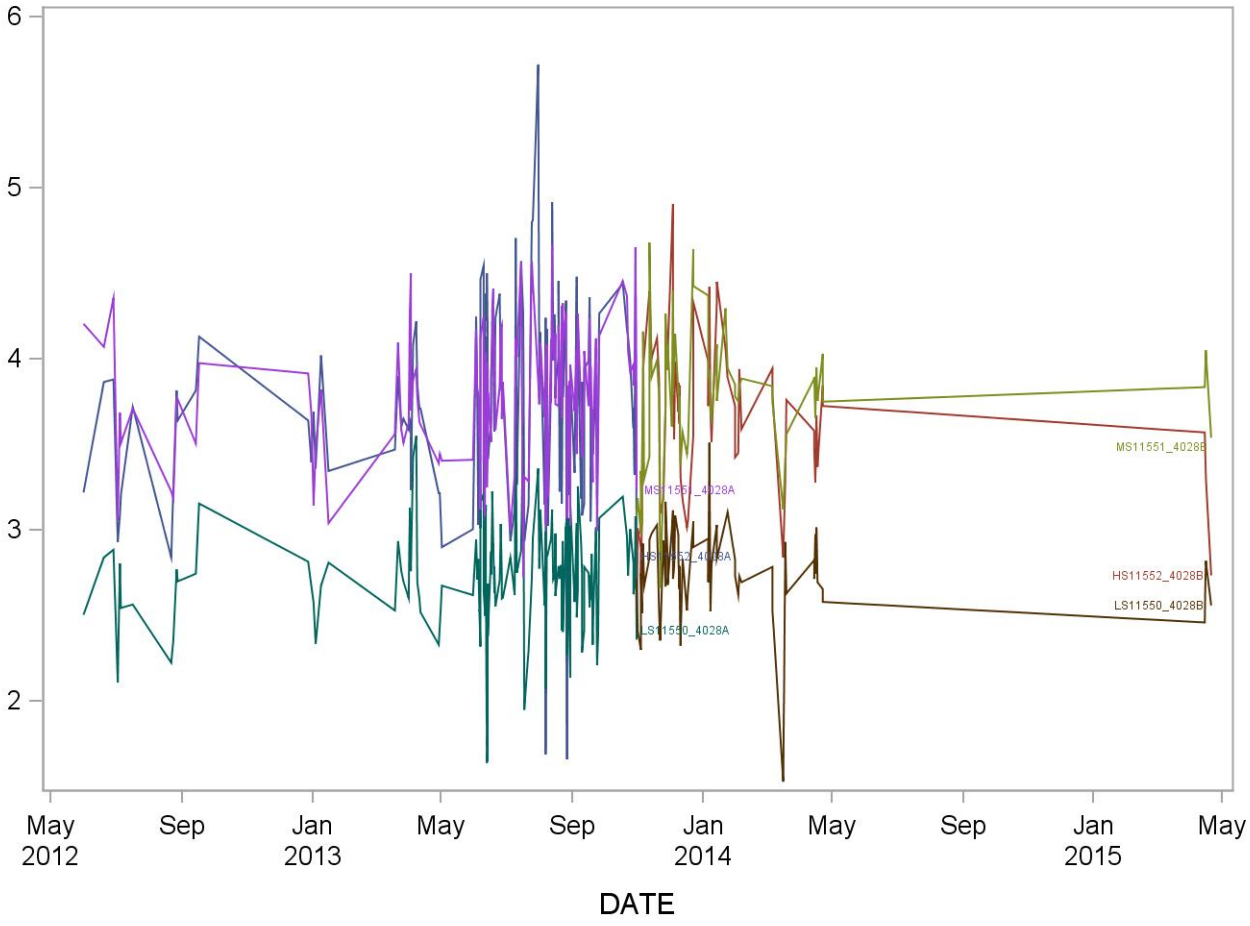
2011-2012 Summary Statistics and QC Chart for Stearic acid (18:0)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	167	01JUN12	30OCT13	1005.4	32.0	3.2
LS11550_4028A	167	01JUN12	30OCT13	504.3	20.1	4.0
MS11551_4028A	167	01JUN12	30OCT13	689.9	24.3	3.5
HS11552_4028B	63	01NOV13	16APR15	1010.3	35.9	3.6
LS11550_4028B	67	01NOV13	16APR15	507.7	17.4	3.4
MS11551_4028B	67	01NOV13	16APR15	694.7	24.3	3.5



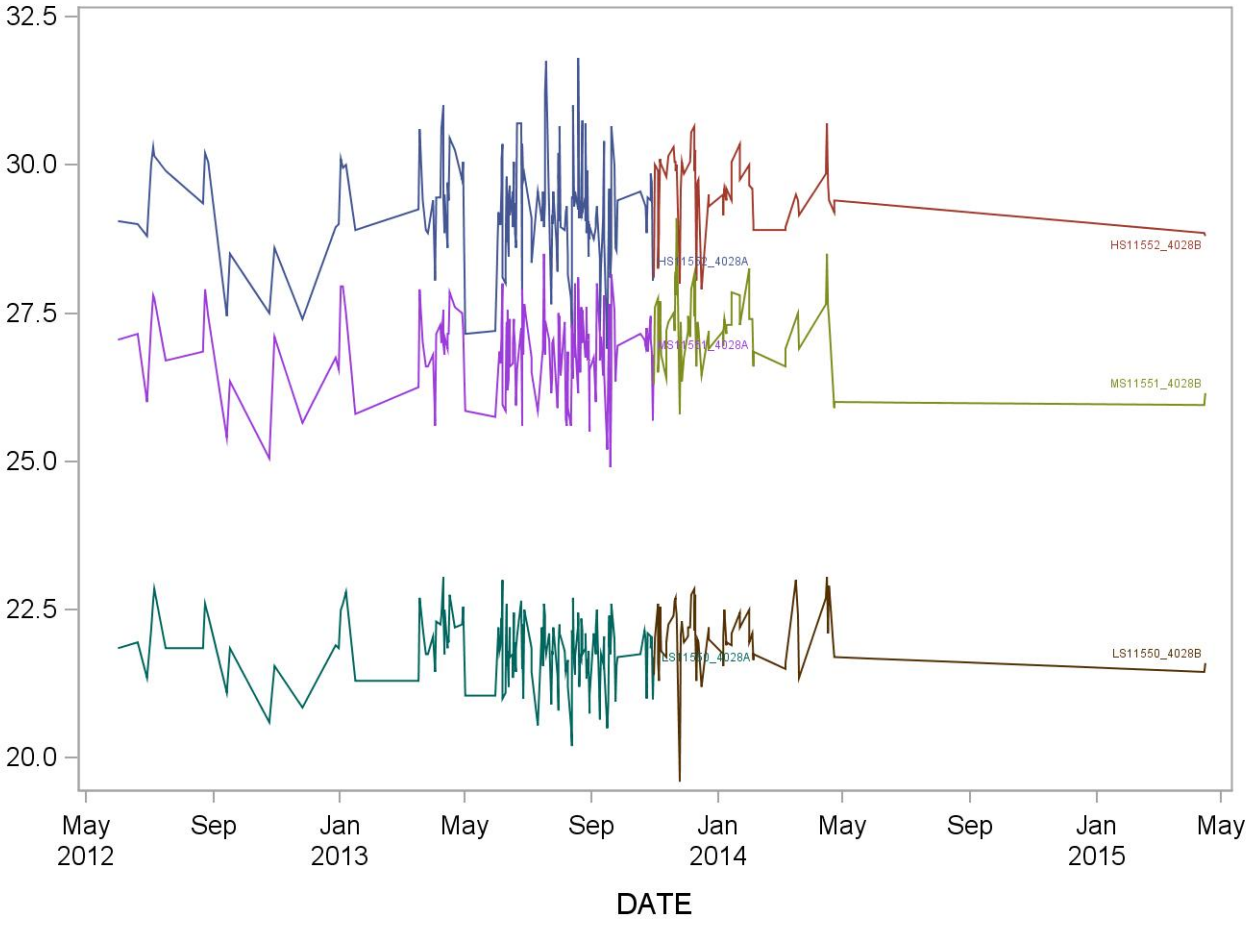
2011-2012 Summary Statistics and QC Chart for Stearidonic acid (C18:4n-3)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	149	01JUN12	31OCT13	3.721	0.558	15.0
LS11550_4028A	149	01JUN12	31OCT13	2.735	0.323	11.8
MS11551_4028A	149	01JUN12	31OCT13	3.800	0.389	10.2
HS11552_4028B	63	31OCT13	21APR15	3.643	0.479	13.2
LS11550_4028B	66	31OCT13	21APR15	2.768	0.268	9.7
MS11551_4028B	66	31OCT13	21APR15	3.771	0.385	10.2



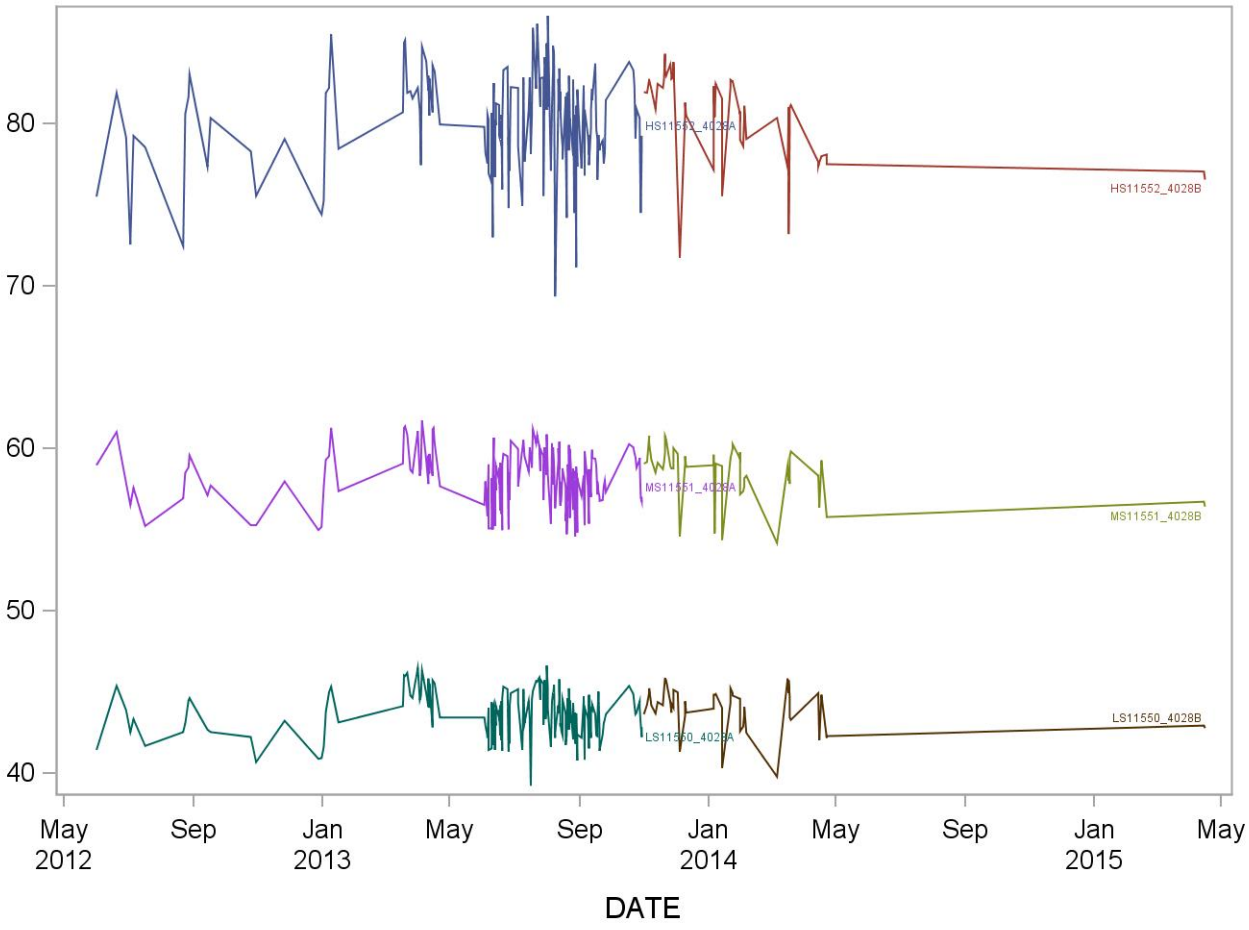
2011-2012 Summary Statistics and QC Chart for Tricosanoic acid (C23:0)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	166	01JUN12	31OCT13	29.229	0.877	3.0
LS11550_4028A	166	01JUN12	31OCT13	21.808	0.548	2.5
MS11551_4028A	166	01JUN12	31OCT13	26.825	0.672	2.5
HS11552_4028B	60	31OCT13	16APR15	29.546	0.638	2.2
LS11550_4028B	64	31OCT13	16APR15	22.056	0.559	2.5
MS11551_4028B	64	31OCT13	16APR15	27.212	0.644	2.4



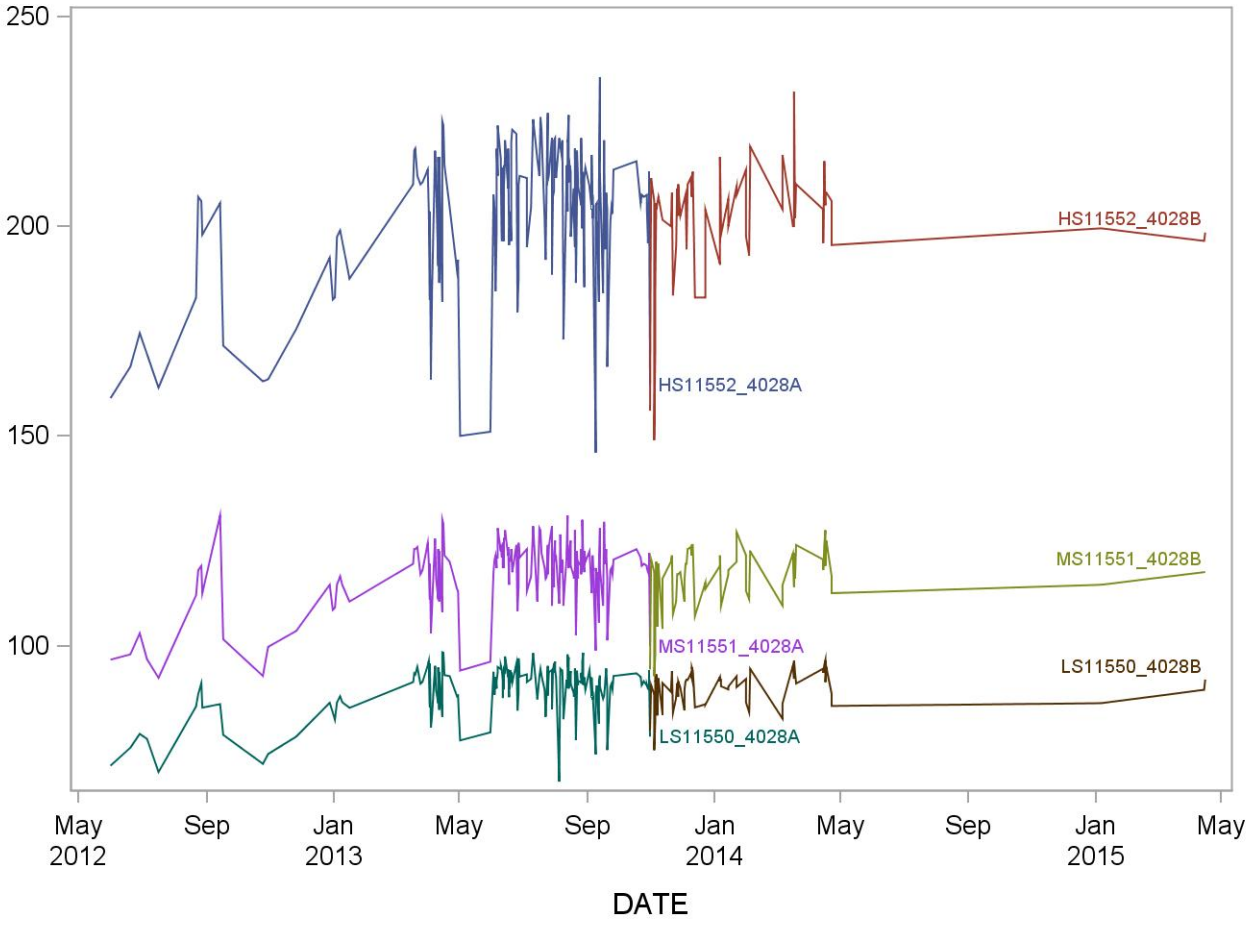
2011-2012 Summary Statistics and QC Chart for alpha-Linolenic acid (18:3n-3)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	175	01JUN12	30OCT13	79.956	3.131	3.9
LS11550_4028A	175	01JUN12	30OCT13	43.618	1.409	3.2
MS11551_4028A	175	01JUN12	30OCT13	58.205	1.682	2.9
HS11552_4028B	54	01NOV13	16APR15	80.381	2.705	3.4
LS11550_4028B	55	01NOV13	16APR15	44.012	1.294	2.9
MS11551_4028B	55	01NOV13	16APR15	58.495	1.608	2.7



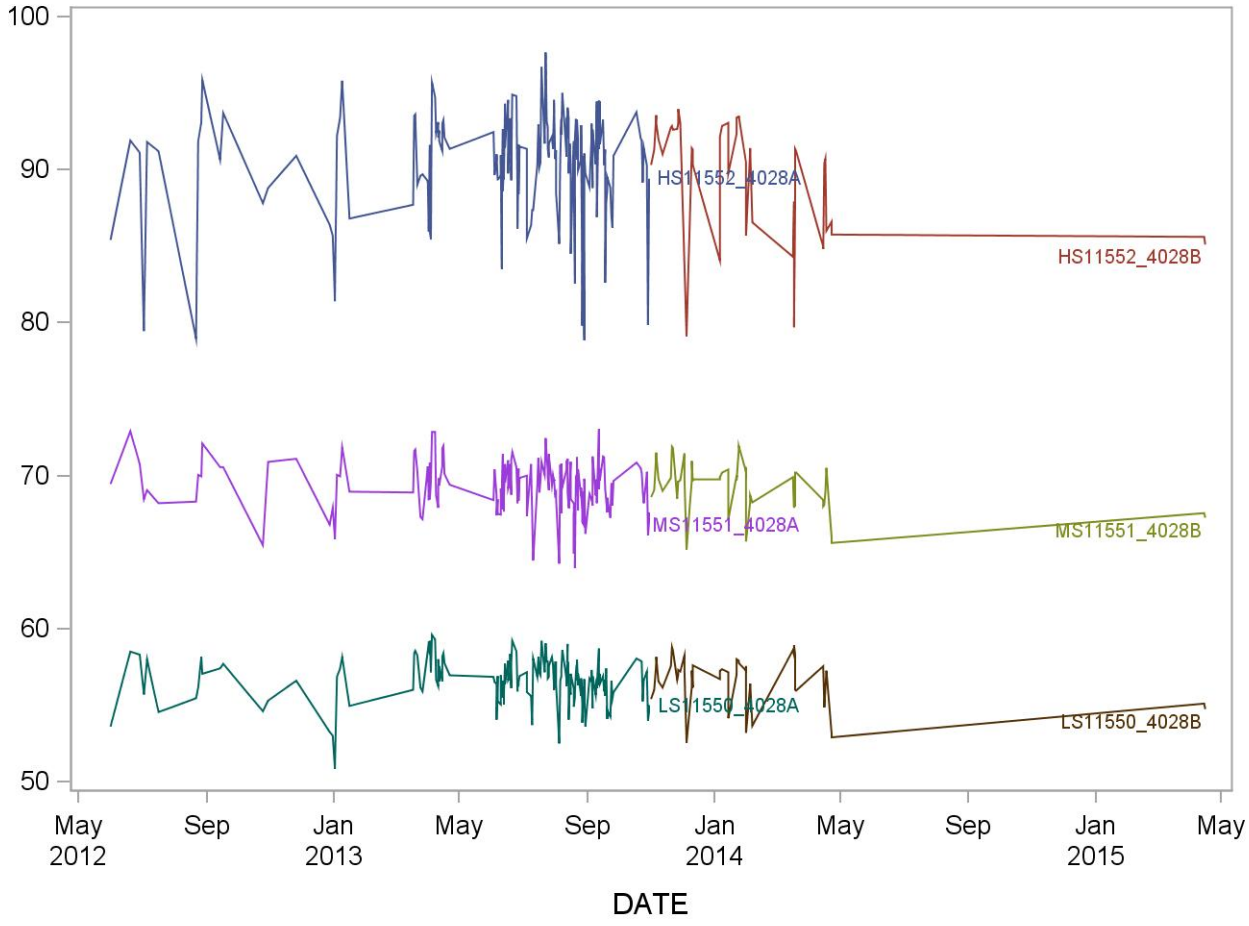
2011-2012 Summary Statistics and QC Chart for cis-Vaccenic acid (18:1n-7)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	177	01JUN12	31OCT13	202.766	17.334	8.5
LS11550_4028A	177	01JUN12	31OCT13	89.650	5.925	6.6
MS11551_4028A	177	01JUN12	31OCT13	117.542	7.929	6.7
HS11552_4028B	60	31OCT13	16APR15	201.450	13.677	6.8
LS11550_4028B	64	31OCT13	16APR15	89.883	4.268	4.7
MS11551_4028B	64	31OCT13	16APR15	116.288	7.192	6.2



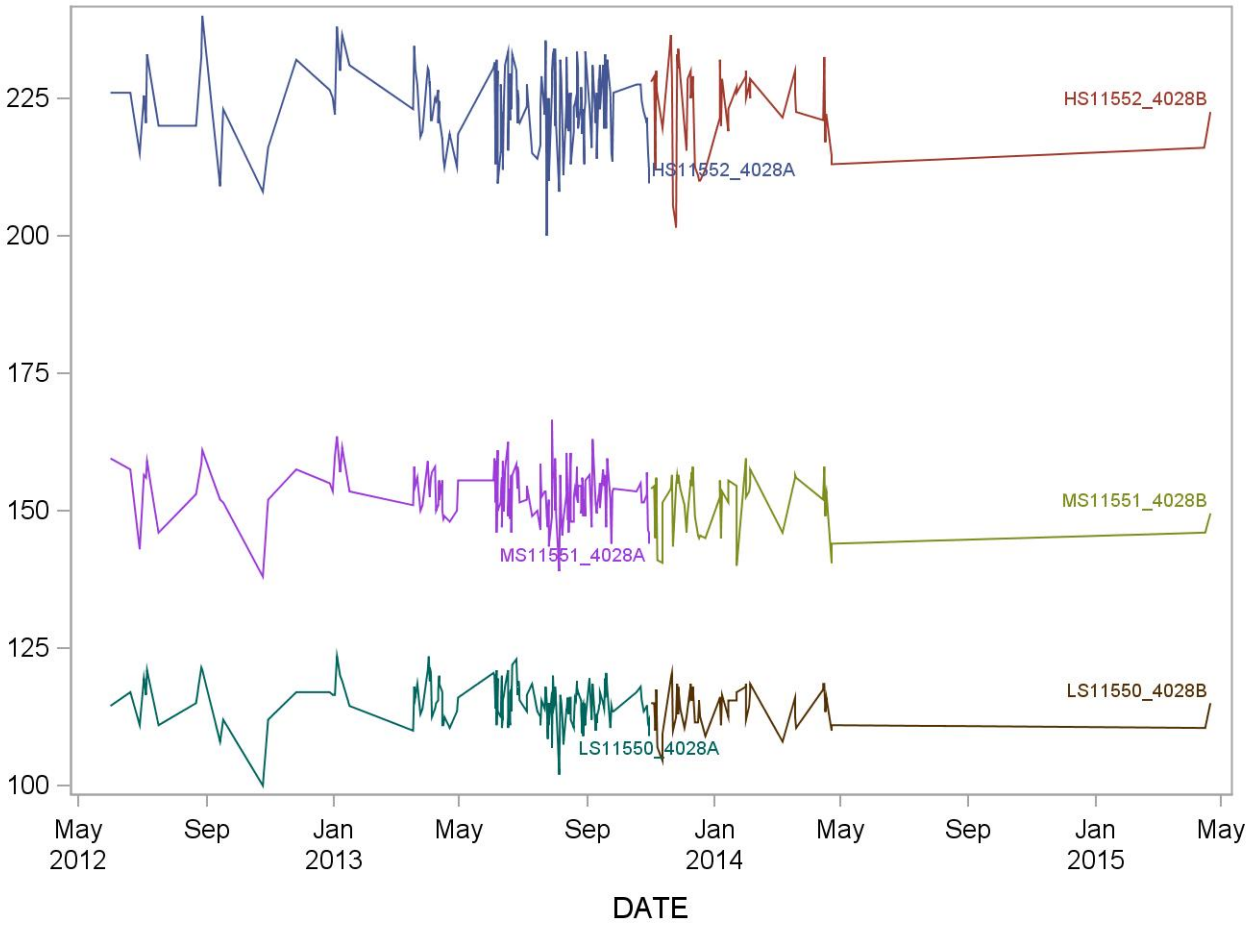
2011-2012 Summary Statistics and QC Chart for gamma-Linolenic acid (18:3n-6)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	175	01JUN12	30OCT13	90.277	3.514	3.9
LS11550_4028A	175	01JUN12	30OCT13	56.563	1.432	2.5
MS11551_4028A	175	01JUN12	30OCT13	69.279	1.644	2.4
HS11552_4028B	49	01NOV13	16APR15	89.641	3.730	4.2
LS11550_4028B	51	01NOV13	16APR15	56.628	1.576	2.8
MS11551_4028B	51	01NOV13	16APR15	69.320	1.623	2.3



2011-2012 Summary Statistics and QC Chart for homo-gamma-Linolenic acid (20:3n-6)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11552_4028A	166	01JUN12	30OCT13	223.120	6.951	3.1
LS11550_4028A	166	01JUN12	30OCT13	114.933	3.766	3.3
MS11551_4028A	166	01JUN12	30OCT13	152.931	4.689	3.1
HS11552_4028B	57	01NOV13	21APR15	222.754	7.674	3.4
LS11550_4028B	60	01NOV13	21APR15	114.125	3.476	3.0
MS11551_4028B	60	01NOV13	21APR15	151.450	5.245	3.5



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Acknowledgments

We gratefully acknowledge the contributions of Leslie F McCoy, PhD who assisted in developing this methodology for the CDC Nutrition Lab.

Appendix A – Method Figures of Merit

Matrix: Serum/Plasma Comparison

A comparison of ~100 matched serum/plasma samples were prepared using the #4026 Fatty Acids Method. The table below gives the equations needed to convert the serum or plasma results for study result comparisons.

No.	Equations
1	ALN Plasma uM = -1.98 + 0.99 * ALN Serum uM
2	AR1 Plasma uM = 1.12 + 0.93 * AR1 Serum uM
3	ARA Plasma uM = 83.9 + 0.85 * ARA Serum uM
4	DA1 Plasma uM = 5.58 + 0.86 * DA1 Serum uM
5	DE1 - not provided
6	DHA Plasma uM = 8.05 + 0.92 * DHA Serum uM
7	DP3 Plasma uM = 5.30 + 0.87 * DP3 Serum uM
8	DP6 Plasma uM = -0.45 + 0.98 * DP6 Serum uM
9	DTA Plasma uM = -0.86 + 0.97 * DTA Serum uM
10	ED1 Plasma uM = 0.23 + 0.92 * ED1 Serum uM
11	EN1 Plasma uM = 0.44 + 0.93 * EN1 Serum uM
12	EPA Plasma uM = 3.72 + 0.94 * EPA Serum uM
13	GLA Plasma uM = 3.55 + 0.93 * GLA Serum uM
14	HGL Plasma uM = 14.3 + 0.84 * HGL Serum uM
15	LG1 Plasma uM = 9.88 + 0.75 * LG1 Serum uM
16	LNA Plasma uM = 488 + 0.86 * LNA Serum uM
17	ML1 Plasma uM = -0.93 + 1.05 * ML1 Serum uM
18	MR1 Plasma uM = 7.63 + 0.93 * MR1 Serum uM
19	NR1 Plasma uM = 19.8 + 0.71 * NR1 Serum uM
20	OL1 Plasma uM = 234 + 0.89 * OL1 Serum uM
21	PL1 Plasma uM = 7.55 + 0.94 * PL1 Serum uM
22	PM1 Plasma uM = 48.2 + 0.93 * PM1 Serum uM
23	ST1 Plasma uM = -6.13 + 0.95 * ST1 Serum uM
24	VC1 Plasma uM = 11.8 + 0.89 * VC1 Serum uM

Recovery, Precision and Limits of Detection

Results of in-house extraction recovery experiments, independent of the IS, showed a mean (± SD) recovery of 68% (± 29) for saturated fatty acids (SFA), 63% (± 19) for monounsaturated fatty acids (MUFA), and 42% (± 15) for polyunsaturated fatty acids (PUFA). Results of in-house recovery studies based on peak area ratios (analyte/IS) showed a mean (± SD) recovery of 101% (± 8) for SFA, 94% (± 9) for MUFA, and 97% (± 11) for PUFA.

Long-term quality control mean CV (± SD) for the 30 reportable analytes including all bench QC pools (LS11550_4028A, MS11551_4028A, and HS11552_4028A)was 5% (± 5) and (LS11550_4028B, MS11551_4028B, and HS11552_4028B) was 5% (± 5).

Limits of detection (LOD) are calculated for the entire measurement system, not just the instrument. In order to include both Type I and Type II errors, a method similar to CLSI EP 17A is used to estimate LOD. This method analyzes the relationship between SD and concentration for blank and near-blank concentrations. Serially diluted serum samples were prepared at the following levels: 1/5, 1/35, 1/70, 1/200, 1/300, 1/500, 1/4000, undiluted. One vial per level was analyzed alongside patient samples for 60 runs. The average (± SD) LOD for all analytes was 6.7 (± 16) µM for the reported analytes.

Individual fatty acid recovery, precision for QC pool ver A and LOD data are also shown in the table below. Pentadecanoic acid (PDE) limits were updated in QC pool ver B due to a mean shift after systematic data review shift removing Std3 vs Std2 from the calibration curve to obtain better linearity on the low end of the calibration curve. The precision CV (SD) for PDE is the same for ver A and ver B limits, 5% ± 2%.

No.	Fatty acids - Saturated	Code	Avg % Recovery ± SD		Precision CV ± SD	LOD (uM)
			Independent of IS	Corrected using IS		
1	Capric acid (C10:0)	CAP	20% ± 6%	97% ± 15%	24% ± 6%	1.59
2	Lauric acid (C12:0)	LAR	42% ± 17%	108% ± 11%	8% ± 3%	2.33
3	Myristic acid (14:0)	MR1	58% ± 18%	100% ± 5%	5% ± 0.7%	4.90
4	Pentadecanoic acid (C15:0)	PDE	78% ± 30%	102% ± 18%	5% ± 2%	0.75
5	Palmitic acid (16:0)	PM1	73% ± 18%	106% ± 9%	4% ± 0.4%	78.1
6	Margaric acid (C17:0)	MRG	99% ± 45%	85% ± 6%	5% ± 1%	3.36
7	Stearic acid (18:0)	ST1	76% ± 25%	116% ± 17%	4% ± 0.2%	39.1
8	Arachidic acid (20:0)	AR1	71% ± 18%	95% ± 7%	3% ± 0.2%	0.82
9	Docosanoic acid (22:0)	DA1	72% ± 18%	99% ± 10%	3% ± 0.2%	0.68
10	Tricosanoic acid (C23:0)	TSA	86% ± 30%	100% ± 19%	3% ± 0.3%	0.90
11	Lignoceric acid (24:0)	LG1	71% ± 21%	98% ± 10%	3% ± 0.3%	1.09
No.	**Fatty acids - Monounsaturated**					
12	Myristoleic acid (14:1n-5)	ML1	39% ± 13%	87% ± 5%	5% ± 0.6%	0.29
13	Palmitoleic acid (16:1n-7)	PL1	58% ± 16%	104% ± 10%	4% ± 1%	6.56
14	*cis*-Vaccenic acid (18:1n-7)	VC1	63% ± 18%	83% ± 10%	9% ± 0.7%	2.31
15	Oleic acid (18:1n-9)	OL1	70% ± 20%	97% ± 5%	4% ± 0.9%	17.7
16	Eicosenoic acid (20:1n-9)	EN1	65% ± 13%	90% ± 6%	6% ± 0.8%	0.87
17	Nervonic acid (24:1n-9)	NR1	81% ± 18%	103% ± 16%	4% ± 0.2%	0.69
No.	**Fatty acids - Polyunsaturated**					
18	Linoleic acid (18:2n-6)	LNA	58% ± 17%	96% ± 5%	4% ± 1%	22.6
19	*alpha*-Linolenic acid (18:3n-3)	ALN	37% ± 7%	100% ± 4%	4% ± 1%	1.54
20	*gamma*-Linolenic acid (18:3n-6)	GLA	39% ± 9%	99% ± 8%	4% ± 1%	0.42
21	Stearidonic acid (C18:4n-3)	SD1	23% ± 5%	78% ± 13%	21% ± 15%	0.24
22	Eicosadienoic acid (20:2n-6)	ED1	62% ± 10%	86% ± 14%	5% ± 0.7%	0.31
23	*homo-gamma*-Linolenic acid (20:3n-6)	HGL	54% ± 11%	105% ± 13%	4% ± 0.3%	1.14
24	Eicosatrienoic acid (C20:3n-9)	ET1	53% ± 10%	81% ± 15%	6% ± 2%	0.39
25	Arachidonic acid (20:4n-6)	ARA	42% ± 10%	103% ± 11%	3% ± 0.5%	7.34
26	Eicosapentaenoic acid (20:5n-3)	EPA	26% ± 4%	101% ± 6%	3% ± 0.1%	0.79
27	Docosatetraenoic acid (22:4n-6)	DTA	53% ± 7%	119% ± 19%	5% ± 0.3%	0.31
28	Docosapentaenoic acid (22:5n-3)	DP3	38% ± 6%	102% ± 6%	4% ± 0.5%	0.55
29	Docosapentaenoic acid (22:5n-6)	DP6	39% ± 4%	102% ± 6%	4% ± 0.5%	0.24
30	Docosahexaenoic acid (22:6n-3)	DHA	25% ± 3%	95% ± 10%	3% ± 0.2%	1.84

Dilution linearity

A simple linear regression comparison of *undiluted versus diluted* patient serum samples was completed using data collected from 1/2012 – 5/2013. The table below summarizes the results. An R^2 <0.9 was considered not adequately linear and therefore any dilutions for those analytes are not to be reported (AR1, DA1, DTA, NR1, SD1, and VC1).

No.	Fatty acids - Saturated	Code	n	Slope	Intercept	R^2
1	Capric acid (C10:0)	CAP	66	0.83	0.82	0.93
2	Lauric acid (C12:0)	LAR	57	0.8	3.78	0.97
3	Myristic acid (14:0)	MR1	25	1.01	-20.9	0.97
4	Pentadecanoic acid (C15:0)	PDE	52	0.93	1.60	0.91
5	Palmitic acid (16:0)	PM1	75	1.06	-427	0.93
6	Margaric acid (C17:0)	MRG	75	0.89	5.45	0.91
7	Stearic acid (18:0)	ST1	76	0.94	14.2	0.91
8	Arachidic acid (20:0)	AR1	72	0.83	5.27	0.86
9	Docosanoic acid (22:0)	DA1	76	0.74	20.2	0.89
10	Tricosanoic acid (C23:0)	TSA	76	0.96	2.00	0.90
11	Lignoceric acid (24:0)	LG1	76	0.78	12.9	0.90
No.	**Fatty acids - Monounsaturated**					
12	Myristoleic acid (14:1n-5)	ML1	76	0.97	0.28	0.98
13	Palmitoleic acid (16:1n-7)	PL1	50	0.98	-30.2	0.96
14	*cis*-Vaccenic acid (18:1n-7)	VC1	72	0.93	17.6	0.89
15	Oleic acid (18:1n-9)	OL1	*	-	-	-
16	Eicosenoic acid (20:1n-9)	EN1	75	0.92	1.14	0.92
17	Nervonic acid (24:1n-9)	NR1	76	0.86	9.41	0.83
No.	**Fatty acids - Polyunsaturated**					
18	Linoleic acid (18:2n-6)	LNA	*	-	-	-
19	*alpha*-Linolenic acid (18:3n-3)	ALN	50	0.99	-9.18	0.96
20	*gamma*-Linolenic acid (18:3n-6)	GLA	*	-	-	-
21	Stearidonic acid (C18:4n-3)	SD1	76	1.11	0.00	0.57
22	Eicosadienoic acid (20:2n-6)	ED1	72	0.88	4.33	0.92
23	*homo-gamma*-Linolenic acid (20:3n-6)	HGL	75	0.91	10.0	0.95
24	Eicosatrienoic acid (C20:3n-9)	ET1	75	0.91	0.94	0.95
25	Arachidonic acid (20:4n-6)	ARA	50	0.92	10.9	0.91
26	Eicosapentaenoic acid (20:5n-3)	EPA	72	0.91	2.21	0.99
27	Docosatetraenoic acid (22:4n-6)	DTA	76	0.78	6.22	0.79
28	Docosapentaenoic acid (22:5n-3)	DP3	76	0.88	-1.21	0.91
29	Docosapentaenoic acid (22:5n-6)	DP6	51	0.82	4.98	0.91
30	Docosahexaenoic acid (22:6n-3)	DHA	76	0.94	2.42	0.98

* Not enough data to evaluate

Method Comparison: #4026 vs #4028

A method comparison consisting of 20 patient samples was completed. Excluding DE1, 23 fatty acids (common to both methods) showed good to excellent agreement with average difference < 1% and excellent correlation with average R^2 of 0.98.

Analyte	Average % Difference*
ALN_N	2.4%
AR1_N	0.6%
ARA_N	2.2%
DA1_N	3.7%
DE1_N	102%
DHA_N	5.5%
DP3_N	0.4%
DP6_N	-4.5%
DTA_N	-12.2%
ED1_N	-3.7%
EN1_N	11.5%
EPA_N	3.7%
GLA_N	1.1%
HGL_N	-6.5%
LG1_N	4.2%
LNA_N	-3.3%
ML1_N	4.8%
MR1_N	-3.2%
NR1_N	18%
OL1_N	-2.8%
PL1_N	3.5%
PM1_N	-3.4%
ST1_N	-10.2%
VC1_N	-7.1%
Mean	-0.1%
SD	6.4%

* (#4028 - #4026)/#4026 [(new method – old method)/old method]

DE1 is a low-level MUFA that is not measured with precision (generally close to the LOD) and thus has been deemed non-reportable in assay #4028.

Appendix B – Ruggedness Testing

Detailed information can be found at:

\\cdc\project\CCEHIP_NCEH_DLS_NBB_LABS\Fatty Acids Documents\Fatty Acids 2 Validation 2008

1. Heating Block Temperature

a. Principle: Esterified fatty acids are hydrolyzed from triglycerides, phospholipids and cholesteryl esters using sequential treatment with mineral acid and base in the presence of heat. The temperature of the heating blocks can vary day to day which could have an effect on hydrolysis.

b. Proposal: To vary the temperature during the hydrolysis phase to determine its effect on area counts for ARA, DHA, EPA, and LNA.

c. Findings: Method specifies 219°F (104°C), lower temps 183°F (84°C) and 201°F (94°C), and higher temps 237°F (114°C) and 255°F (124°C) were tested

Analyte	QCID	219°F uM	183°F uM	201°F uM	237°F uM	255°F uM
ARA	HP06+03_6209_QC04	1215	1220	1190	1190	1205
	LP_06553_QC04	528	547	543	527	543
	MP06+02_6208_QC04	893	876	882	874	876
DHA	HP06+03_6209_QC04	187	187	186	184	184
	LP_06553_QC04	123	127	127	122	128
	MP06+02_6208_QC04	196	190	190	191	191
EPA	HP06+03_6209_QC04	67	68	67	66.5	67
	LP_06553_QC04	38.5	39.5	39	38.5	39.5
	MP06+02_6208_QC04	107	104.5	104	105	104
LNA	HP06+03_6209_QC04	5520	5600	5490	5465	5505
	LP_06553_QC04	3010	3125	3110	2995	3080
	MP06+02_6208_QC04	3250	3185	3200	3190	3180

d. Heating Block Temperature Summary: Varying the temperature of the heating blocks within 183 – 255°F does not appear to affect ARA, DHA, EPA or LNA concentrations.

2. Heating Block versus Oven for Hydrolysis

a. Principle: The heating block temperature can fluctuate causing high temperature spikes, which can lead to evaporation of the solvent during the hydrolysis step. This loss of solvent can affect the recovery of some of the analytes causing a higher sample repeat rate.

b. Proposal: To compare the high calibrator, high and low QC in triplicate in the oven and heating block and measure the peak area ratio to determine if there are any differences between using an oven or a heating block. Both the heating block and oven were set at 104°C.

c. Findings:

Table1: Effect of using a heating block versus an oven during the hydrolysis step on the mean peak area ratio (PAR)

Analyte Code	cal 40			HP06+03_6209_QC04			LP_06553_QC04			Average
	Heating block	Oven	% diff	Heating block	Oven	% diff	Heating block	Oven	% diff	% diff PAR
ALN	2.51	2.52	0%	0.97	0.95	-2%	0.22	0.22	1%	0%
AR1	1.61	1.69	5%	0.21	0.22	6%	0.13	0.15	11%	7%
ARA	4.76	4.79	1%	2.68	2.65	-1%	1.15	1.18	2%	1%
DA1	1.26	1.35	8%	0.42	0.46	10%	0.25	0.29	15%	11%
DE1	0.37	0.42	15%	0.11	0.15	44%	0.08	0.1	28%	29%
DHA	4.3	4.34	1%	0.76	0.76	-1%	0.5	0.51	3%	1%
DP3	1.23	1.28	4%	0.34	0.35	2%	0.17	0.18	5%	3%
DP6	0.61	0.64	5%	0.15	0.15	1%	0.06	0.06	5%	3%
DTA	0.75	0.8	7%	0.23	0.24	4%	0.11	0.12	12%	7%
ED1	0.13	0.13	4%	0.1	0.1	3%	0.04	0.05	7%	5%
EN1	0.15	0.16	8%	0.09	0.1	5%	0.03	0.03	8%	7%
EPA	3.27	3.3	1%	0.25	0.25	0%	0.14	0.14	1%	0%
GLA	1.38	1.38	0%	0.45	0.45	-1%	0.35	0.35	2%	0%
HGL	1.53	1.57	2%	1.02	1.02	1%	0.79	0.83	5%	2%
LG1	1.21	1.35	12%	0.33	0.38	14%	0.21	0.25	17%	14%
LNA	3.37	3.39	1%	2.39	2.37	-1%	1.3	1.32	2%	0%
ML1	0.51	0.5	-1%	0.05	0.05	-3%	0.06	0.06	-1%	-2%
MR1	4.36	4.39	1%	1.63	1.63	0%	0.95	0.97	2%	1%
NR1	7.66	8.52	11%	0.47	0.52	11%	0.39	0.46	17%	13%
OL1	4.06	4.09	1%	3.32	3.31	0%	1.08	1.09	1%	1%
PL1	6.53	6.55	0%	1.34	1.26	-7%	0.55	0.54	-3%	-3%
PM1	5.13	5.15	0%	3.17	3.14	-1%	1.29	1.31	2%	0%
ST1	6.17	6.2	1%	3.48	3.45	-1%	1.71	1.72	1%	0%
VC1	0.77	0.78	1%	0.38	0.39	1%	0.16	0.16	-1%	0%

d. Using a heating block versus an oven for hydrolysis summary: The overall average percent difference for all analytes was 4%. There appears to be no difference in using the heating blocks verus the oven on any analyte, therefore either can be used. The oven is preferred because the temperature is easier to maintain and there are fewer samples evaporating as compared to when the heating blocks were used.

3. Derivitization

a. Principle: The extract is derivatized with pentaflurobenzyl bromide in the presence of triethylamine (TEA) to form pentaflurobenzyl (PFB) esters. The current method specifies to add 10uL of TEA to the bottom of the tube and then add 100uL of 7% PFBBr in acetonitrile and wait 15 minutes to allow sufficient time for reaction.

b. Proposal: To compare adding TEA and PFBBr solution separately or mixed together prior to addition to extract. To allow reaction to take place for an additional 10 minutes to verify that 15 minutes is sufficient. Only one set of calibrators (set defined as cal 40a, 30a, 20a, 10a, and blank) per factor was used.

c. Findings:

Table1: Effect of mixing TEA with PFBBr solution or adding separately on the concentrations of ARA, DHA, EPA, and LNA

Analyte	Factor	cal 40a (uM)	cal 30a (uM)	cal 20a (uM)	cal 10a (uM)	blank (uM)
ARA	TEA added separate; rxn time 15 min	2120	470	198	80.9	12.1
	TEA mixed prior to addition; rxn time 15 min	2100	462	191	83.5	11.7
	TEA added separate; rxn time 25 min	2105	461	189	79.8	11.7
	TEA mixed prior to addition; rxn time 25 min	2069	472	196	80.3	11.8
DHA	TEA added separate; rxn time 15 min	1042	240	99.2	40.3	6.60
	TEA mixed prior to addition; rxn time 15 min	1032	236	97	41.2	6.30
	TEA added separate; rxn time 25 min	1058	236	95.6	38.8	6.40
	TEA mixed prior to addition; rxn time 25 min	1036	242	98.8	39.4	6.40
EPA	TEA added separate; rxn time 15 min	829	192	79.9	32.2	5.10
	TEA mixed prior to addition; rxn time 15 min	831	189	77.9	33.1	5.00
	TEA added separate; rxn time 25 min	848	191	77.3	31.2	5.00
	TEA mixed prior to addition; rxn time 25 min	828	192	80.2	31.7	5.00
LNA	TEA added separate; rxn time 15 min	8072	1954	814	319	12.2
	TEA mixed prior to addition; rxn time 15 min	7941	1920	794	328	10.0
	TEA added separate; rxn time 25 min	8224	1956	779	310	9.70
	TEA mixed prior to addition; rxn time 25 min	8022	1945	806	314	10.5

d. Adding TEA separately or mixed with PFBBr solution summary: Mixing the TEA with the PFBBr solution prior to adding to the extract does not appear to affect ARA, DHA, EPA, or LNA, however in the past if TEA is not properly added to the bottom of the tube containing the extract, then the derivatization is not complete. So since it yields similar results, TEA will be added to the PFBBr solution just prior to aliquotting to achieve the most consistent derivatization possible.

Table 2: Effect of longer reaction time of TEA and PFBBr has on the peak area of ARA, DHA, EPA, and LNA in calibrators

Analyte	Factor	cal 40a (analyte peak area)	cal 30a (analyte peak area)	cal 20a (analyte peak area)	cal 10a (analyte peak area)	blank (analyte peak area)
ARA	TEA added separate; rxn time 15 min	10,342,800	2,423,610	690,931	272,022	2,418
	TEA mixed prior to addition; rxn time 15 min	11,474,800	1,841,460	799,494	297,310	704
	TEA added separate; rxn time 25 min	7,828,580	871,874	608,026	231,309	488
	TEA mixed prior to addition; rxn time 25 min	10,408,500	1,854,230	763,023	258,501	1,080
DHA	TEA added separate; rxn time 15 min	16,794,800	4,197,870	1,181,700	460,744	5,053
	TEA mixed prior to addition; rxn time 15 min	21,171,200	3,372,700	1,465,720	519,113	1,757
	TEA added separate; rxn time 25 min	13,903,800	846,652	1,033,040	380,262	1,841
	TEA mixed prior to addition; rxn time 25 min	18,192,600	3,370,860	1,389,410	438,293	2,848
EPA	TEA added separate; rxn time 15 min	14,293,900	3,506,330	971,106	375,213	3,419
	TEA mixed prior to addition; rxn time 15 min	17,305,900	2,731,130	1,195,730	423,339	2,067
	TEA added separate; rxn time 25 min	11,268,600	879,146	864,994	311,999	1,149
	TEA mixed prior to addition; rxn time 25 min	15,188,900	2,722,590	1,136,990	356,939	2,323
LNA	TEA added separate; rxn time 15 min	2,192,640	479,788	128,988	50,473	860
	TEA mixed prior to addition; rxn time 15 min	2,225,670	338,265	145,263	53,237	434
	TEA added separate; rxn time 25 min	1,545,730	221,902	111,004	41,484	358
	TEA mixed prior to addition; rxn time 25 min	2,087,520	337,335	134,607	46,523	540

e. **Increasing reaction time summary:** Increasing the reaction time, does not appear to increase the peak area for ARA, DHA, EPA or LNA, therefore either 15 or 25 minutes are equivalent and may be used.

4. Time length before injection

a. Principle: Occasionally it is necessary to store the prepared samples at -20°C until they can be run on an instrument at a later time.

b. Proposal: To vary storage time of prepared samples at -80°C to determine if there are effects on analyte concentrations. Experiment 1: 325 patient samples, quality control materials and calibrators were analyzed in March 2010 and re-injected May 2011. Experiment 2: 266 patient samples, quality control materials and calibrators were analyzed in March- April 2010 and re-injected June 2013.

c. Findings: Tables 1 and 2 show the comparison of linear regressions for each experiment.

Table 1: 14 months at -80°C

Analyte	n	R^2	Intercept	Slope
ALN	325	1.00	0.67	0.99
AR1	325	1.00	-0.68	1.01
ARA	325	1.00	-9.96	1.01
DA1	325	0.99	-0.70	1.00
DE1	325	1.00	0.12	1.00
DHA	325	1.00	-2.14	1.00
DP3	325	1.00	-0.02	1.01
DP6	325	1.00	-0.01	1.00
DTA	325	1.00	0.00	1.01
ED1	325	1.00	0.07	1.00
EN1	325	1.00	-0.13	1.00
EPA	325	1.00	-0.74	1.01
GLA	325	1.00	0.26	0.99
HGL	325	0.99	-0.81	0.99
LG1	325	0.99	-0.07	1.00
LNA	325	1.00	-102	1.02
ML1	323	1.00	0.2	1.00
MR1	325	1.00	-1.22	1.00
NR1	325	1.00	-1.25	1.02
OL1	325	1.00	-29.4	1.01
PL1	325	0.99	4.41	1.02
PM1	325	1.00	45.0	0.98
ST1	325	1.00	-18.1	1.02
VC1	325	0.92	29.2	0.93

Table 2: 3 years and 3 months at -80°C

Analyte	n	R^2	Intercept	Slope
ALN	265	1.00	0.36	1.00
AR1	266	1.00	-0.67	1.02
ARA	264	1.00	-24.0	1.01
DA1	266	0.99	-2.30	1.03
DE1	266	1.00	-0.11	1.01
DHA	264	1.00	-2.34	1.01
DP3	264	1.00	0.69	1.02
DP6	262	1.00	-0.23	1.00
DTA	262	1.00	-0.11	1.01
ED1	262	1.00	0.37	1.02
EN1	265	1.00	0.00	1.00
EPA	263	1.00	-0.25	1.01
GLA	263	1.00	0.73	1.00
HGL	263	1.00	-0.23	1.00
LG1	265	0.99	-1.98	1.03
LNA	266	1.00	10.5	1.01
ML1	262	1.00	0.21	1.01
MR1	266	1.00	-6.55	1.02
NR1	264	1.00	0.48	1.03
OL1	266	1.00	-11.6	1.01
PL1	264	0.99	3.91	1.01
PM1	266	0.99	-117	1.08
ST1	266	0.98	-43.1	1.01
VC1	266	0.95	29.4	0.91

d. Stored extracted samples for varying lengths of time summary: There is essentially no difference in prepared samples tested up to 3 years later.

5. Sonication of calibrators/samples prior to aliquotting

a. Principle: When preparing stock standard solutions (p.6), it is necessary to sonicate some of the individual analytes to go into toluene prior to preparing the working standards. Determine how long the sonication should be to solubilize the material but not to destroy it.

b. Proposal: To vary sonication time (15 – 90 minutes) of the high calibrator to determine if there is an effect on analyte concentration.

c. Findings: The table shows the average of 4 replicates across 4 sonication time points (15, 30, 60, and 90 minutes)

Analyte	Avg (uM)	SD	CV
Fatty acids - Saturated			
AR1	164	15	9%
DA1	236	27	11%
LG1	123	16	13%
MR1	473	9	2%
PM1	5537	100	2%
ST1	1710	33	2%
Fatty acids - Monounsaturated			
EN1	62.9	3	5%
DE1	63.5	5	8%
ML1	50.8	2	3%
NR1	131	11	9%
OL1	6591	129	2%
PL1	380	7	2%
VC1	961	26	3%
Fatty acids - Polyunsaturated			
ALN	361	7	2%
ARA	2542	46	2%
DHA	801	17	2%
DP3	158	4	2%
DP6	79.9	2	2%
DTA	77.9	2	3%
ED1	64.3	2	4%
EPA	1101	23	2%
GLA	181	4	2%
HGL	383	7	2%
LNA	7898	125	2%

d. Sonication time summary: There does not appear to be a difference in concentration for any analyte with increased sonication time. Therefore a minimum of 15 minutes sonication time will be done for all standards, QC and samples prior to aliquotting.