Laboratory Procedure Manual

Analyte: **Vitamins A, E, and carotenoids**

Matrix: **Serum or Plasma**

Method: Liquid Chromatography Photodiode Array

Method No: 4020.05

Revised: 6/2020

as performed by:

Fat-soluble Micronutrients 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.

Public Release Data Set Information

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

Data File Name	Variable Name	SAS Label
VITAEC_J	LBXALC	alpha-carotene (ug/dL)
	LBDALCSI	alpha-carotene (umol/L)
	LBXARY	alpha-crypotoxanthin (ug/dL)
	LBXARYSI	alpha-crypotoxanthin (umol/dL)
	LBXBEC	trans-beta-carotene (ug/dL)
	LBDBECSI	trans-beta-carotene (umol/L)
	LBXCBC	cis-beta-carotene (ug/dL)
	LBDCBCSI	cis-beta-carotene (umol/L)
	LBXCRY	beta-cryptoxanthin (ug/dL)
	LBDCRYSI	beta-cryptoxanthin (umol/L)
	LBXGTC	gamma-tocopherol (ug/dL)
	LBDGTCSI	gamma-tocopherol (umol/L)
	LBXLUZ	Lutein and zeaxanthin (ug/dL)
	LBDLUZSI	Lutein and zeaxanthin (umol/L)
	LBXLYC	trans-lycopene (ug/dL)
	LBDLYCSI	trans-lycopene (umol/L)
	LBXRPL	Retinyl palmitate (ug/dL)
	LBDRPLSI	Retinyl palmitate (umol/L)

	LBXRST	Retinyl stearate (ug/dL)
	LBDRSTSI	Retinyl stearate (umol/L)
	LBXLCC	Total Lycopene (ug/dL)
	LBDLCCSI	Total Lycopene (umol/L)
	LBXVIA	Retinol (ug/dL)
	LBDVIASI	Retinol (umol/L)
	LBXVIE	alpha-tocopherol (ug/dL)
	LBDVIESI	alpha-tocopherol (umol/L)

1. Summary of Test Principle and Critical Relevance

A. Clinical Relevance

This HPLC fat-soluble vitamins assay measures three classes of compounds (vitamins A (retinol) and E and carotenoids), with each class of nutrients being measured at a different wavelength. While there are some common characteristics, each class of analytes has distinct chemical properties and physiological functions. The following five analytes: retinol, alpha-tocopherol, lutein/zeaxanthin, lycopene, and beta-carotene are generally present in measurable amounts in most sera. These analytes are either required nutrients or have been associated with health effects in epidemiological studies. Less is known about health effects associated with the other analytes (lycopene, alpha-cryptoxanthin, beta-cryptoxanthin, cis-beta-carotene, and gamma-tocopherol).

Worldwide, vitamin A deficiency is the leading cause of preventable blindness. Although vitamin A deficiency is uncommon in the US, it is associated with excess morbidity and mortality from infectious disease in developing countries. Toxicity related to excess consumption of vitamin A can lead to permanent liver damage and death. Serum retinyl esters are of interest generally only in fasting specimens and are used to indicate potential hepatotoxicity in subjects with elevated serum retinol concentrations.

Vitamin E has low potential for toxicity. Elevated serum vitamin E concentrations are only of concern in people receiving anticoagulant therapy. Low serum concentrations are rarely observed, except in those with malabsorption syndromes.

A physiological need for the carotenoids, except as vitamin A precursors, has not been established. Excess consumption of carotenoids may cause red or orange discoloration of the skin as a result of carotenoid deposits in subcutaneous fat. Several xanthophylls are found in the macular pigment in the eye where they may protect against macular degeneration.

B. Test Principle

Serum concentrations of retinol and vitamin E (alpha- and gamma -tocopherol), two retinyl esters, and seven carotenoids are measured using a modification of a high performance liquid chromatography with photodiode array detection method (1). A small volume (100 uL) of serum is mixed with an ethanol solution containing two internal standards- retinyl butyrate and nonapreno-beta-carotene (C45). The micronutrients are extracted from the aqueous phase into hexane and dried under vacuum. The extract is re-dissolved in ethanol and acetonitrile, and it is filtered to remove any insoluble material. An aliquot of the filtrate is injected onto a C18 reversed phase column and isocratically eluted with a mobile phase consisting of equal parts of ethanol and acetonitrile. Absorbance of these substances in solution is linearly proportional to concentration (within limits), thus spectrophotometric methods are used for quantitative analysis. Three wavelengths, approximately corresponding to absorption maxima, namely, 300, 325, and 450 nm, are simultaneously monitored and chromatograms are recorded. Quantitation is accomplished by comparing the peak height or peak area of the analyte in the unknown with the peak height or peak area of a known amount of the same analyte in a calibrator solution. Calculations are corrected based on the peak height or peak area of the internal standard in the unknown compared with the peak height or peak area of the internal standard in the calibrator solution. Retinol and the retinyl esters are compared with retinyl butyrate at 325 nm, alpha- and gamma -tocopherol are compared with retinyl butyrate at 300 nm, and the carotenoids are compared with C45 at 450 nm.

2. Safety Precautions

Consider all serum specimens received for analysis 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 serum. Observe universal precautions; wear laboratory coats, safety glasses, and protective gloves during all steps of this method. Disposable face shields are highly recommended when working with acids or bases. Discard any residual sample material by autoclaving after analysis is completed. Place all plastic and glassware that contacts serum in an autoclave bag for disposal. Handle acids and bases with extreme care; they are corrosive or caustic and damaging to living tissues.

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

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 photodiode array 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 manual integration in some cases. 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 **4020_SOP Computerization & Data Management** for a step-by-step description of data transfer, review and approval.

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 and care should be taken to avoid exposure of the serum or plasma to sunlight or other sources of full spectrum radiation.

B. Specimens for fat soluble vitamin analysis may be fresh or frozen. Serum should be harvested from blood collected in red-top or royal blue-top Vacutainer® tubes by using standard venipuncture procedures. EDTA- or heparinized- plasma may be used instead of serum.

C. A 500-uL sample of serum is preferable to allow sufficient material to conduct initial analysis and repeats, but a sample volume of as little as 200 uL may be accepted.

D. Specimens collected in the field are frozen and then shipped on dry ice by overnight carrier. Frozen samples are stored at -70°C. All analytes for this assay are stable in matrix stored for at least 5 years at -70°C. Sample quality may degrade with successive freeze thaw cycles.

E. Specimens should arrive frozen. Refrigerated samples may be used provided that they are kept cold and brought promptly (within 2 hours) from the site of collection.

F. Improperly handled specimens which have been through more than five freeze thaw cycles, have been refrigerated for more than 24 hours, or have undergone hemolysis may give inaccurate results for one or more primary analytes, particularly the carotenoids (i.e., beta carotene, lycopene, or lutein/zeaxanthin). The retinyl ester concentration of non-fasting serum is generally non-informative.

G. Specimen handling conditions are outlined in the DLS Policies and Procedures Manual. The protocol discusses in general collection and transport of specimens and the special equipment required. If there is more than one test of interest in the specimen and it needs to be divided, the appropriate amount of serum or plasma should be transferred into a Nalge cryovial labeled with a new specimen ID linked to the participant's ID; avoid cross-contamination.

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) **Mobile Phase**

Component 1: 100% ethanol (200 proof) with 0.1% triethylamine (TEA)
Component 2: 100% acetonitrile (HPLC grade) with 0.1% TEA
The solvents for each reservoir are filtered separately using 0.45-um pore size membranes (Cat. no. HVHP04700; Millipore, Medford, MA). Prior to filtering, TEA is added to each solvent at approximately 32 drops per liter using a glass Pasteur pipette to make 0.1% TEA solutions.

2) **10% Ascorbic Acid and 10% NaCl**

Weigh 0.5 g L-Ascorbic Acid (ACS Certified) and 0.5 g NaCl (Analytical Grade) and dissolved in 5.00 mL deionized water in a 12 x 75-mm test tube. The ascorbic acid is relatively labile but normally can be used for 2 weeks or until there is a noticeable yellow color to the solution. The solution is covered with parafilm and stored at room temperature under gold fluorescent lights.

B. Standards Preparation (performed under gold fluorescent lights)

(1) **Purified Stock Standard Solutions**
Stock solutions of all analytes are prepared by further purifying commercially purchased materials. For each analyte, a small amount of the concentrated material dissolved in chloroform or ethanol (zeaxanthin) is repeatedly injected on to a HPLC column that is selective for that analyte (see Table 1 for list of columns). A segment of the eluting peak is collected, pooled, and then measured for concentration via spectrophotometer at 300nm (tocopherols), 325nm (retinols), or 450nm (carotenoids). Dilutions to the purified materials are done with mobile phase or ethanol to prepare calibration materials to approximately the concentration shown in Table 1. See section 6b (3) below for final working standards solution preparation. The

final working solution is aliquotted into pre-labeled amber vials and stored at -70ºC. See **4020_SOP Purification of AECar Isocratic standards** for detailed step by step instructions on the purification process.

(2) **Purified Stock Internal Standard Solutions**

Stock solutions of the internal standard analytes (retinyl butyrate and nonapreno-beta-carotene) are prepared by further purifying the synthesized materials. Retinyl butyrate was internally synthesized and nonapreno-beta-carotene was contractually synthesized. For each analyte, a small amount of the concentrated material dissolved in chloroform is repeatedly injected on to a column that is selective for that analyte (see Table 1 for list of columns). A segment of the eluting peak is collected and pooled then measured for concentration via a spectrophotometer at 325 nm (retinyl butyrate) or 482 nm (nonapreno-beta-carotene). Dilutions to the purified materials are done with ethanol with 0.1% TEA to prepare internal standard materials at approximate concentrations of 0.32 AU for nonapreno-beta-carotene and 0.25 AU for retinyl butyrate. These two solutions are mixed 1:1 to make the final internal standard. The internal standard mixture is aliquoted into 20 mL glass vials with teflon-lined caps (KIMBLE Glass Inc., Vineland, NJ. part # 74500-20) with minimal headspace and stored at -70˚C. See **4020_SOP Purification of AECar Isocratic standards** for detailed step-by-step instructions on the purification process.

Table 1. Extinction coefficients (EC) used to calculate concentrations in standard stock solutions (EC provided by NIST from materials dissolved in ethanol, unless otherwise indicated.

Analyte	Extinction Coefficient* (dL/g·cm)	CDC Wavelength (nm) (NIST)	Target Concentrations of Stock Solutions (ug/dL)	Column used for purification	Solvent used for value assignment
Standards					
Retinol	1850 CDC (1843 NIST)	325 (325)	50	Burdick and Jackson OD5 octadecylsilane 150 x 4.6 mm, 5 um	50:50 ethanol:acetonitrile with 0.1% TEA
Retinyl palmitate	1850 CDC (975 NIST)	325 (325)	20**	"	50:50 ethanol:acetonitrile with 0.1% TEA
Retinyl stearate	1850 CDC	325	20**	"	50:50 ethanol:acetonitrile with 0.1% TEA
gamma-Tocopherol	91.4 CDC (91.4 NIST)	298 (298)	1350	"	50:50 ethanol:acetonitrile with 0.1% TEA
alpha-Tocopherol	75.8 CDC (75.8 NIST)	292 (292)	3165	"	50:50 ethanol:acetonitrile with 0.1% TEA
beta-Cryptoxanthin	2370 CDC (2356 NIST)	454 (452)	57	"	50:50 ethanol:acetonitrile with 0.1% TEA
Lycopene (NIST in hexane)	3450 CDC (3450 NIST)	474 (472)	24	"	50:50 ethanol:acetonitrile with 0.1% TEA
alpha-Carotene	2725 CDC (2800 NIST)	447 (444)	36	"	50:50 ethanol:acetonitrile with 0.1% TEA
beta-Carotene (NIST in hexane)	2560 CDC (2560 NIST)	454 (450)	23	"	50:50 ethanol:acetonitrile with 0.1% TEA
Zeaxanthin	2540 CDC (2540 NIST)	454 (450)	63	YMC C30 Carotenoid 150 x 4.6 mm column	50:50 ethanol:acetonitrile with 0.1% TEA
Internal Standards					
Nonapreno-beta-carotene (C45)	NA (absorbance only)	482	NA for this method (only absorbance used)	Luna C18 250 x 10 mm, 5um	100% ethanol with 0.1% TEA
Retinyl butyrate (RB)	NA (absorbance only)	325	NA for this method (only absorbance used)	Luna C18 250 x 10 mm, 5um	100% ethanol with 0.1% TEA

*$A^{1\%}_{1cm}$ is defined as the theoretical absorbance of a 1% solution (1g/100 mL) in a cell of 1 cm pathlength.

** Concentrations of retinyl esters are reported as retinol equivalents by the NHANES lab.

(3) Mixed Standards (Working Standards)
Refer to Table 2 in section 7 for working standard concentrations.
Retinyl Ester: A retinyl ester standard is prepared by mixing equal volumes of retinyl palmitate and retinyl stearate stock solutions using one volumetric pipette and rinsing the pipette with 50:50 ethanol:acetonitrile between each standard. Retinyl stearate is synthesized in-house.

Tocopherols: A mixed vitamin E standard is prepared by mixing equal parts of alpha-tocopherol and gamma-tocopherol using one volumetric pipette and rinsing the pipette with 50:50 ethanol:acetonitrile between each standard.

Carotenoids: A carotenoid mixed standard is prepared by mixing equal volumes of alpha-carotene, zeaxanthin and beta-cryptoxanthin using one volumetric pipette and rinsing the pipette with 50:50 ethanol:acetonitrile between each standard.

(4) Individual Un-Mixed Standards (Working Standards)
Refer to Table 2 in section 7 for working standard concentrations.
Some standards are aliquotted as individual compound standards and are not mixed with any other solutions. These standards can be periodically reassessed for concentration changes on the spectrophotomer. These working standard standards are retinol, lycopene and beta-carotene. The working standards are stable for at least 26 weeks up to 2 years.

(5) Retinyl stearate synthesis: See **4020_SOP Purification of AECar Isocratic standards** for step by step instructions.

(6) Retinyl butyrate (RB) synthesis: See **4020_SOP Purification of AECar Isocratic standards** for step by step instructions.

(7) nonapreno-beta-carotene (C45) synthesis: Synthesized under contract to CDC

C. Preparation of Quality Control Materials

1) Bench Quality Control

All serum is filtered through sterile gauze to remove fibrin prior to aliquotting into 2-mL Nalge cryovials. Typical aliquot volumes are 500uL per vial. The vials are ideally blanketed with nitrogen or argon before sealing, but this is not required. The QC pools are stored at -70°C and are stable with little degradation for more than 10 years. Approximate QC target values for serum fat-soluble micronutrients are the lower third of population distribution for each analyte and the upper third of population distribution for each analyte. The most current distributions can be found in the latest National Report on Biochemical Indicators of Diet and Nutrition in the US Population [1]. Pools can be prepared by selecting and blending sera that contain appropriate levels of the analyte or diluting the sera with a saline solution to the desired concentration. Spiking is generally successful only for retinol. People who eat very large quantities of fresh fruits and vegetables and have high serum lipid concentrations are most likely to have high concentrations of fat-soluble micronutrients. In some instances dog serum, which typically has a high retinyl ester concentration, is added to the pools. Other types of subjects useful for blending into the high pool are Type 2 diabetics who, in the absence of good glycemic control, may have high concentrations of lutein/zeaxanthin, beta-cryptoxanthin and

alpha-tocopherol. Sera from individuals taking vitamin supplements are also used. Limits for all pools are established by analyzing duplicates for at least 20 runs.

2) Blind Quality Control

All serum is filtered through sterile gauze before being stabilized with 6 g/dL MPA. A 1:5 dilution of serum in 6.0 g/dL MPA are aliquoted into sterile 2-mL Nalge cryovial, sealed, and vortexed. The blind QC pools are stored at -70°C and are stable with little degradation for more than 10 years. Screen serum blood bank donors for endogenous fat-soluble micronutrients. Donor sera are typically blended to achieve appropriate concentrations. Limits for all pools are established by analyzing duplicates for at least 20 runs.

D. Other Materials

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

1) General Supplies

a) Autosampler 12x31mm widemouth vials (TCW224626, Wheaton, Millville, NJ)
b) 250-uL polypropylene inserts (TCW225259, Wheaton, Millville, NJ)
c) screw caps with teflon/silicone septa (TCW242762, Wheaton, Millville, NJ)
d) Assay Column: 15cm x 4.6mm Phenomenex Ultracarb 3 C18, 3µm particle size column (Phenomenex, Torrance, CA)
e) Serum extract filters: 0.45 um syringe tip PVDF hydrophilic filter (4 mm diameter) (Millipore Corp, Medford, MA)
f) Solvent filters: 0.45 µm pore size, PVDF (HVHP04700, Millipore Corp, Medford, MA)
g) Plastic tuberculin syringes (obtained from various sources)
h) 2 mL polypropylene cryovials (Fisher Scientific, Inc, Fairlawn, NJ)
i) 12 x 75 mm disposable glass culture tubes (Fisher Scientific, Inc, Fairlawn, NJ)
j) 13 x 100 mm disposable glass culture tubes (Fisher Scientific, Inc, Fairlawn, NJ)
k) 5¾ inch pasteur pipettes (Fisher Scientific, Inc, Fairlawn, NJ)
l) Combitip Plus (0.5 mL) for Eppendorf repeater pipette (Eppendorf)
m) Combitip Plus (2.5 mL) for Eppendorf repeater pipette (Eppendorf)
n) Rainin tips for LTS pipette (Rainin, Woburn, MA)
o) Various glass beakers, graduated cylinders and glass bottles, class A glassware and actinic glassware
p) Waters Guard-Pak Module (cat. no. WAT 88141) with Guard Pak filters (cat. no. WAT032472)
q) Aluminum foil (Fisher)

2) Purification supplies
    a) Columns:
        1. 15 cm x 4.6 mm Burdick and Jackson OD5 C18 5µm particle size column (Burdick and Jackson Laboratories, Muskegan, MI)
        2. 15 cm x 4.6 mm YMC C30 Carotenoid column (YMC, Wilmington, NC)
        3. 25 cm x 10 mm Phenomenx 5 um C18 Luna column (Phenomenex, Torrance, CA)
    b) Autosampler vials 12x31mm widemouth vials (cat. no. TCW224626; Wheaton)
    c) 250-uL glass inserts with spring foot (for any chloroform diluted materials)
    d) Screw caps with teflon/silicone septa (cat. no. TCW242762, Millville, NJ)

3) Chemicals
    a) Hexane UV (Burdick and Jackson Laboratories, Muskegan, MI)
    b) Acetonitrile HPLC grade (Burdick and Jackson Laboratories, Muskegan, MI)
    c) Ethanol, absolute (USP), glass bottles only, (Pharmco Products, Brookfield, CT)
    d) Chloroform, spectrophotometric grade (Mallinckrodt, St. Louis, MO)
    e) Triethylamine, 'Baker' grade (Fisher Scientific, Inc, Fairlawn, NJ)
    f) L-Ascorbic acid, ACS grade (Fisher Scientific, Inc, Fairlawn, NJ)
    g) Sodium Chloride, ACS grade (Fisher Scientific, Inc, Fairlawn, NJ)
    h) Stearic anhydride (Sigma Chemical Co, St. Louis, MO) for synthesis of retinyl stearate
    i) Butyric anhydride (Sigma Chemical Co, St. Louis, MO) for synthesis of retinyl butyrate
    j) Alumina, Grade III (obtained from various sources)
    k) Methanol, HPLC grade, (Fisher Scientific, Inc, Fairlawn, NJ)
    l) Argon, Ultrapure (Air Products, Inc, Chamblee, GA)
    m) Nitrogen (Air Products, Inc, Chamblee, GA)

4) Standards
    a) Retinol (Sigma Chemical Co, St. Louis, MO)
    b) Retinyl palmitate (Sigma Chemical Co, St. Louis, MO)
    c) alpha-Tocopherol (Sigma Chemical Co, St. Louis, MO)
    d) gamma-Tocopherol (Eastman Chemical Co, Kingsport, TN)
    e) Zeaxanthin (Extrasynthese, Genay, France)
    f) beta-Cryptoxanthin (Extrasynthese, Genay, France)
    g) Lycopene (Sigma Chemical Co, St. Louis, MO)
    h) alpha-Carotene (Sigma Chemical Co, St. Louis, MO)
    i) beta-Carotene (Sigma Chemical Co, St. Louis, MO)
    j) alpha-cryptoxanthin (Dr. Fred Khachik, Kemin Industries, Des Moines, IA)
    k) nonapreno-beta-carotene (Georgia State University)

E. Instrumentation

In the case of simple laboratory instrumentation (e.g., pipettes, vortex mixer, analytical balance, etc.) a product listed herein may be substituted with equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed. In the case of analysis instrumentation (e.g., LC components, photodiode array) equivalent performance must be demonstrated experimentally in accordance with DLS Policies and Procedures Manual if a product substitution is made. Equivalent performance must also be demonstrated in accordance with DLS policies and procedures when multiple analysis systems are used in parallel, even if they are of the exact same type.

1) Waters HPLC system (Waters Chromatography Division, Milford, MA)
    a) Alliance HPLC model 2695
    b) Waters photodiode array detector (PDA) model 2996
    c) Cera column cooler 250 (Cera, Inc. Baldwin Park, CA)
2) Rack-type vortex mixer
3) Speedvac SC200 and SC210A systems
4) Precision Model VP 190 Direct Drive Vacuum Pump
5) Refrigerated vapor trap, model RVT-4104
6) Magnetic stirrer
7) Digiflex Automatic Diluter/Dispenser, with 200uL sampling and 2.0mL dispensing syringes
8) Rainin LTS single channel pipettes
9) Eppendorf repeater pipettes
10) Gilson positive displacement pipettes
11) Mettler XPE205 analytical balance
12) Cary 300 Bio UV-visible spectrophotometer (Varian instruments)

7. Calibration and Calibration Verification Procedures

A. Method Calibration

At the beginning of each run, a single set of calibrators is prepared. For each calibration solution, 100 uL of the internal standard is combined with 100 uL of the working standard solution using a positive displacement pipette. Thus, each calibrator contains half as much of each component as the working concentrations (**Table 2**).

Table 2. Calibrator concentrations for fat-soluble micronutrient assay

Analyte	Stock Concentration (purified unmixed)	True Final Concentration (Std mixed + IS)	Working Concentration (Instrument Value)	Internal Standard (IS)
Individual Standards				
Retinol	50 ug/dL ± 10%	25 ug/dL ± 10%	50 ug/dL ± 10%	Retinyl butyrate
beta-Carotene, trans-	36 ug/dL ± 10%	6 ug/dL ± 10%	12 ug/dL ± 10%	nonapreno-beta-carotene
Lycopene, trans-	24 ug/dL ± 10%	12 ug/dL ± 10%	24 ug/dL ± 10%	nonapreno-beta-carotene
Mixed Standards				
Retinyl palmitate (Cal: retinyl ester)	20 ug/dL ± 10%	5 ug/dL ± 10%	10 ug/dL ± 10%	Retinyl butyrate
Retinyl stearate (Cal: retinyl ester)	20 ug/dL ± 10%	5 ug/dL ± 10%	10 ug/dL ± 10%	Retinyl butyrate
alpha-Tocopherol (Cal: tocopherols)	3165 ug/dL ± 10%	528 ug/dL ± 10%	1055 ug/dL ± 10%	Retinyl butyrate
gamma-Tocopherol (Cal: tocopherols)	1350 ug/dL ± 10%	225 ug/dL ± 10%	450 ug/dL ± 10%	Retinyl butyrate
Zeaxanthin (Cal: carotenoids)	63 ug/dL ± 10%	10.5 ug/dL ± 10%	21 ug/dL ± 10%	nonapreno-beta-carotene
beta-Cryptoxanthin (Cal: carotenoids)	57 ug/dL ± 10%	9.5 ug/dL ± 10%	19 ug/dL ± 10%	nonapreno-beta-carotene
alpha-Carotene (Cal: carotenoids)	23 ug/dL ± 10%	11.5 ug/dL ± 10%	23 ug/dL ± 10%	nonapreno-beta-carotene

The working concentrations are used to calibrate, this accounts for the fact that the serum or plasma extract is prepared using the starting volume of 100 uL to a final volume of 100 uL (no dilution factor) while the combined standard and internal standard in the calibrator are prepared by blending 100 uL of calibrator + 100 uL of IS (2x dilution factor). Using the working concentration for the calibration curve allows for the direct measurement of unknown concentrations off the curve without having to multiply the unknown concentration by 2x. The standards are read as calibrators at the beginning of each run. The calibrator value is used to generate a one-point standard curve forced through zero for each analyte. The concentrations of unknowns are calculated from the regression equation based on the area or height ratios between the analyte and internal standard. There are two analytes (alpha-cryptoxanthin and cis-beta-carotene) that use an appropriate equivalent standard for calibration. The beta-cryptoxanthin calibrator is used to calculate alpha-cryptoxanthin. The exctinction coefficient at 454nm for beta-cryptoxanthin is 2320 and similarly

2520 alpha-cryptoxanthin; both analytes have a molecular weight of 552.872 g/mol. The beta-carotene calibrator is used to calculate cis-beta-carotene. The exctinction coefficient at 454nm for beta-carotene is 2725 and is used for cis-beta-carotene; both analytes have a molecular weight of 536.88 g/mol.

The instrument software (Empower 3) performs all calculations. Calibration curves are linear and based on single injection analysis of a single standard concentration.

Calibration verification is conducted at least twice a year. Since the calibration procedure in this method does not include three or more levels of calibration material and does not includes a low calibrator near the LOD, mid, and high value, an additional requirement for calibration verification must be performed. For detailed instructions, see **4020_SOP Calibration and Calibration Verification.**

This method uses ethanol or ethanol:acetonitrile as the matrix for the calibrators. It is well known information from CDC and NIST that spiking serum does not work well for this method.

For troubleshooting and accuracy verification, NIST SRM 968e (Level I, II, and III) is available.

We participate in an external proficiency testing program for retinol, alpha- and gamma-tocopherol, lutein/zeaxanthin, beta-cryptoxanthin, trans- and total lycopene (cis- plus trans-), and alpha- and beta-carotene sponsored by the National Institute of Standards and Technology (NIST, Gaithersburg, MD). Round Robin materials are sent by NIST to assess laboratory performance up to one time per year. At the same time, certified reference materials (SRM) for retinol, alpha-tocopherol and beta-carotene, currently NIST SRM 968e (Certificate of Analysis kept by analyst), are analyzed to determine the agreement between results obtained with the CDC laboratory method and the certified values. Details can be found in the proficiency testing form. For general information on the handling, analysis, review, and reporting of proficiency testing materials see **NBB_SOP Proficiency Testing Procedure.** Additionally, an in-house proficiency testing program has been developed and is conducted one to two times per year. For details, see **4020_SOP In-House Proficiency Testing.**

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

C. Balances
Balances are calibrated annually and verified as used using calibrated weights.

D. Cary UV/vis spectrophotometer
Proficiency testing is done three times per year by participation in the CAP instrumentation survey. Additionally, every time the instrument is turned on there are internal diagnostics that are run. Calibration verification using certified filters is performed twice per year. Calibration verification of the certified filters are done externally every other year.

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 unknowns, quality controls, and standards to reach ambient temperature for less than 1 hour. Visually check each sample for unusual specimen color or debris/precipitate. All sample and calibrator handling is performed under gold fluorescent lights.
   3) Set up Excel run sheet containing sample IDs prior to starting sample preparation. This will be used to keep track of any problems that may occur during the sample preparation.
   4) Set-up and label one 12 x 75 mm glass culture tube, one 13x100 mm glass culture tube, and one HPLC vial per unknown/quality control. Set-up one 12x75mm glass culture tube and one HPLC vial per calibrator.
   5) Prepare a shallow (2-3" deep) ethanol and dry ice bath with temperatures reaching ≤ -70°C for proper freezing of the samples.
   6) A typical run consists of 6 calibrators, 3 bench QC samples (first set), 60 patient samples, 2 blind QC samples, 3 bench QC samples (second set).

B. Calibrator preparation
   1) Prepare calibrators by mixing 100 uL internal standard solution and 100 uL calibrator using a positive displacement pipette.
   2) Agitate the prepared calibrators and transfer to an HPLC vial using a Pasteur pipette. Cap immediately.

C. Sample preparation
   1) Extraction phase
      a. Dispense 10 uL of the 10% L Ascorbic Acid/20% NaCl solution into the bottom of each 13 x 100 mm glass culture tube using a repeater pipette.
      b. Add 100uL serum to each 13 x 100 mm glass culture tube.
      c. Cover the tubes with heavy duty aluminum foil and vortex for 60 seconds; do not allow liquid to touch foil during vortexing.
      d. Add 100uL internal standard solution to each 13 x 100 mm glass culture tube.
      e. Cover the tubes with heavy duty aluminum foil and vortex for 60 seconds; do not allow liquid to touch foil during vortexing.
      f. Add 1 mL hexane to each tube.
      g. Cover the tubes with heavy duty aluminum foil and vortex for six cycles at 60 seconds each cycle; do not allow liquid to touch foil during vortexing.
      h. Allow the samples to rest for 10 minutes in order for the aqueous and organic phases to fully separate.
      i. Transfer the rack to the ethanol and dry ice bath which is at least -70°C. Allow the aqueous phase to freeze (≥ 25 min).
      j. Pour the hexane (upper) layer of each tube into the second set of labeled 12x75 mm glass culture tubes.
      k. Evaporate the hexane in the Speedvac (without heat). Dry times will vary according to the number of tubes (9 minutes for 40 - 60 tubes, 10 minutes for 60 - 100 tubes, and 12 minutes for >100 tubes). Each Speedvac has slight differences in dry times which each analyst has documented. Make sure not to over-dry the extracts.

2) Reconstitution phase
   l. Add 50 uL of ethanol to the tubes containing the dried extracts.
   m. Cover the tubes with heavy duty aluminum foil and vortex the tubes for 2 cycles of 60 seconds each.
   n. Add 50 uL of acetonitrile to each dissolved extract.
   o. Cover the tubes with heavy duty aluminum foil and vortex the tubes for 2 cycles of 60 seconds each.
   p. Draw each extract into a 1.0-mL tuberculin syringe taking care to leave an air space between the plunger tip and the solution, place a 0.45-um pore-size syringe filter on the loaded syringe, and filter the extract into a prepared autosampler vial.
   q. Cap the vials, tap the vials to remove any bubbles, and place them in the autosampler compartment set to 20°C.

D. HPLC-PDA Instrument Preparation
   1) Refill solvents and/or mobile phase
   2) Pump: Wet prime solvent and seal wash lines: 5mL/min for 5 minutes
   3) Instrument method should contain the following parameters:
      a. Run time: 13.0 min (dependent upon column and column age)
      b. Pump pressure limits: 20-3000 psi
      c. Pump flow ramp: 3 min
      d. Degas mode: continuous
      e. PDA sampling frequency: 1.0 points per sec
      f. PDA resolution bandwidth: 4.8 nm
      g. PDA wavelengths: 270-480 nm
      h. Data rate: 10.56 KB/min
      i. Column temperature: 25°C
      j. Autosampler compartment temp: 20°C
      k. Pump Mode: Isocratic flow (see Table 3)

Table 3. Isocratic conditions

Time	Flow	% Ethanol	% Acetonitrile	Curve
	1.0	50	50	
16.0	1.0	50	50	6
20.0	0	50	50	1

   4) Collect baseline absorbance data while equilibrating with mobile phase (isocratic conditions 50:50 ethanol with 0.1% TEA:acetonitrile w/0.1% TEA, 1mL/min) for at least 1 hour before collecting sample chromatography.
   5) Start the analytical run after equilibration is finished.

E. Processing and reporting a run
   1) The Waters Empower 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 of chromatography review, see **4020_SOP Processing and reporting a run**.
      a) Reviewing the chromatography
         i. When the run is finished acquiring the data, the data is reviewed in Empower. Chromatograms for vitamins A, E, the carotenoids, and internal standards

(retinyl butyrate and nonapreno-beta-carotene) are checked for retention times, peak shapes, peak separation, intensity and/or potential interferences.

b) Quantitation and integration of the completed data file
   i. Generate results using auto integration.
   ii. Review integrations and make any necessary integration corrections either using the manual or auto integration option. Auto integration is preferred over manual integration.
   iii. Print the results for each analyte as a PDF to allow future review and documentation (routine procedure) or print hardcopies (exception).
   iv. Save the results in an ASCII file to import into the LIMS database.
   v. Import the results file into the LIMS database for further data review.

Calculations

The Empower software performs all calculations. Calibration curves are linear and are based on a single point. For each analyte not present in a standard solution, linkage to an appropriate standard is made and used to calculate concentrations. The concentration of the components of the mixed standards is equal to the concentration of the purified stock divided by the number of components of the solution, excluding the internal standards. Vitamins A, E, and the various carotenoid concentration in unknown samples are calculated using the regression parameters.

F. System Maintenance (other than daily maintenance)
Waters HPLC-PDA – Preventative maintenance is performed on an annual basis by a qualified service engineer. Routine maintenance should be performed as indicated in this document and in the Waters User Manuals.

G. CDC Modifications
This method is a modification of a method described by Sowell et al. (2).

9. Reportable Range of Results

This method is linear for the approximate ranges as follows: 1-100 ug/dL for the carotenoids, 30-100 ug/dL for retinol, 1-10 ug/dL for the retinyl esters, 50-500 ug/dL for gamma-tocopherol, and 500-2500 ug/dL for alpha-tocopherol. The coefficients of variation (CV) for vitamins A and E and beta-carotene are generally less than 5%. The CV for the minor carotenoids are generally less than 20%.

A repeat analysis with dilution may be performed to improve internal standard recovery or to confirm a result greater than the 97.5th percentile of the population (see Section 13 Table 4). If repeat analysis with dilution is required, mix a 50-uL aliquot of a diluent (saline or 4% albumin in PBS) plus a 50-uL aliquot of serum. The combined mixture should be used as the sample aliquot. All results from this aliquot must be multiplied by two. Note that dilutions do not provide accurate results for lutein/zeaxanthin or beta-cryptoxanthin.

10. Quality Control (QC) Procedures

A. Blind Quality Control

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 10 different pools and the analyte concentrations are similar to those found in patient samples.

B. Bench Quality Controls

Bench QC specimens are prepared from four serum pools, which represent low, intermediate, and high levels of fat-soluble micronutrients in serum. These pools are prepared in the same manner as patient samples and analyzed in duplicate at the beginning and end of each run.

The results from 2-3 pools per analyte are checked after each run. In 2 cases, the 3rd pool is <LOD. The system is declared "in control" if the results pass the following tests:

A. Multi-rule quality control system: quality control rules for two QC pools per run
    1. One QC result per pool
        a. If both QC run results are within 2 Si limits, accept the run
        b. If one of the two QC run results is outside a 2 Si limit, reject run if:
            i. 1 3S Rule—Run result is outside a 3 Si limit or
            ii. 2 2S Rule—Both run results are outside the same 2 Si limit or
            iii. 10 Xbar Rule—Current and previous nine run results are on the same side of the characterization mean or
            iv. R 4S Rule—Two consecutive standardized run results differ by more than 4 Si
    2. Two QC results per pool
        a. If both QC run means are within 2 Sm limits and individual results are within 2 Si limits, accept the run
        b. If one of the two QC run means is outside a 2 Sm limit, reject run if:
            i. 1 3S Rule—Run mean is outside a 3 Sm limit or
            ii. 2 2S Rule—Both run means are outside the same 2 Sm limit or
            iii. 10 Xbar Rule—Current and previous nine run means are on the same side of the characterization mean
        c. If one of the four QC individual results is outside a 2 Si limit, reject run if:
            i. Outlier—One individual result is beyond the characterization mean ±4 Si or
            ii. R 4S Rule—Within-run ranges for both pools in the same run exceed 4 Sw (i.e. 95 percent range limit)

B. Multi-rule quality control system: quality control rules for three QC pools per run
    1. One QC result per pool
        a. If all three QC run results are within 2 Si limits, accept the run
        b. If one of the three QC run results is outside a 2 Si limit, reject run if:
            i. 1 3S Rule—Run result is outside a 3 Si limit or
            ii. 2 2S Rule—Two or more of the three run results are outside the same 2 Si limit or
            iii. 10 Xbar Rule—Current and previous nine run results are on the same side of the characterization mean or
            iv. R 4S Rule—Two consecutive standardized run results differ by more than 4 Si
    2. Two QC results per pool
        a. If all three QC run means are within 2 Sm limits and individual results are within 2 Si limits, accept the run
        b. If one of the three QC run means is outside a 2 Sm limit, reject run if:
            i. 1 3S Rule—Run mean is outside a 3 Sm limit or
            ii. 2 2S Rule—Two or more of the three run means are outside the same 2 Sm limit or

iii. 10 Xbar Rule—Current and previous nine run means are on the same side of the characterization mean

c. If one of the six QC individual results is outside a 2 Si limit, reject run if:
   i. Outlier—One individual result is beyond the characterization mean ±4 Si or
   ii. R 4S Rule—Two or more of the within-run ranges in the same run exceed 4 Sw (i.e. 95 percent range limit)

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 (3). 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. Look for sample preparation errors, e.g., vial spilled during preparation or analyst forgot or under-pipetted the internal standard.

B. Look for extraction errors, e.g., isolated poor recovery suggests a sample spill. Poor recovery for the entire run suggests analyte degradation. Carotenoids are sensitive to air, light and heat, e.g. room too warm, samples at room temperature for longer than necessary, samples dried in SpeedVac too long, or samples exposed to full-spectrum light for longer than necessary. Additionally, verify the ascorbic acid antioxidant was prepared fresh and added to the serum samples according to directions.

C. Check the calibration of the pipettes.

D. Check to make sure that the hardware is functioning properly. Make sure the PDA status light is solid green. Check the autosampler to make sure the injections are being made as programmed. Make sure the pump is operating at an appropriate pressure with steady delivery.

E. 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.

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

12. Limitations of Method; Interfering Substances and Conditions

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

B. Calibrators, internal standards, quality control pools, and specimens should be mixed thoroughly via vortex prior to aliquotting.

C. Handling calibrators and the internal standard in step-wise sequential manner will minimize the chances of cross-contaminations.

D. Also changing of gloves after preparations of stock and working standards and internal standards is recommended to avoid any contamination.

E. Phytofluene, is a UV absorbing carotenoid (~maximum at 300 nm) found in tomatoes which can be measured in sera. It had been proposed that phytofluene co-elutes with retinyl palmitate (~maximum at 325 nm) and spills over from 300 nm to 325 nm causing elevated retinyl palmitate without elevation of other retinyl esters. CDC investigation has shown that there is no contribution from phytofluene to retinyl palmitate using the current CDC method.

F. In this method the retention times for cis-beta-carotene and retinyl palmitate are similar. Under the conditions where cis-beta-carotene is elevated, the carotenoid absorption at 325 nm may cause overestimation of retinyl palmitate. The palmitate value is unreliable in these samples.

G. Most methodological problems within a run affect all analytes in a class in a similar manner, though not necessarily all classes of analytes.

H. Ideally, the column cooler should be at 25°C for 24 hours to allow the column to stabilize. The autosampler refrigeration unit needs approximately 45 minutes to stabilize. The lamp should have 1 hour to stabilize. The column should be under flow for at least 60 minutes before the first injection is made. In actual practice the system is only turned completely off if it will be idle for more than three days, except for the lamp, which is turned off when not in use.

I. The following substitution may be made for the specified instrumentation: Instead of drying the hexane extracts with a Speedvac system, the samples may be dried under a stream of nitrogen without heating.

13. Reference Ranges (Normal Values)

Reference ranges for serum retinol, alpha- and gamma-tocopherol, the carotenoids and the retinyl esters are in Table 4. These values are based on the more than 8,000 specimens analyzed for NHANES 2005-2006.

Table 4. Geometric means and reference ranges for US population (NHANES 2005-2006) in ug/dL

Analyte	Age Group	Geometric Mean **(NHANES 2005-2006)**	97.5% Reference Range **(NHANES 2005-2006)**
Retinol (vitamin A)	6-11 yr	36.4	23-54
	12-19 yr	46.5	29-76
	20-39 yr	54.3	31-89
	40-59 yr	58.7	33-100
	60+ yr	64.4	36-108
alpha-Tocopherol (vitamin E)	6-11 yr	820	546-1360
	12-19 yr	770	497-1320
	20-39 yr	1020	590-1940
	40-59 yr	1230	702-2510
	60+ yr	1400	704-2900
gamma-Tocopherol	6+ yr	188	54-489
alpha-Carotene	6+ yr	2.76	<LOD-20
beta-Carotene	6+ yr	12.1	2-74
cis-beta-Carotene	6+ yr	<LOD	<LOD-5
Lutein/Zeaxanthin	6+ yr	13.8	5-39
beta-Cryptoxanthin	6+ yr	7.7	2-31
trans-Lycopene	6+ yr	21.2	6-48
Total-Lycopene	6+ yr	39.4	11-91
alpha-Carotene	6+ yr	3	<LOD-20
Retinyl palmitate	6+ yr	2.11	<LOD-7
Retinyl stearate	6+ yr	<LOD	<LOD-2

See http://www.cdc.gov/nutritionreport/ for results stratified by gender and/or race/ethnicity (3).

14. Critical Call Results ("Panic Values")

Any NHANES samples with serum vitamin A levels < 20 ug/dL or > 100ug/dL, vitamin E levels < 500 ug/dL, and suspected hypervitaminosis A are considered to require follow-up. Hypervitaminosis A is defined as a retinyl ester to retinol ratio > 0.4 and retinyl palmitate ≥ 15ug/dL. The retinyl ester to retinol ratio is calculated as follows: (retinyl palmitate + retinyl stearate)/(retinol + retinyl palmitate + retinyl stearate). Since survey data are transmitted approximately every 2 months to Westat, abnormal reports are automatically forwarded to the NCHS survey physician for follow-up. For smaller, non-NHANES studies, abnormal values are identified to the study principal investigator. Emails sent concerning abnormal results are maintained by the supervisor for the duration of the study. Most of these studies are epidemiological in nature.

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 frozen storage (typically -70^{o}C) as soon as possible. Once the samples are ready to run, they are placed in the autosampler at 20°C.

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

Since the analysis of serum for fat-soluble vitamins is inherently complex and challenging, there are no acceptable alternative methods of analysis in the NHANES laboratory. If the analytical system fails, then storage at ≤ 4°C of the extracted specimens is recommended until the analytical system is restored to functionality.

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.
For NHANES, all data are reported electronically approximately every two months to Westat who then transfer the results to NCHS. For some smaller studies, electronic copies of a data report are sent and upon request hard copies can be sent as well.

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.

CDC recommends 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. Method Performance Documentation

Method performance documentation for this method including accuracy, precision, sensitivity, specificity and stability is provided in **Appendix A** of this method documentation. **The signatures of the branch chief and director of the Division of Laboratory Sciences on the first page of this procedure denote that the method performance is fit for the intended use of the method.**

References

1. U.S. Centers for Disease Control and Prevention. Second National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population 2012. Atlanta (GA): National Center for Environmental Health; April 2012.
2. Sowell AL, Huff DL, Yeager PR, Caudill SP, and Gunter EW. Retinol, alpha-tocopherol, lutein/zeaxanthin, beta-cryptoxanthin, lycopene, beta-carotene, trans-beta-carotene, and four retinyl esters in serum determined simultaneously by reversed-phase HPLC with multiwavelength detection. Clin Chem. 1994; 40(3): 411-416.
3. Caudill SP, Schleicher RL, Pirkle JL. 2008. Multi-rule quality control for the age-related eye disease study. Stat Med 27:4094-4106.

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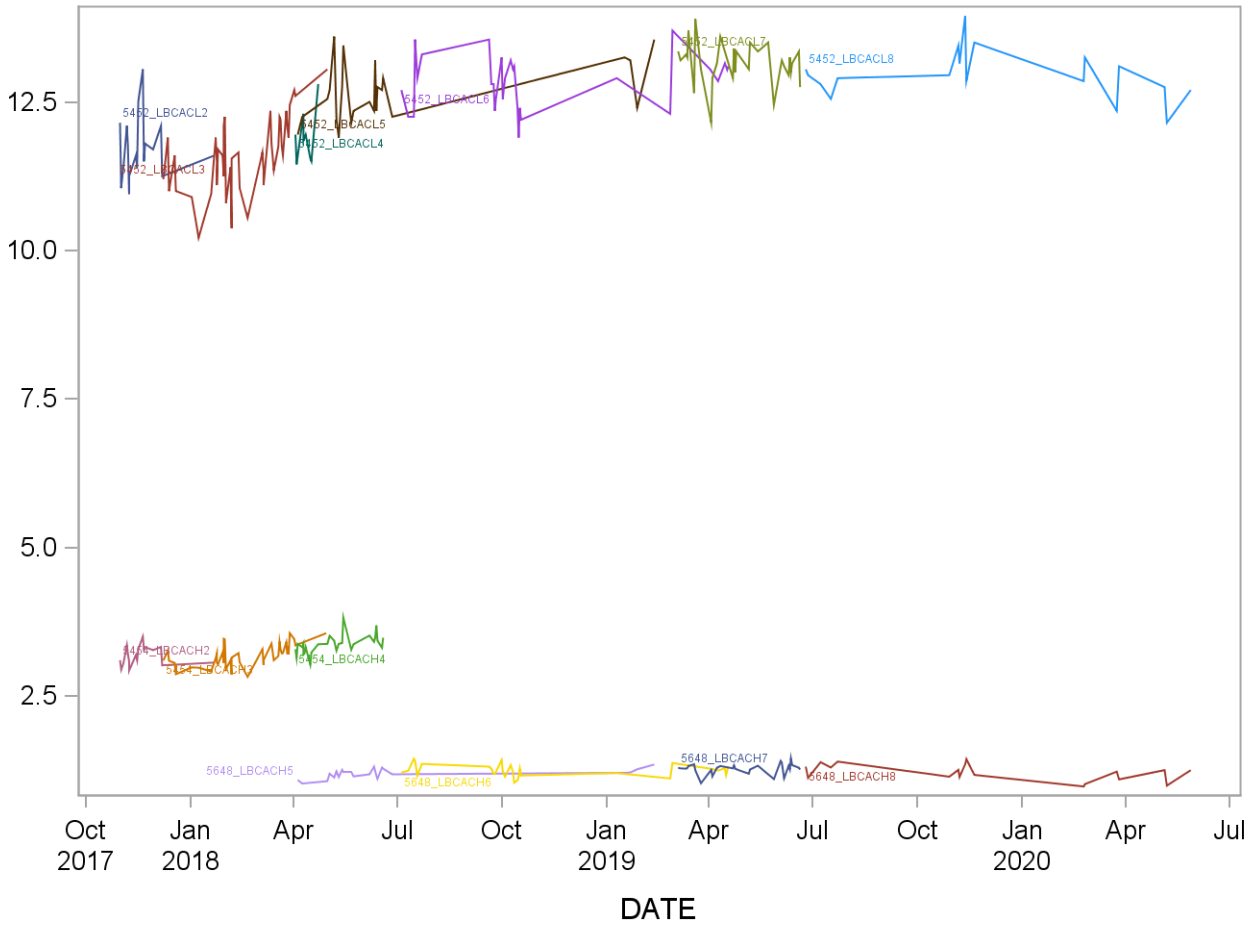
20. Summary Statistics and QC Graph

Please see following pages.

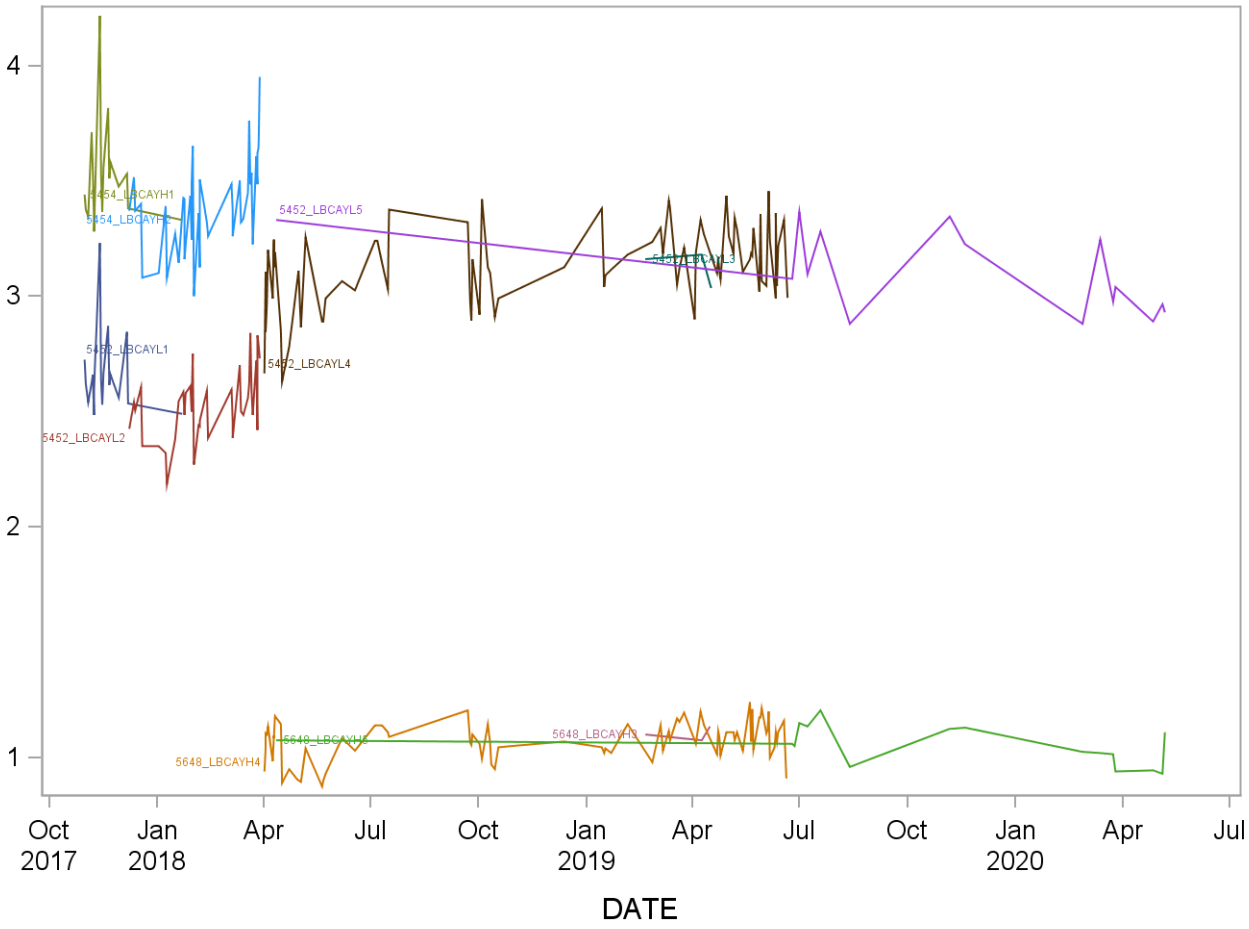
2017-2018 Summary Statistics and QC Chart
LBXALC (a-Carotene (µg/dL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
5454_LBCACH2	17	31OCT17	22JAN18	3.181	0.159	5.0
5452_LBCACL2	17	31OCT17	22JAN18	11.715	0.538	4.6
5454_LBCACH3	42	08DEC17	30APR18	3.186	0.188	5.9
5452_LBCACL3	42	08DEC17	01MAY18	11.615	0.633	5.5
5454_LBCACH4	27	03APR18	19JUN18	3.374	0.159	4.7
5452_LBCACL4	9	03APR18	23APR18	11.917	0.427	3.6
5648_LBCACH5	23	05APR18	12FEB19	1.199	0.081	6.7
5452_LBCACL5	23	05APR18	12FEB19	12.663	0.504	4.0
5648_LBCACH6	29	05JUL18	18APR19	1.246	0.101	8.1
5452_LBCACL6	29	05JUL18	18APR19	12.829	0.452	3.5
5648_LBCACH7	30	05MAR19	20JUN19	1.275	0.089	7.0
5452_LBCACL7	30	05MAR19	20JUN19	13.152	0.358	2.7
5648_LBCACH8	18	25JUN19	28MAY20	1.216	0.140	11.5
5452_LBCACL8	18	25JUN19	28MAY20	12.958	0.422	3.3



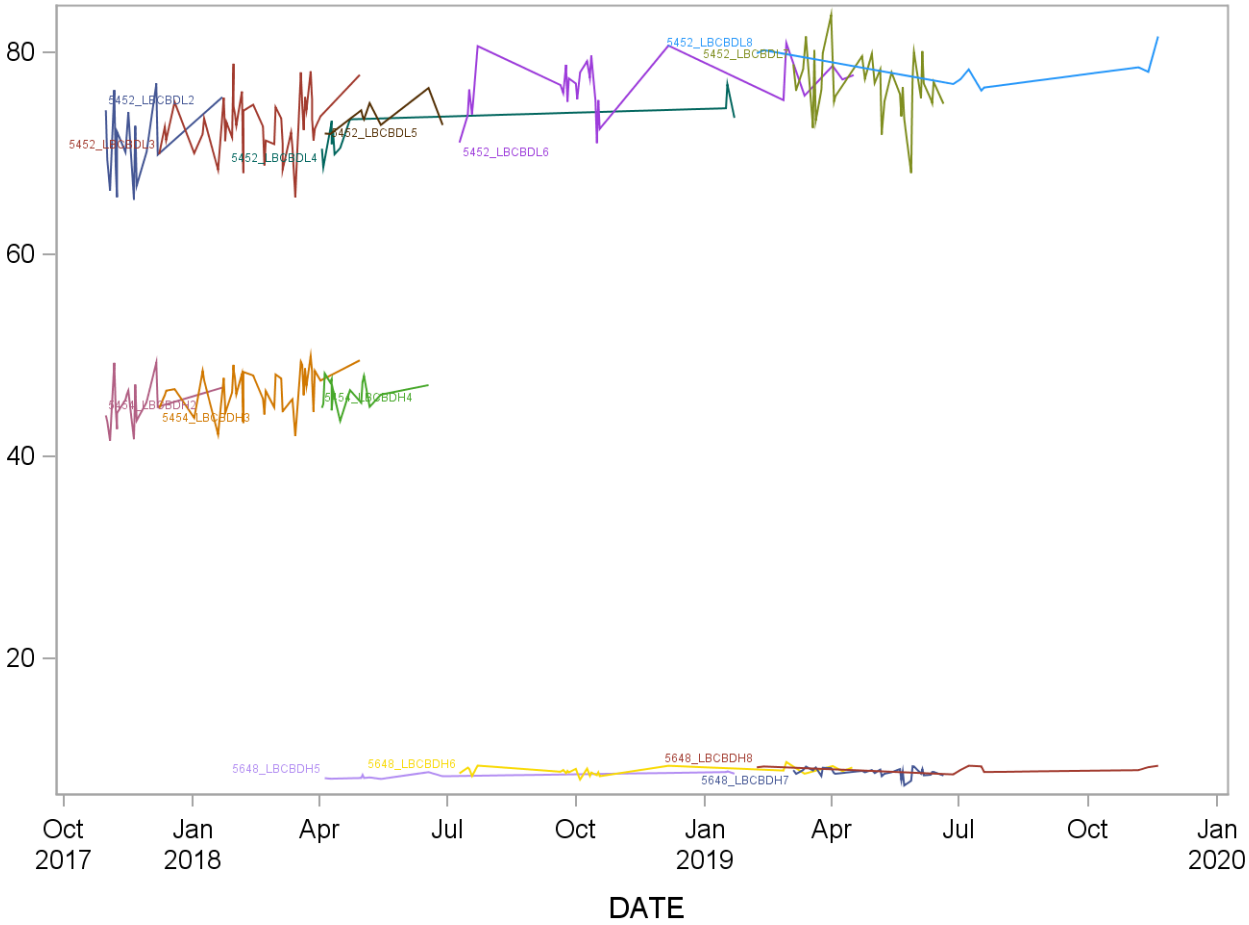
2017-2018 Summary Statistics and QC Chart
LBXARY (alpha Cryptoxanthin (µg/dL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
5454_LBCAYH1	18	31OCT17	22JAN18	3.516	0.223	6.3
5452_LBCAYL1	18	31OCT17	22JAN18	2.661	0.177	6.7
5454_LBCAYH2	40	08DEC17	29MAR18	3.384	0.199	5.9
5452_LBCAYL2	40	08DEC17	29MAR18	2.525	0.149	5.9
5648_LBCAYH4	80	02APR18	20JUN19	1.073	0.085	7.9
5452_LBCAYL4	81	02APR18	21JUN19	3.128	0.175	5.6
5648_LBCAYH5	16	12APR18	07MAY20	1.055	0.084	8.0
5452_LBCAYL5	16	12APR18	07MAY20	3.106	0.176	5.7
5648_LBCAYH3	3	20FEB19	16APR19	1.103	0.030	2.7
5452_LBCAYL3	3	20FEB19	17APR19	3.125	0.079	2.5



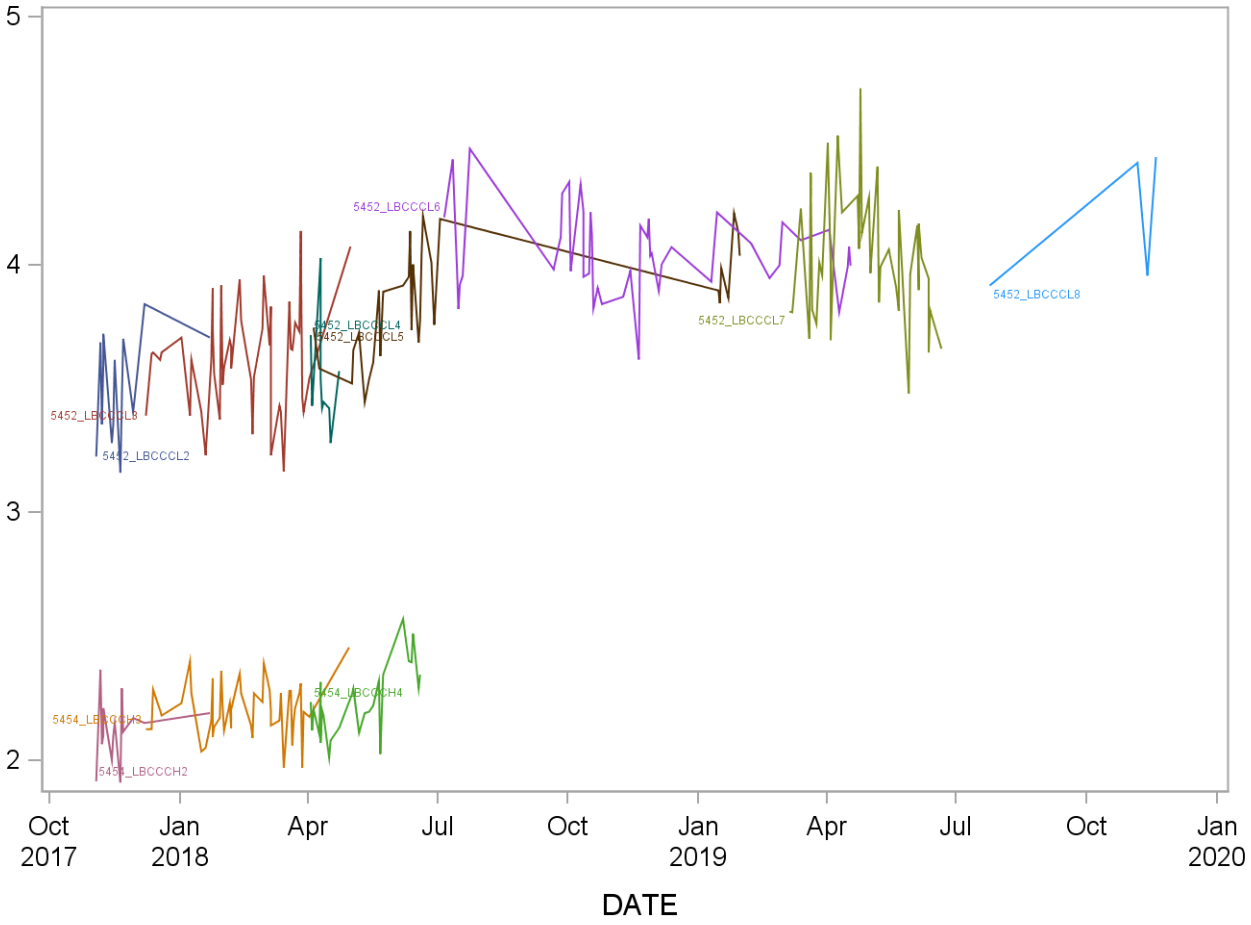
2017-2018 Summary Statistics and QC Chart
LBXBEC (trans-b-Carotene (µg/dL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
5454_LBCBDH2	17	31OCT17	22JAN18	45.112	2.266	5.0
5452_LBCBDL2	17	31OCT17	22JAN18	70.947	3.695	5.2
5454_LBCBDH3	44	08DEC17	30APR18	46.547	2.021	4.3
5452_LBCBDL3	44	08DEC17	30APR18	72.842	2.835	3.9
5454_LBCBDH4	16	03APR18	18JUN18	46.175	1.375	3.0
5452_LBCBDL4	11	03APR18	22JAN19	72.164	2.369	3.3
5648_LBCBDH5	12	05APR18	22JAN19	8.379	0.281	3.4
5452_LBCBDL5	9	05APR18	28JUN18	73.567	1.471	2.0
5648_LBCBDH6	27	10JUL18	16APR19	8.831	0.393	4.4
5452_LBCBDL6	27	10JUL18	17APR19	76.520	2.676	3.5
5648_LBCBDH8	10	07FEB19	20NOV19	9.098	0.294	3.2
5452_LBCBDL8	10	07FEB19	20NOV19	78.335	1.750	2.2
5648_LBCBDH7	35	05MAR19	20JUN19	8.727	0.416	4.8
5452_LBCBDL7	35	05MAR19	20JUN19	76.699	3.035	4.0



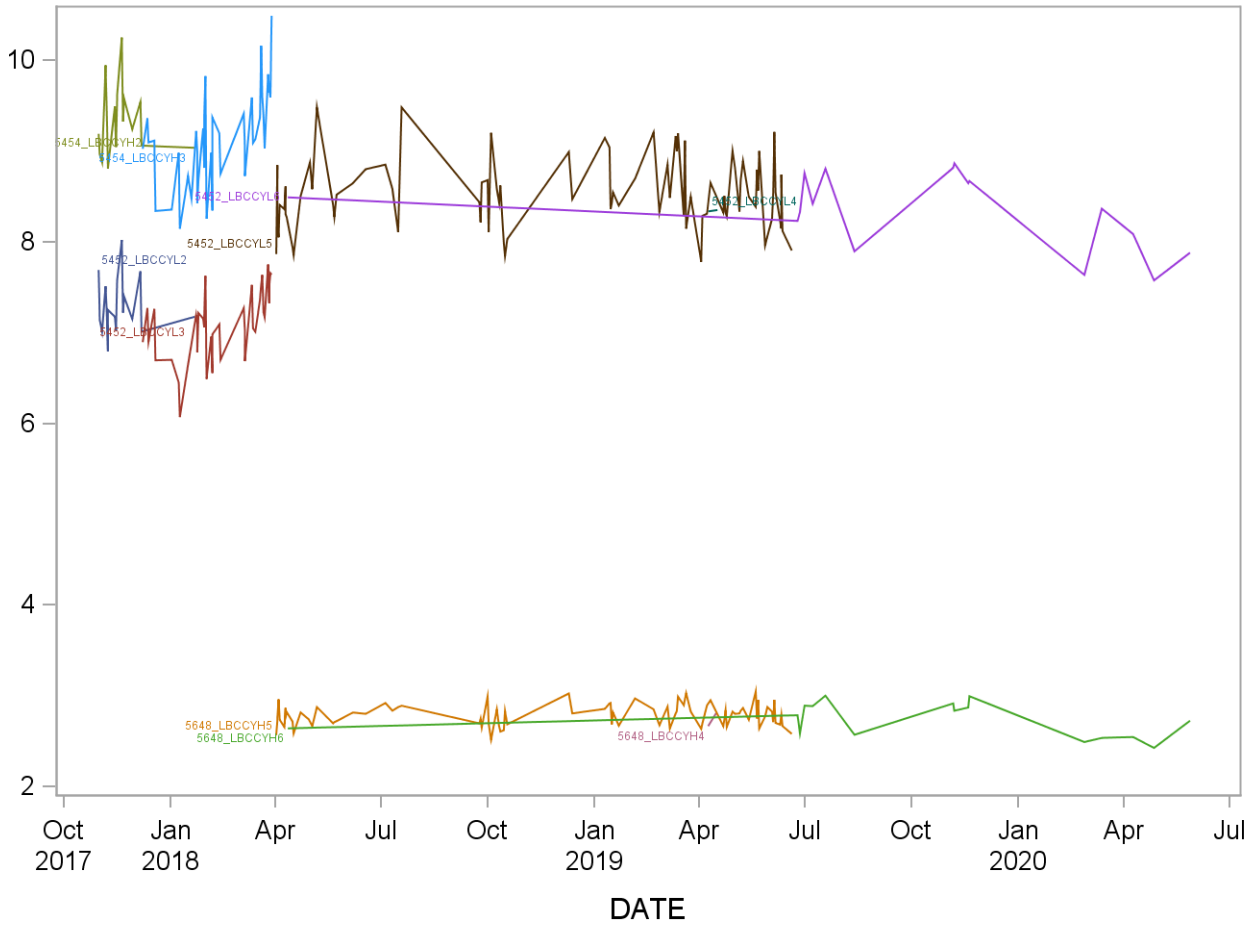
2017-2018 Summary Statistics and QC Chart
LBXCBC (cis-b-Carotene (µg/dL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
5454_LBCCCH2	14	03NOV17	22JAN18	2.1236	0.1272	6.0
5452_LBCCCL2	14	03NOV17	22JAN18	3.5061	0.2143	6.1
5454_LBCCCH3	45	08DEC17	30APR18	2.2040	0.1078	4.9
5452_LBCCCL3	45	08DEC17	01MAY18	3.6212	0.2169	6.0
5454_LBCCCH4	26	03APR18	19JUN18	2.2354	0.1410	6.3
5452_LBCCCL4	9	03APR18	23APR18	3.5383	0.2192	6.2
5452_LBCCCL5	30	05APR18	30JAN19	3.8518	0.2152	5.6
5452_LBCCCL6	44	06JUL18	18APR19	4.0512	0.1711	4.2
5452_LBCCCL7	37	06MAR19	21JUN19	4.0205	0.2708	6.7
5452_LBCCCL8	4	25JUL19	19NOV19	4.1783	0.2816	6.7



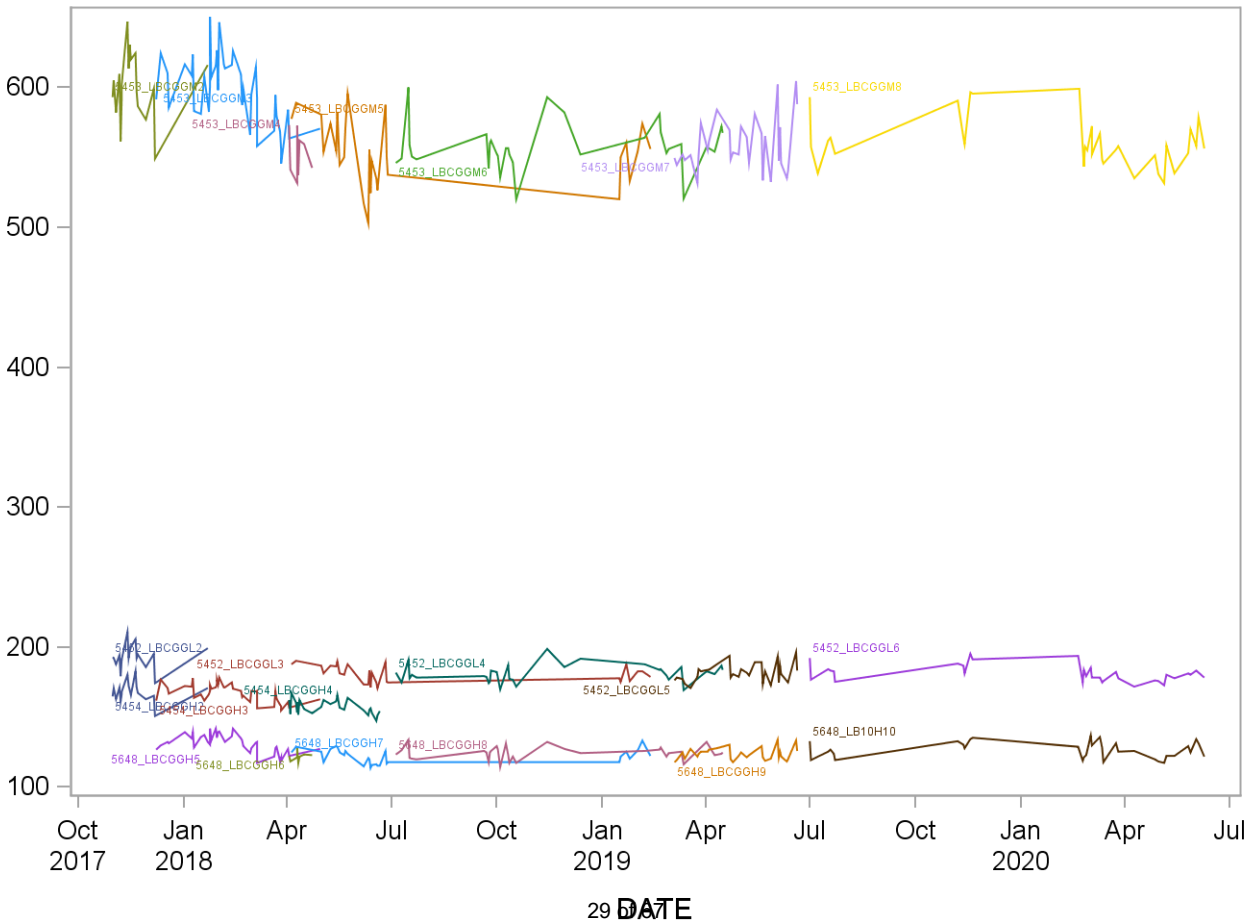
2017-2018 Summary Statistics and QC Chart
LBXCRY (b-Cryptoxanthin (µg/dL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
5454_LBCCYH2	17	31OCT17	22JAN18	9.308	0.403	4.3
5452_LBCCYL2	17	31OCT17	22JAN18	7.298	0.309	4.2
5454_LBCCYH3	40	08DEC17	29MAR18	9.123	0.528	5.8
5452_LBCCYL3	40	08DEC17	29MAR18	7.080	0.381	5.4
5648_LBCCYH5	84	02APR18	20JUN19	2.785	0.120	4.3
5452_LBCCYL5	84	02APR18	20JUN19	8.522	0.391	4.6
5648_LBCCYH6	16	12APR18	28MAY20	2.731	0.189	6.9
5452_LBCCYL6	16	12APR18	28MAY20	8.341	0.424	5.1
5648_LBCCYH4	2	09APR19	16APR19	2.738	0.103	3.7
5452_LBCCYL4	2	09APR19	17APR19	8.343	0.011	0.1



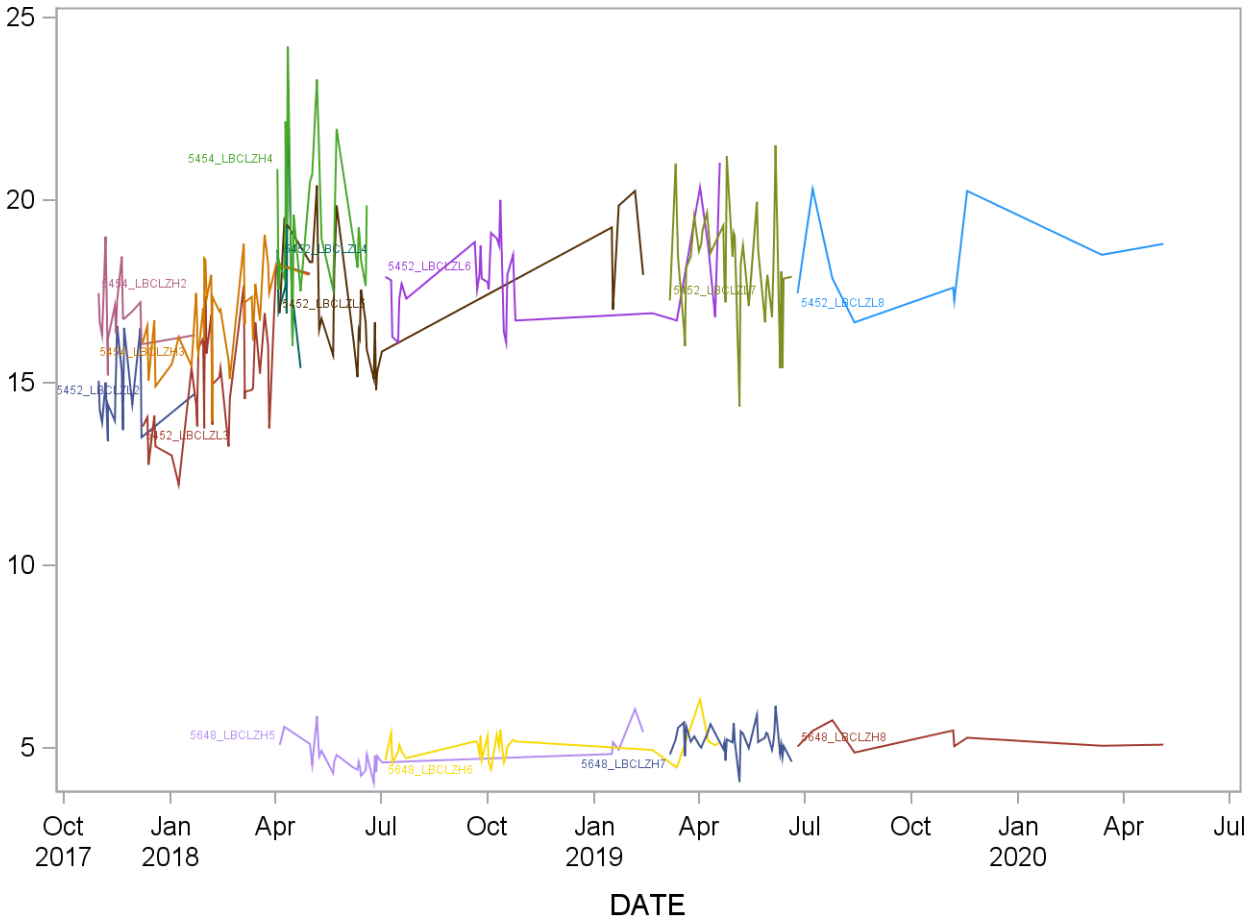
2017-2018 Summary Statistics and QC Chart
LBXGTC (g-Tocopherol (µg/dL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
5454_LBCGGH2	18	31OCT17	22JAN18	167.75	7.06	4.2
5452_LBCGGL2	18	31OCT17	22JAN18	192.89	8.49	4.4
5453_LBCGGM2	18	31OCT17	22JAN18	600.31	24.18	4.0
5454_LBCGGH3	40	08DEC17	30APR18	166.99	6.33	3.8
5648_LBCGGH5	41	08DEC17	30APR18	131.06	6.50	5.0
5453_LBCGGM3	40	08DEC17	30APR18	597.71	24.66	4.1
5454_LBCGGH4	27	03APR18	21JUN18	156.85	4.95	3.2
5648_LBCGGH6	8	03APR18	23APR18	121.56	3.21	2.6
5453_LBCGGM4	8	03APR18	23APR18	552.44	16.29	2.9
5648_LBCGGH7	29	05APR18	12FEB19	121.72	5.21	4.3
5452_LBCGGL3	29	05APR18	12FEB19	180.64	6.00	3.3
5453_LBCGGM5	29	05APR18	12FEB19	553.43	23.41	4.2
5648_LBCGGH8	33	05JUL18	16APR19	123.95	4.72	3.8
5452_LBCGGL4	33	05JUL18	16APR19	180.88	6.32	3.5
5453_LBCGGM6	32	05JUL18	16APR19	557.30	16.96	3.0
5648_LBCGGH9	31	05MAR19	20JUN19	123.39	4.28	3.5
5452_LBCGGL5	31	05MAR19	20JUN19	181.29	6.45	3.6
5453_LBCGGM7	31	05MAR19	20JUN19	558.76	19.15	3.4
5648_LB10H10	34	01JUL19	09JUN20	125.97	5.84	4.6
5452_LBCGGL6	34	01JUL19	09JUN20	180.93	5.89	3.3
5453_LBCGGM8	34	01JUL19	09JUN20	560.32	17.95	3.2



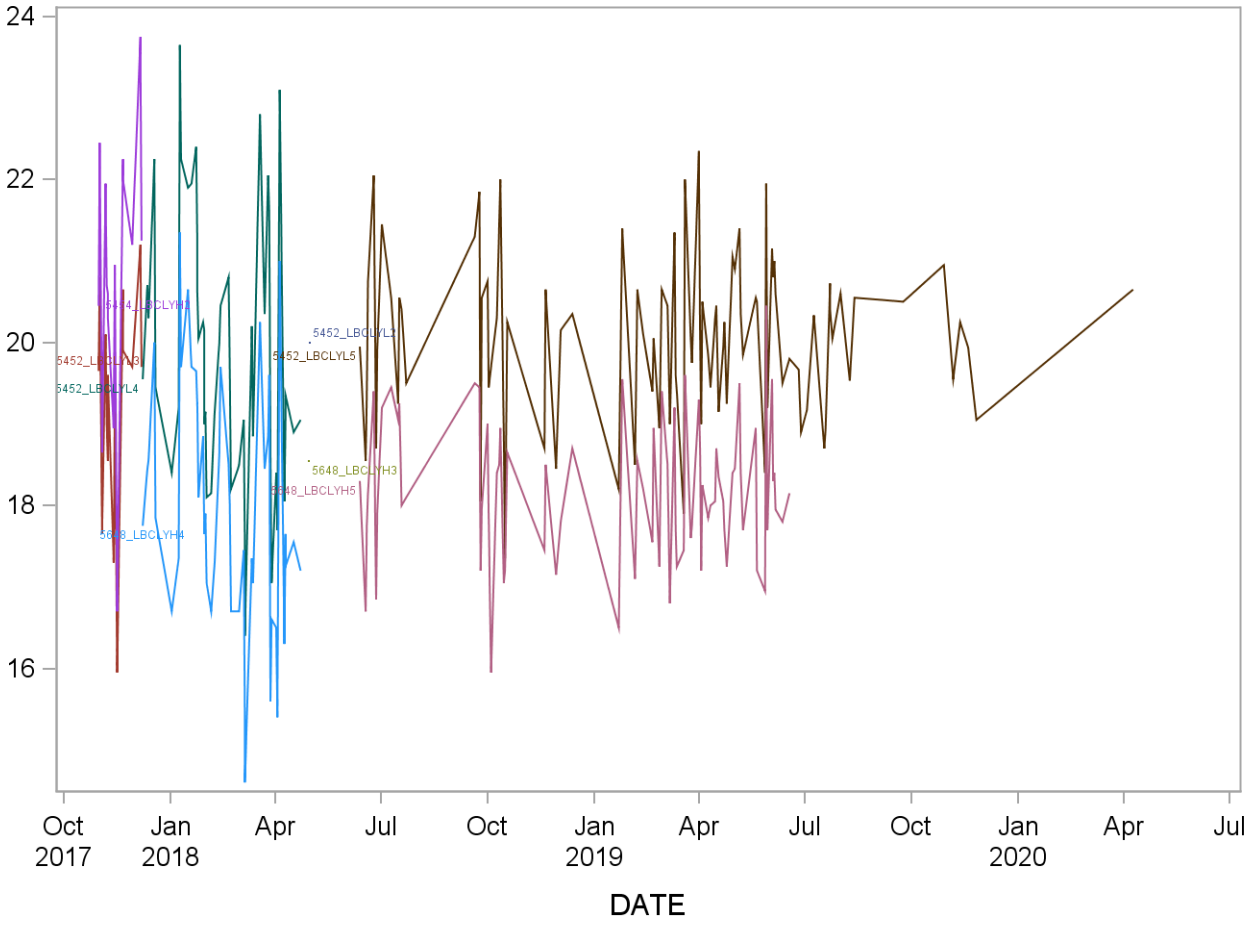
2017-2018 Summary Statistics and QC Chart
LBXLUZ (cis-lutein zeaxanthin (µg/dL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
5454_LBCLZH2	17	31OCT17	22JAN18	16.900	0.895	5.3
5452_LBCLZL2	17	31OCT17	22JAN18	14.747	1.024	6.9
5454_LBCLZH3	37	08DEC17	30APR18	16.849	1.208	7.2
5452_LBCLZL3	37	08DEC17	01MAY18	15.044	1.506	10.0
5454_LBCLZH4	26	03APR18	19JUN18	19.517	1.927	9.9
5452_LBCLZL4	9	03APR18	23APR18	17.961	2.354	13.1
5648_LBCLZH5	27	05APR18	12FEB19	4.807	0.485	10.1
5452_LBCLZL5	27	05APR18	12FEB19	17.276	1.688	9.8
5648_LBCLZH6	29	05JUL18	18APR19	5.016	0.389	7.8
5452_LBCLZL6	29	05JUL18	19APR19	17.916	1.250	7.0
5648_LBCLZH7	38	07MAR19	20JUN19	5.186	0.384	7.4
5452_LBCLZL7	38	07MAR19	20JUN19	18.200	1.538	8.5
5648_LBCLZH8	9	25JUN19	05MAY20	5.224	0.284	5.4
5452_LBCLZL8	9	25JUN19	05MAY20	18.294	1.291	7.1



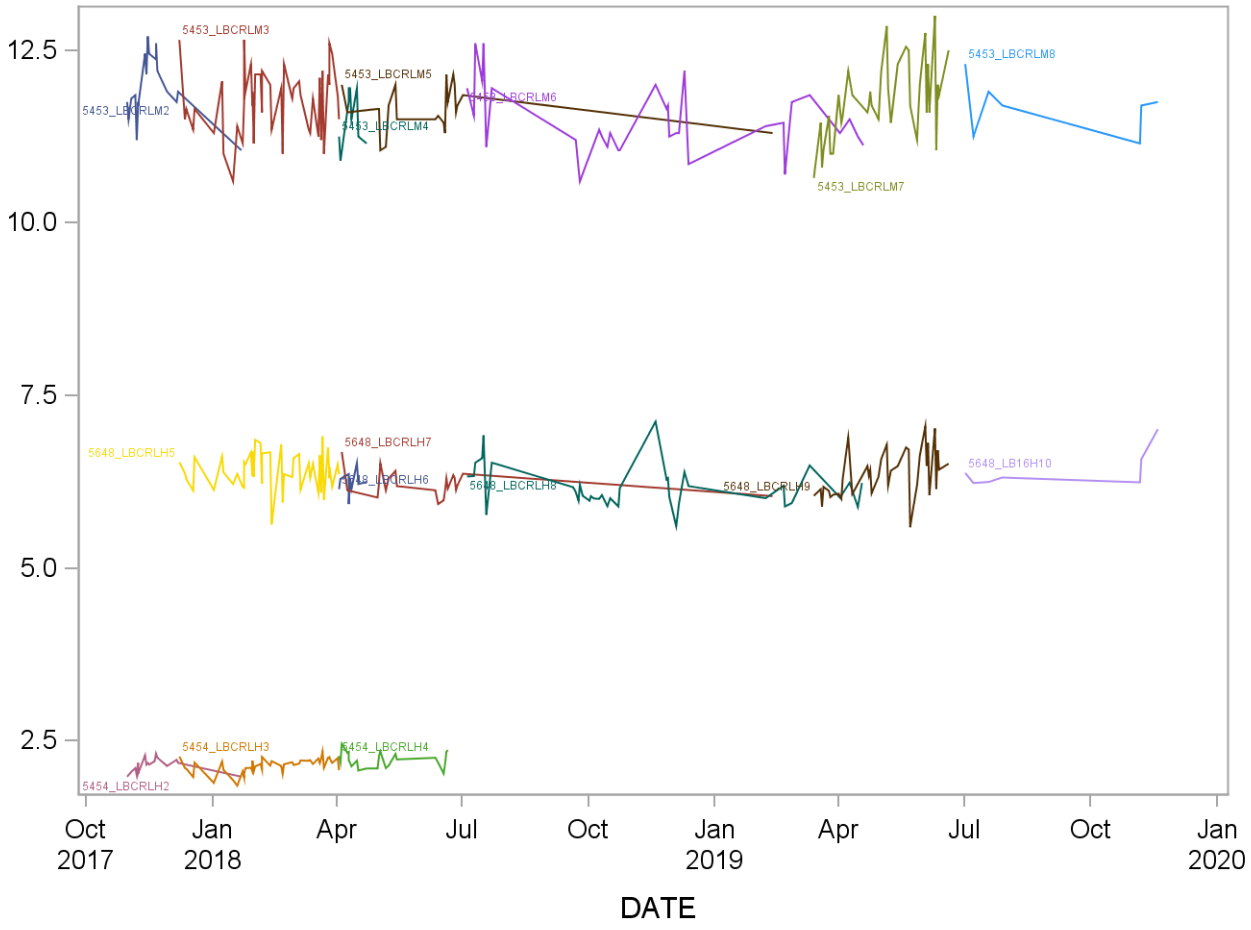
2017-2018 Summary Statistics and QC Chart
LBXLYC (trans-Lycopene (µg/dL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
5454_LBCLYH2	15	31OCT17	07DEC17	20.810	1.731	8.3
5452_LBCLYL3	15	31OCT17	07DEC17	19.270	1.409	7.3
5648_LBCLYH4	48	08DEC17	23APR18	17.968	1.516	8.4
5452_LBCLYL4	48	08DEC17	23APR18	19.834	1.674	8.4
5648_LBCLYH5	77	13JUN18	18JUN19	18.176	0.882	4.9
5452_LBCLYL5	96	13JUN18	09APR20	20.048	1.010	5.0



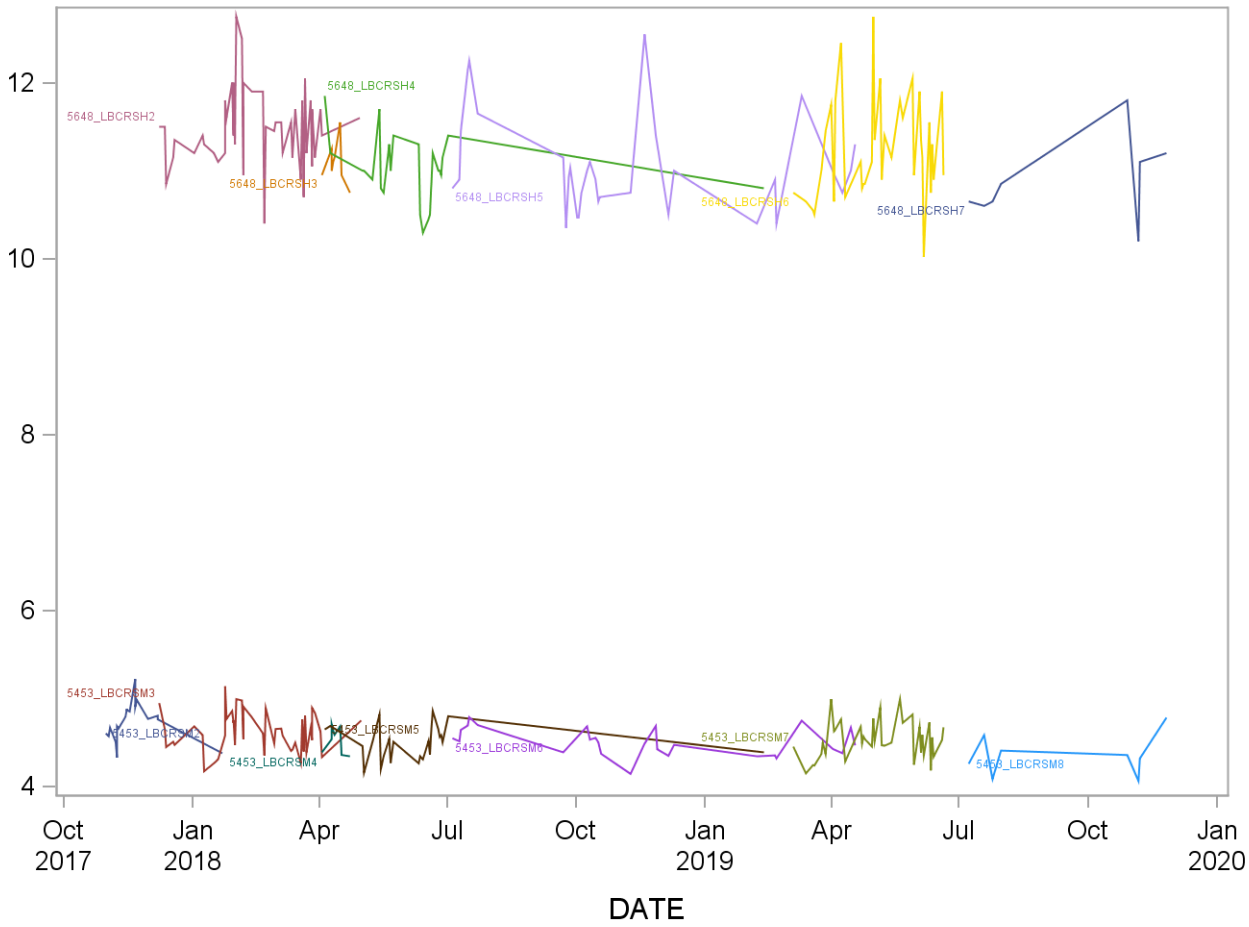
2017-2018 Summary Statistics and QC Chart
LBXRPL (Retinyl Palmitate (µg/dL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
5454_LBCRLH2	18	31OCT17	22JAN18	2.1297	0.1065	5.0
5453_LBCRLM2	18	31OCT17	22JAN18	11.9481	0.4574	3.8
5454_LBCRLH3	45	08DEC17	03APR18	2.1330	0.1067	5.0
5648_LBCRLH5	45	08DEC17	03APR18	6.4048	0.2692	4.2
5453_LBCRLM3	45	08DEC17	03APR18	11.7500	0.4852	4.1
5454_LBCRLH4	24	03APR18	21JUN18	2.2174	0.1090	4.9
5648_LBCRLH6	9	03APR18	23APR18	6.2378	0.1650	2.6
5453_LBCRLM4	9	03APR18	23APR18	11.5167	0.3992	3.5
5648_LBCRLH7	21	05APR18	12FEB19	6.2167	0.1812	2.9
5453_LBCRLM5	21	05APR18	12FEB19	11.6452	0.3106	2.7
5648_LBCRLH8	38	05JUL18	18APR19	6.1639	0.2991	4.9
5453_LBCRLM6	32	05JUL18	19APR19	11.4773	0.4857	4.2
5648_LBCRLH9	36	14MAR19	20JUN19	6.3590	0.3377	5.3
5453_LBCRLM7	36	14MAR19	20JUN19	11.7819	0.5791	4.9
5648_LB16H10	7	02JUL19	19NOV19	6.4264	0.2843	4.4
5453_LBCRLM8	7	02JUL19	19NOV19	11.6786	0.3882	3.3



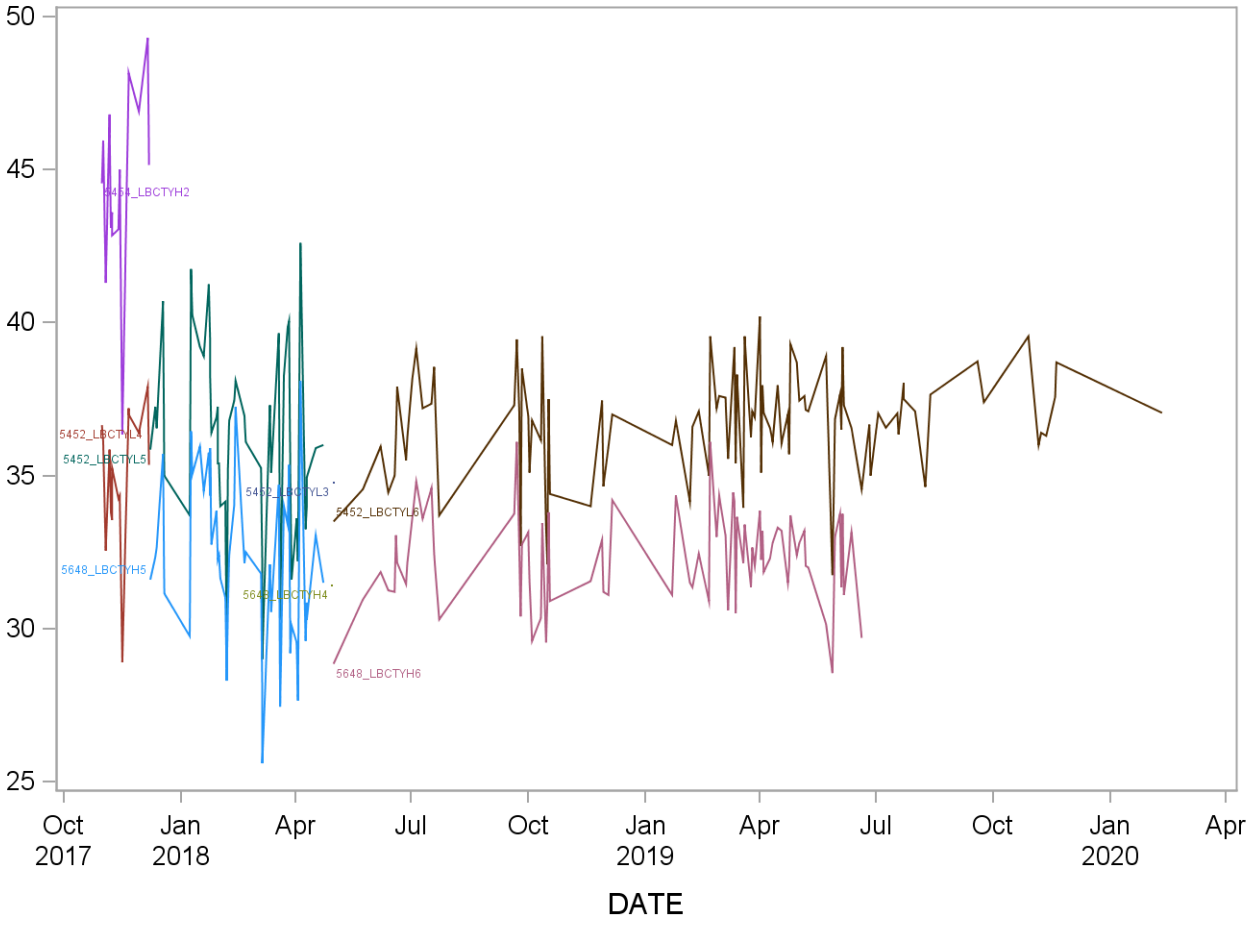
2017-2018 Summary Statistics and QC Chart
LBXRST (Retinyl Stearate (µg/dL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
5453_LBCRSM2	18	31OCT17	22JAN18	4.7373	0.2161	4.6
5648_LBCRSH2	44	08DEC17	30APR18	11.4761	0.4466	3.9
5453_LBCRSM3	44	08DEC17	01MAY18	4.6229	0.2246	4.9
5648_LBCRSH3	8	03APR18	23APR18	11.0875	0.2402	2.2
5453_LBCRSM4	8	03APR18	23APR18	4.5313	0.1474	3.3
5648_LBCRSH4	24	05APR18	12FEB19	11.0123	0.3781	3.4
5453_LBCRSM5	24	05APR18	12FEB19	4.4988	0.1946	4.3
5648_LBCRSH5	32	05JUL18	18APR19	11.0563	0.5490	5.0
5453_LBCRSM6	26	05JUL18	18APR19	4.5090	0.1588	3.5
5648_LBCRSH6	37	05MAR19	20JUN19	11.2397	0.5806	5.2
5453_LBCRSM7	37	05MAR19	20JUN19	4.5364	0.2153	4.7
5648_LBCRSH7	8	08JUL19	26NOV19	10.8813	0.4847	4.5
5453_LBCRSM8	8	08JUL19	26NOV19	4.3588	0.2414	5.5



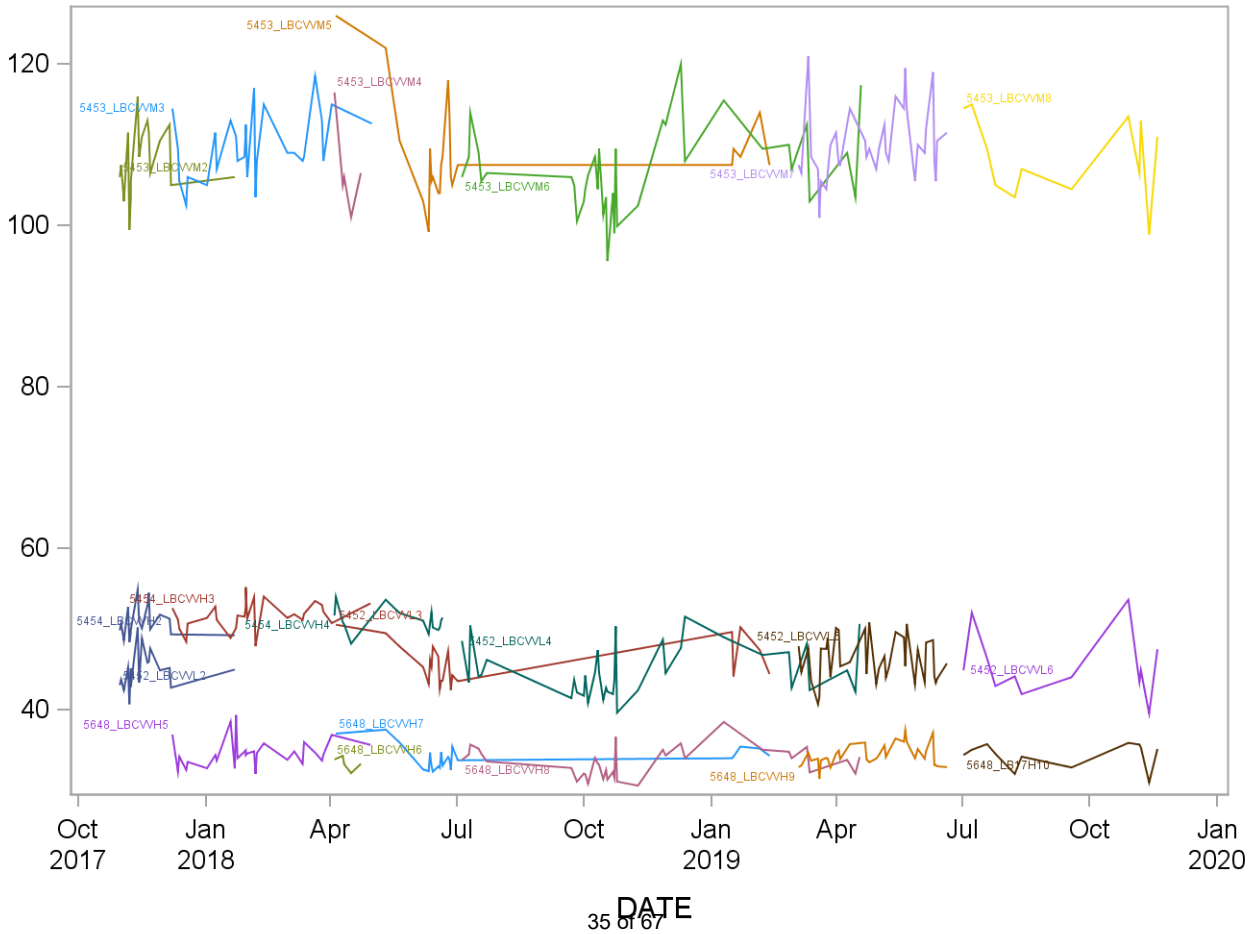
2017-2018 Summary Statistics and QC Chart
LBXLCC (Combined Lycopene)

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
5454_LBCTYH2	15	31OCT17	07DEC17	44.663	3.225	7.2
5452_LBCTYL4	15	31OCT17	07DEC17	35.007	2.264	6.5
5648_LBCTYH5	47	08DEC17	23APR18	32.282	2.744	8.5
5452_LBCTYL5	47	08DEC17	23APR18	36.244	3.139	8.7
5648_LBCTYH6	79	01MAY18	20JUN19	32.302	1.526	4.7
5452_LBCTYL6	99	01MAY18	11FEB20	36.717	1.728	4.7



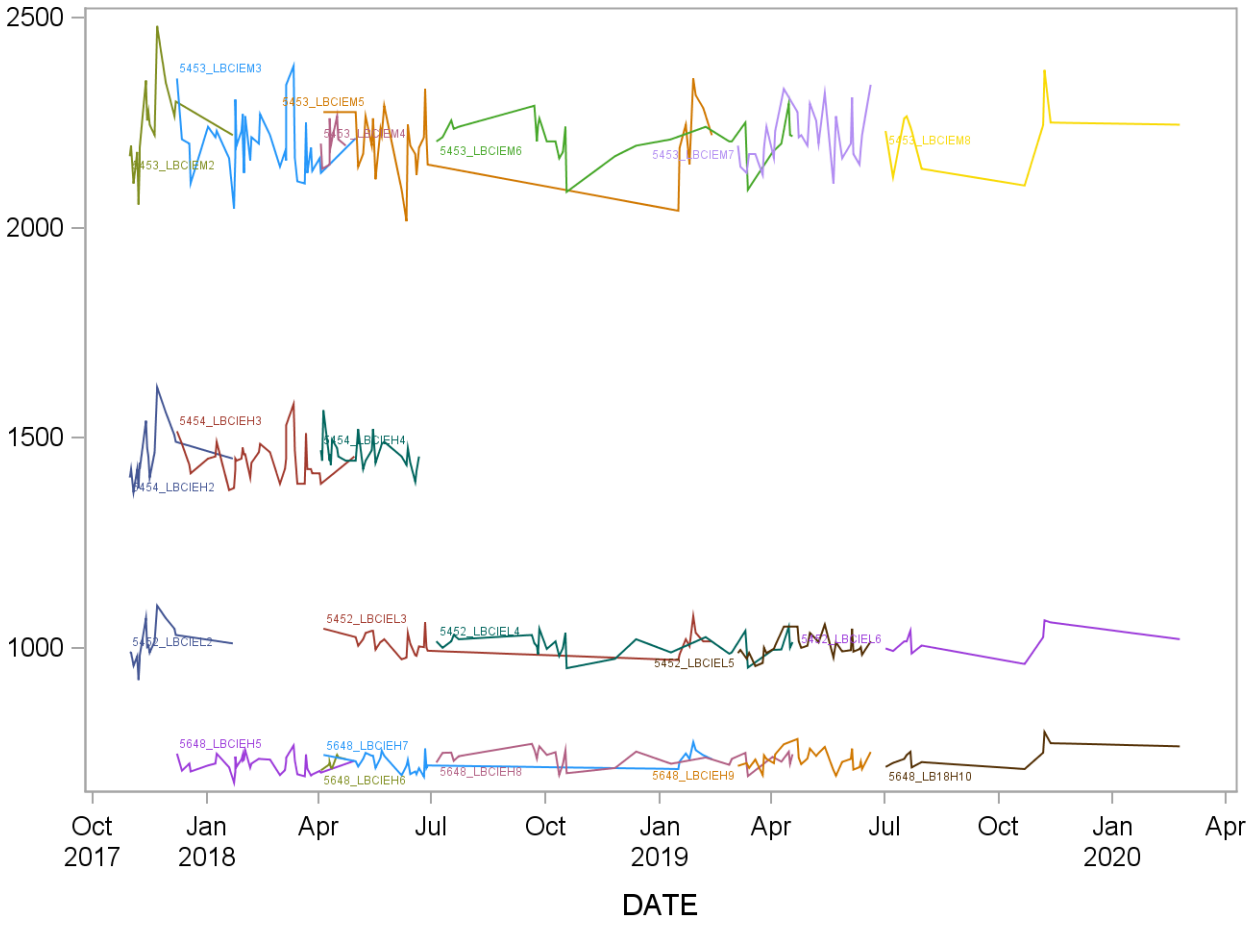
2017-2018 Summary Statistics and QC Chart
LBXVIA (Vitamin A (µg/dL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
5454_LBCVVH2	17	31OCT17	22JAN18	50.832	1.935	3.8
5452_LBCVVL2	17	31OCT17	22JAN18	44.826	2.407	5.4
5453_LBCVVM2	17	31OCT17	22JAN18	108.056	4.281	4.0
5454_LBCVVH3	28	08DEC17	30APR18	51.513	1.741	3.4
5648_LBCVVH5	29	08DEC17	30APR18	34.593	1.713	5.0
5453_LBCVVM3	28	08DEC17	01MAY18	109.915	4.024	3.7
5454_LBCVVH4	16	04APR18	21JUN18	50.959	1.496	2.9
5648_LBCVVH6	5	04APR18	23APR18	33.320	0.807	2.4
5453_LBCVVM4	5	04APR18	23APR18	107.000	5.734	5.4
5648_LBCVVH7	22	05APR18	12FEB19	34.141	1.469	4.3
5452_LBCVVL3	22	05APR18	12FEB19	45.880	2.540	5.5
5453_LBCVVM5	22	05APR18	12FEB19	109.214	6.263	5.7
5648_LBCVVH8	36	05JUL18	18APR19	33.438	1.813	5.4
5452_LBCVVL4	36	05JUL18	18APR19	44.913	3.185	7.1
5453_LBCVVM6	36	05JUL18	19APR19	107.040	5.242	4.9
5648_LBCVVH9	35	05MAR19	20JUN19	34.326	1.322	3.9
5452_LBCVVL5	35	05MAR19	20JUN19	46.177	2.734	5.9
5453_LBCVVM7	35	05MAR19	20JUN19	109.970	4.384	4.0
5648_LB17H10	12	02JUL19	19NOV19	34.279	1.571	4.6
5452_LBCVVL6	12	02JUL19	19NOV19	45.408	4.017	8.8
5453_LBCVVM8	12	02JUL19	19NOV19	108.492	5.067	4.7



2017-2018 Summary Statistics and QC Chart
LBXVIE (Vitamin E (ug/dl))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
5454_LBCIEH2	17	31OCT17	22JAN18	1460.9	66.1	4.5
5452_LBCIEL2	17	31OCT17	22JAN18	1007.3	44.7	4.4
5453_LBCIEM2	17	31OCT17	22JAN18	2235.9	99.6	4.5
5454_LBCIEH3	37	08DEC17	30APR18	1447.6	44.3	3.1
5648_LBCIEH5	37	08DEC17	30APR18	722.2	19.9	2.8
5453_LBCIEM3	37	08DEC17	01MAY18	2200.2	72.7	3.3
5454_LBCIEH4	27	03APR18	21JUN18	1462.2	35.1	2.4
5648_LBCIEH6	9	03APR18	23APR18	723.6	13.4	1.8
5453_LBCIEM4	9	03APR18	23APR18	2202.8	42.0	1.9
5648_LBCIEH7	30	05APR18	12FEB19	729.6	20.9	2.9
5452_LBCIEL3	30	05APR18	12FEB19	1012.8	25.8	2.5
5453_LBCIEM5	30	05APR18	12FEB19	2206.5	80.5	3.7
5648_LBCIEH8	29	05JUL18	18APR19	736.9	19.4	2.6
5452_LBCIEL4	29	05JUL18	18APR19	1005.4	24.8	2.5
5453_LBCIEM6	28	05JUL18	18APR19	2211.0	47.3	2.1
5648_LBCIEH9	29	05MAR19	20JUN19	733.1	21.5	2.9
5452_LBCIEL5	29	05MAR19	20JUN19	1003.3	25.2	2.5
5453_LBCIEM7	29	05MAR19	20JUN19	2213.6	63.4	2.9
5648_LB18H10	12	02JUL19	24FEB20	742.8	26.6	3.6
5452_LBCIEL6	12	02JUL19	24FEB20	1015.2	30.1	3.0
5453_LBCIEM8	12	02JUL19	24FEB20	2225.0	74.4	3.3



Appendix A: Method Performance

Accuracy using Spike Recovery

Since this method has three levels of SRM for 11/13 analytes, the accuracy using spike recovery was only necessary for two analytes (shown below). Spiking with standards in solvents was not successful (recovery < 50%). Therefore, canine serum (high in these analytes) was used for spiking. A repeat of the spike recovery experiment using standards in solvent will be repeated with higher concentrations.

1. Retinyl palmitate (RPL)

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: **retinyl palmitate (RPL)**

		Low QC					High QC						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		1.29	1.10				2.02	1.98				
	2	0	1.14	1.28	1.20		0	2.13	2.16	2.13		94.8	5.0
	3		1.15	1.23				2.20	2.29				
Sample + Spike 1	1		2.05	1.97				3.00	2.84				
	2	0.916	2.14	2.01	2.07	95.1	0.916	3.04	2.75	2.97	92.0		
	3		2.11	2.14				3.10	3.10				
Sample + Spike 2	1		2.45	2.37				3.16	3.35				
	2	1.28	2.48	2.25	2.43	95.9	1.28	3.34	3.23	3.31	92.1		
	3		2.47	2.54				3.43	3.35				
Sample + Spike 3	1		2.86	2.83				3.67	3.39				
	2	1.65	2.86	3.00	2.91	103.9	1.65	3.63	3.56	3.61	89.8		
	3		2.95	2.97				3.77	3.64				

2. Retinyl stearate (RST)

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: **retinyl stearate (RST)**

		Low QC					High QC						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		0.342	0.245				0.670	0.651				
	2	0	0.443	0.212	0.293		0	0.671	0.584	0.633		103.4	2.2
	3		0.365	0.149				0.684	0.535				
Sample + Spike 1	1		2.09	2.04				2.51	2.52				
	2	1.79	2.20	2.10	2.12	101.8	1.79	2.43	2.43	2.49	103.8		
	3		2.22	2.07				2.48	2.60				
Sample + Spike 2	1		2.81	2.78				3.21	3.40				
	2	2.51	2.82	2.66	2.80	99.9	2.51	3.12	3.34	3.29	105.9		
	3		2.87	2.88				3.36	3.33				
Sample + Spike 3	1		3.67	3.49				3.97	3.95				
	2	3.23	3.61	3.78	3.66	104.3	3.23	4.09	3.98	4.01	104.5		
	3		3.48	3.93				4.07	3.99				

Accuracy compared to Reference Material

This method has three levels of SRM 968e for 11/13 analytes (shown below).

1. alpha-carotene (ALC)

Accuracy compared to Reference Material - fill in yellow shaded cells
Mean concentration should be within ±15% of the nominal value except at 3*LOD, where it should be within ± 20%

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Reference material: **SRM968e**
Analyte: **α-carotene (ALC)**

Reference material	Replicate	Nominal value	Measured concentration Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	Difference from nominal value (%)
Level 1	1	1.1	0.851	0.773	0.597	0.607	0.594	0.727	0.11	15.16	-34
	2		0.863	0.809	0.797	0.758	0.619				
Level 2	1	3.1	3.37	3.49	2.75	3.03	3.38	3.23	0.31	9.55	4.3
	2		3.17	3.71	2.88	3.02	3.51				
Level 3	1	1.5	1.33	1.85	1.15	1.17	1.31	1.33	0.21	15.95	-11.2
	2		1.40	1.19	1.49	1.22	1.22				

NOTE: Level 1 is a low concentration of α-carotene; CDC within certificate of analysis U95 (1.1 ug/dL ± 0.5 ug/dL)

2. alpha-cryptoxanthin (ARY)

Accuracy compared to Reference Material - fill in yellow shaded cells
Mean concentration should be within ±15% of the nominal value except at 3*LOD, where it should be within ± 20%

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Reference material: **SRM968e**
Analyte: **α-cryptoxanthin (ARY)**

Reference material	Replicate	Nominal value	Measured concentration Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	Difference from nominal value (%)
Level 1	1	1.6	1.80	1.80	2.01	1.94	1.84	1.85	0.19	10.10	15.7
	2		1.74	1.42	2.01	2.07	1.89				
Level 2	1	2	2.01	2.06	2.31	1.85	2.14	2.2	0.30	13.98	7.8
	2		2.09	2.04	2.95	2.00	2.10				
Level 3	1	1.5	1.76	1.59	1.90	1.67	1.75	1.8	0.22	11.97	20.7
	2		1.66	1.69	1.86	2.35	1.88				

3. beta-carotene (BEC)

Accuracy compared to Reference Material - fill in yellow shaded cells
Mean concentration should be within ±15% of the nominal value except at 3*LOD, where it should be within ± 20%

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Reference material: **SRM968e**
Analyte: **β-carotene (BEC)**

Reference material	Replicate	Nominal value	Measured concentration Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	Difference from nominal value (%)
Level 1	1	8.8	8.63	8.42	8.65	7.70	7.54	8.32	0.55	6.66	-5.4
	2		8.81	8.66	9.17	7.79	7.86				
Level 2	1	20.3	24.3	22.3	20.5	20.5	20.9	21.9	1.34	6.11	7.9
	2		23.5	23.0	22.0	20.8	21.3				
Level 3	1	36.3	39.5	36.9	35.8	34.5	34.9	37.4	2.65	7.09	3.0
	2		39.4	36.9	43.2	36.2	36.4				

4. cis-beta-carotene (CBC) – These values are <3*LOD and are close to ±20%.

Accuracy compared to Reference Material - fill in yellow shaded cells

Mean concentration should be within ±15% of the nominal value except at 3*LOD, where it should be within ± 20%

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Reference material: **SRM968e**
Analyte: ***cis*-β-carotene (CBC)**

Reference material	Replicate	Nominal value	Measured concentration					Mean	SD	CV (%)	Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5				
Level 1	1	0.5	0.616	0.346	0.402	0.357	0.271	0.41	0.10	23.94	-17.6
	2		0.483	0.446	0.488	0.360	0.350				
Level 2	1	1.3	1.247	1.008	0.816	0.98	0.843	1.0	0.14	13.58	-22.8
	2		1.212	1.004	0.960	0.97	1.001				
Level 3	1	1.6	1.84	1.71	1.35	1.21	1.24	1.5	0.27	17.72	-6.2
	2		1.82	1.20	1.78	1.33	1.54				

5. beta-cryptoxanthin (CRY) – Comparing our results to the average NIST assigned value (NAV) from the 2011-2017 NIST round robin (RR) for levels 1 and 3 (only levels used as unknowns in RR) CRY is within ±15%. It appears that the average NIST assigned value from 2011-2017 has shifted upwards compared to the certificate (30% for level 1 and 68% for level 3).

Accuracy compared to Reference Material - fill in yellow shaded cells

Mean concentration should be within ±15% of the nominal value except at 3*LOD, where it should be within ± 20%

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Reference material: **SRM968e**
Analyte: **β-cryptoxanthin (CRY)**

Reference material	Replicate	Nominal value	Measured concentration					Mean	SD	CV (%)	Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5				
Level 1	1	4.1	5.09	4.90	5.56	5.44	5.19	5.21	0.38	7.25	27.0
	2		5.03	4.46	5.47	5.78	5.13				
Level 2	1	4	5.27	5.03	5.88	5.16	5.44	5.49	0.50	9.06	37.2
	2		5.41	5.09	6.72	5.53	5.36				
Level 3	1	2.1	3.80	3.44	4.20	3.74	3.65	3.92	0.49	12.56	86.6
	2		3.76	3.45	4.16	5.12	3.88				

Reference material	Replicate	2011-2017 RR NAV avg	Measured concentration					Mean	SD	CV (%)	Difference from avg RR (%)
			9/29/2017	10/4/2017	10/5/2017	10/12/2017	10/19/2017				
Level 1	1	5.3	5.09	4.90	5.56	5.44	5.19	5.21	0.38	7.25	-1.5
	2		5.03	4.46	5.47	5.78	5.13				
Level 3	1	3.5	3.80	3.44	4.20	3.74	3.65	3.92	0.49	12.56	10.9
	2		3.76	3.45	4.16	5.12	3.88				

6. gamma-tocopherol (GTC)

Accuracy compared to Reference Material - fill in yellow shaded cells

Mean concentration should be within ±15% of the nominal value except at 3*LOD, where it should be within ± 20%

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Reference material: **SRM968e**
Analyte: **γ-tocopherol (GTC)**

Reference material	Replicate	Nominal value	Measured concentration					Mean	SD	CV (%)	Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5				
Level 1	1	186	191	191	202	202	195	195	7.38	3.79	4.7
	2		191	179	203	200	193				
Level 2	1	143	147	141	157	158	162	154	6.76	4.38	7.8
	2		151	151	163	156	157				
Level 3	1	227	243	238	258	254	246	248	6.99	2.82	9.2
	2		243	242	257	254	245				

7. lutein/zeaxanthin (LUZ)

Accuracy compared to Reference Material - fill in yellow shaded cells

Mean concentration should be within ±15% of the nominal value except at 3*LOD, where it should be within ± 20%

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Reference material: **SRM968e**
Analyte: **lutein/zeaxanthin (LUZ)**

Reference material	Replicate	Nominal value	Measured concentration					Mean	SD	CV (%)	Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5				
Level 1	1	9.8	10.08	9.53	12.98	11.27	9.76	10.72	1.46	13.62	9.3
	2		10.42	8.68	12.50	12.28	9.66				
Level 2	1	12.27	11.6	10.7	15.5	13.2	11.3	12.5	1.58	12.67	1.7
	2		11.7	12.8	14.7	12.0	11.2				
Level 3	1	15.3	15.5	15.3	18.2	19.1	14.9	17.1	3.11	18.20	11.8
	2		16.0	14.7	18.1	24.7	14.8				

8. trans-lycopene (LYC)

Accuracy compared to Reference Material - fill in yellow shaded cells

Mean concentration should be within ±15% of the nominal value except at 3*LOD, where it should be within ± 20%

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Reference material: **SRM968e**
Analyte: ***trans*-lycopene (LYC)**

Reference material	Replicate	Nominal value	Measured concentration					Mean	SD	CV (%)	Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5				
Level 1	1	13.5	13.60	14.16	10.29	10.87	10.97	12.15	1.88	15.44	-10.0
	2		14.44	14.87	10.10	10.99	11.22				
Level 2	1	30.7	31.4	36.3	26.3	29.0	29.4	30.1	3.28	10.89	-2.0
	2		33.7	*	26.1	29.0	29.6				
Level 3	1	49	44.4	55.8	35.7	39.8	39.6	43.6	7.36	16.87	-11.0
	2		54.8	49.9	37.0	38.0	41.1				

9. total lycopene (TLY)

Accuracy compared to Reference Material - fill in yellow shaded cells

Mean concentration should be within ±15% of the nominal value except at 3*LOD, where it should be within ± 20%

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Reference material: **SRM968e**
Analyte: **total lycopene (TLY)**

Reference material	Replicate	Nominal value	Measured concentration					Mean	SD	CV (%)	Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5				
Level 1	1	23.4	27.9	28.0	20.7	21.6	21.7	24.20	3.68	15.22	3.4
	2		27.9	29.7	20.3	21.9	22.3				
Level 2	1	52	51.8	64.2	52.9	58.0	58.0	57.4	5.96	10.39	10.3
	2		50.2	69.5	52.8	57.9	58.3				
Level 3	1	86	77.4	108.4	80.5	88.4	89.9	88.9	11.33	12.74	3.4
	2		76.8	108.1	83.1	85.2	91.5				

10. retinol (VIA)

Accuracy compared to Reference Material - fill in yellow shaded cells

Mean concentration should be within ±15% of the nominal value except at 3*LOD, where it should be within ± 20%

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Reference material: **SRM968e**
Analyte: **retinol (VIA)**

Reference material	Replicate	Nominal value	Measured concentration					Mean	SD	CV (%)	Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5				
Level 1	1	34.1	36.0	34.5	39.3	34.0	34.2	36.3	3.83	10.55	6.3
	2		35.1	34.5	46.2	33.8	35.2				
Level 2	1	48.2	49.5	55.8	54.5	49.3	48.3	50.9	2.60	5.11	5.7
	2		49.3	52.1	49.0	49.3	52.4				
Level 3	1	64.7	62.3	63.6	71.2	63.5	65.9	64.8	4.19	6.46	0.2
	2		62.4	62.2	73.6	61.7	62.0				

11. alpha-tocopherol (VIE)

Accuracy compared to Reference Material - fill in yellow shaded cells

Mean concentration should be within ±15% of the nominal value except at 3*LOD, where it should be within ± 20%

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Reference material: **SRM968e**
Analyte: **Vitamin E (VIE)**

Reference material	Replicate	Nominal value	Measured concentration					Mean	SD	CV (%)	Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5				
Level 1	1	653	688	668	769	709	733	716.05	56.61	7.91	9.7
	2		687	662	851	698	697				
Level 2	1	1033	1040	962	1063	1081	1095	1062.2	43.09	4.06	2.8
	2		1038	1055	1084	1087	1117				
Level 3	1	1937	1876	1857	1902	1943	2001	1922.6	60.13	3.13	-0.7
	2		1878	1843	2015	1947	1963				

Precision

The method precision of the 13 analytes in the low QC and high QC is shown below.

1. alpha-carotene (ALC)

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	Vitamins A and E and carotenoids in serum
Method #:	4020
Matrix:	Serum
Units:	µg/dL
Analyte:	**α-carotene (ALC)**

Quality material 1 (2010 Low QC)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	12.4	13.7	13.05	0.4225	0.4225	340.605
2	11.2	11.8	11.50	0.09	0.09	264.5
3	11.5	12.1	11.80	0.09	0.09	278.48
4	10.7	11.1	10.90	0.04	0.04	237.62
5	11.2	12.2	11.70	0.25	0.25	273.78
6	11.3	12.1	11.70	0.16	0.16	273.78
7	10.4	10.5	10.45	0.0025	0.0025	218.405
8	11.3	11.5	11.40	0.01	0.01	259.92
9	12.1	12.1	12.10	0	0	292.82
10	11.1	11.4	11.25	0.0225	0.0225	253.125
Grand sum	231.7	Grand mean	11.585			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	2.175	0.2175	0.466368953	4.03
Between Run	8.7905	0.976722222	0.616125889	5.32
Total	10.9655		0.772729649	**6.67**

Quality material 2 (2010 High QC)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	3.35	3.65	3.50	0.0225	0.0225	24.5
2	3.28	3.29	3.29	2.5E-05	2.5E-05	21.58245
3	3.25	3.40	3.33	0.005625	0.005625	22.11125
4	3.06	3.18	3.12	0.0036	0.0036	19.4688
5	2.96	3.59	3.28	0.099225	0.099225	21.45125
6	2.86	3.00	2.93	0.0049	0.0049	17.1698
7	3.09	2.85	2.97	0.0144	0.0144	17.6418
8	3.03	3.05	3.04	0.0001	1E-04	18.4832
9	3.44	3.22	3.33	0.0121	0.0121	22.1778
10	2.97	3.07	3.02	0.0025	0.0025	18.2408
Grand sum	63.59	Grand mean	3.1795			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	0.32995	0.032995	0.181645259	5.71
Between Run	0.642745	0.071416111	0.138602148	4.36
Total	0.972695		0.228485351	**7.19**

2. alpha-cryptoxanthin (ARY)

Precision - fill in yellow shaded cells

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	Vitamins A and E and carotenoids in serum
Method #:	4020
Matrix:	Serum
Units:	µg/dL
Analyte:	**α-cryptoxanthin (ARY)**

Quality material 1 (Low QC:LS10493)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	2.77	2.97	2.87	0.01	0.01	16.4738
2	2.52	2.71	2.62	0.009025	0.009025	13.67645
3	2.63	2.69	2.66	0.0009	0.0009	14.1512
4	2.59	2.49	2.54	0.0025	0.0025	12.9032
5	2.47	2.65	2.56	0.0081	0.0081	13.1072
6	2.59	2.75	2.67	0.0064	0.0064	14.2578
7	2.34	2.42	2.38	0.0016	0.0016	11.3288
8	2.58	2.48	2.53	0.0025	0.0025	12.8018
9	2.69	3.00	2.85	0.024025	0.024025	16.18805
10	2.52	2.55	2.54	0.000225	0.000225	12.85245
Grand sum	52.41	Grand mean	2.6205			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.13055	0.013055	0.114258479	4.36
Between Run	0.400345	0.044482778	0.125355051	4.78
Total	0.530895		0.169613941	6.47

Quality material 2 (High QC:HS10492)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	3.71	3.92	3.82	0.011025	0.011025	29.10845
2	3.67	3.35	3.51	0.0256	0.0256	24.6402
3	3.46	3.70	3.58	0.0144	0.0144	25.6328
4	3.29	3.44	3.37	0.005625	0.005625	22.64645
5	3.28	3.67	3.48	0.038025	0.038025	24.15125
6	3.16	3.44	3.30	0.0196	0.0196	21.78
7	3.32	3.10	3.21	0.0121	0.0121	20.6082
8	3.37	3.38	3.38	2.5E-05	2.5E-05	22.78125
9	3.67	3.39	3.53	0.0196	0.0196	24.9218
10	3.29	3.47	3.38	0.0081	0.0081	22.8488
Grand sum	69.08	Grand mean	3.454			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	0.3082	0.03082	0.175556259	5.08
Between Run	0.51688	0.057431111	0.11534971	3.34
Total	0.82508		0.210060838	6.08

3. beta-carotene (BEC)

Precision - fill in yellow shaded cells

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	Vitamins A and E and carotenoids in serum
Method #:	4020
Matrix:	Serum
Units:	µg/dL
Analyte:	**β-carotene (BEC)**

Quality material 1 (Low QC:LS10493)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	62.7	68.1	65.4	7.29	7.29	8554.32
2	70.5	74.9	72.7	4.84	4.84	10570.58
3	64.8	68.8	66.8	4	4	8924.48
4	65.4	68.2	66.8	1.96	1.96	8924.48
5	67.4	72.8	70.1	7.29	7.29	9828.02
6	72.5	76.9	74.7	4.84	4.84	11160.18
7	66.2	67.5	66.9	0.4225	0.4225	8937.845
8	71.1	73.0	72.1	0.9025	0.9025	10382.405
9	77.0	76.8	76.9	0.01	0.01	11827.22
10	69.3	70.4	69.9	0.3025	0.3025	9758.045
Grand sum	1404.3	Grand mean	70.215			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	63.715	6.3715	2.524183036	3.59
Between Run	264.6505	29.40561111	3.393678764	4.83
Total	328.3655		4.229486441	**6.02**

Quality material 2 (High QC:HS10492)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	40.6	42.8	41.7	1.21	1.21	3477.78
2	48.4	45.8	47.1	1.69	1.69	4436.82
3	42.1	45.0	43.6	2.1025	2.1025	3793.205
4	43.4	45.9	44.7	1.5625	1.5625	3987.245
5	43.4	47.3	45.4	3.8025	3.8025	4113.245
6	44.5	46.7	45.6	1.21	1.21	4158.72
7	46.0	43.3	44.7	1.8225	1.8225	3987.245
8	46.1	46.3	46.2	0.01	0.01	4268.88
9	51.0	47.6	49.3	2.89	2.89	4860.98
10	43.9	45.9	44.9	1	1	4032.02
Grand sum	906	Grand mean	45.3			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	34.6	3.46	1.860107524	4.11
Between Run	74.34	8.26	1.549193338	3.42
Total	108.94		2.420743687	**5.34**

4. cis-beta-carotene (CBC)

Precision - fill in yellow shaded cells

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	Vitamins A and E and carotenoids in serum
Method #:	4020
Matrix:	Serum
Units:	µg/dL
Analyte:	*cis* -β-carotene (CBC)

Quality material 1 (Low QC:LS10493)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	2.86	3.35	3.11	0.061504	0.061504	19.28205
2	2.96	3.41	3.19	0.05040025	0.05040025	20.3203125
3	2.88	3.49	3.18	0.09150625	0.09150625	20.2693445
4	3.02	3.34	3.18	0.02544025	0.02544025	20.1930125
5	3.39	3.70	3.54	0.023716	0.023716	25.105698
6	3.21	3.65	3.43	0.04995225	0.04995225	23.5366605
7	3.11	3.23	3.17	0.003721	0.003721	20.110482
8	2.99	3.08	3.03	0.001849	0.001849	18.398178
9	3.21	3.57	3.39	0.03150625	0.03150625	22.9503125
10	3.71	3.97	3.84	0.0169	0.0169	29.4912
Grand sum	66.117	Grand mean	3.30585			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.7129905	0.07129905	0.26701882	8.08
Between Run	1.08436605	0.120485117	0.156821661	4.74
Total	1.79735655		0.309664469	**9.37**

Quality material 2 (High QC:HS10492)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	1.71	2.02	1.87	0.024336	0.024336	6.971378
2	1.87	1.83	1.85	0.00034225	0.00034225	6.8265125
3	1.88	1.82	1.85	0.000961	0.000961	6.822818
4	1.83	2.05	1.94	0.0121	0.0121	7.550498
5	1.99	2.31	2.15	0.024649	0.024649	9.253602
6	1.79	2.17	1.98	0.035721	0.035721	7.856648
7	2.00	2.03	2.01	0.00034225	0.00034225	8.1164205
8	1.98	2.01	1.99	0.000169	0.000169	7.944098
9	1.99	2.00	2.00	4E-06	4E-06	7.968032
10	1.93	2.42	2.18	0.060025	0.060025	9.46125
Grand sum	39.632	Grand mean	1.9816			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	0.317299	0.0317299	0.178128886	8.99
Between Run	0.2364858	0.0262762	0	0.00
Total	0.5537848		0.178128886	**8.99**

5. beta-cryptoxanthin (CRY)

Precision - fill in yellow shaded cells

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	Vitamins A and E and carotenoids in serum
Method #:	4020
Matrix:	Serum
Units:	µg/dL
Analyte:	**β-cryptoxanthin (CRY)**

Quality material 1 (Low QC:LS10493)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	7.63	8.41	8.02	0.1521	0.1521	128.6408
2	7.01	7.43	7.22	0.0441	0.0441	104.2568
3	7.18	7.61	7.40	0.046225	0.046225	109.37205
4	6.69	6.91	6.80	0.0121	0.0121	92.48
5	6.84	7.46	7.15	0.0961	0.0961	102.245
6	7.05	7.66	7.36	0.093025	0.093025	108.19205
7	6.49	6.66	6.58	0.007225	0.007225	86.46125
8	7.05	7.15	7.10	0.0025	0.0025	100.82
9	7.47	7.88	7.68	0.042025	0.042025	117.81125
10	6.92	7.10	7.01	0.0081	0.0081	98.2802
Grand sum	144.6	Grand mean	7.23			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	1.007	0.1007	0.317332633	4.39
Between Run	3.1014	0.3446	0.349213402	4.83
Total	4.1084		0.471858029	6.53

Quality material 2 (High QC:HS10492)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	10.0	10.5	10.3	0.0625	0.0625	210.125
2	9.66	8.99	9.3	0.112225	0.112225	173.91125
3	9.26	9.89	9.6	0.099225	0.099225	183.36125
4	8.69	9.24	9.0	0.075625	0.075625	160.74245
5	8.76	9.72	9.2	0.2304	0.2304	170.7552
6	8.63	9.12	8.9	0.060025	0.060025	157.53125
7	8.94	8.40	8.7	0.0729	0.0729	150.3378
8	8.98	8.96	9.0	1E-04	1E-04	160.9218
9	9.90	9.18	9.5	0.1296	0.1296	182.0232
10	8.82	9.30	9.1	0.0576	0.0576	164.1672
Grand sum	184.94	Grand mean	9.247			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	1.8004	0.18004	0.424311207	4.59
Between Run	3.73622	0.415135556	0.342852414	3.71
Total	5.53662		0.545516066	5.90

6. gamma-tocopherol (GTC)

Precision - fill in yellow shaded cells

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: **γ-tocopherol (GTC)**

Quality material 1 (Med QC:MS10491)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	619	630	625	30.25	30.25	780000.5
2	607	587	597	100	100	712818
3	581	592	587	30.25	30.25	687964.5
4	559	558	559	0.25	0.25	623844.5
5	575	579	577	4	4	665858
6	517	511	514	9	9	528392
7	590	552	571	361	361	652082
8	566	535	551	240.25	240.25	606100.5
9	609	589	599	100	100	717602
10	552	546	549	9	9	602802
Grand sum	11454	Grand mean	572.7			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	1768	176.8	13.29661611	2.32
Between Run	17758.2	1973.133333	29.96942887	5.23
Total	19526.2		32.78668429	**5.72**

Quality material 2 (High QC:HS10492)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	183	182	183	0.25	0.25	66612.5
2	183	159	171	144	144	58482
3	163	171	167	16	16	55778
4	157	163	160	9	9	51200
5	161	164	163	2.25	2.25	52812.5
6	148	147	148	0.25	0.25	43512.5
7	170	159	165	30.25	30.25	54120.5
8	151	151	151	0	0	45602
9	171	159	165	36	36	54450
10	152	149	151	2.25	2.25	45300.5
Grand sum	3243	Grand mean	162.15			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	480.5	48.05	6.93181073	4.27
Between Run	2018.05	224.2277778	9.385568118	5.79
Total	2498.55		11.66785708	**7.20**

7. lutein/zeaxanthin (LUZ)

Precision - fill in yellow shaded cells

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: **lutein/zeaxanthin (LUZ)**

Quality material 1 (Low QC:LS10493)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	11.0	11.1	11.05	0.0025	0.0025	244.205
2	10.3	10.8	10.55	0.0625	0.0625	222.605
3	9.29	9.91	9.60	0.0961	0.0961	184.32
4	9.33	9.63	9.48	0.0225	0.0225	179.7408
5	9.68	9.81	9.75	0.004225	0.004225	189.93005
6	9.33	11.4	10.37	1.071225	1.071225	214.86645
7	9.93	9.44	9.69	0.060025	0.060025	187.59845
8	10.8	9.90	10.35	0.2025	0.2025	214.245
9	11.0	10.1	10.55	0.2025	0.2025	222.605
10	10.7	10.3	10.50	0.04	0.04	220.5
Grand sum	203.75	Grand mean	10.1875			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	3.52815	0.352815	0.593982323	5.83
Between Run	4.912625	0.545847222	0.310670422	3.05
Total	8.440775		0.670321648	**6.58**

Quality material 2 (High QC:HS10492)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	17.9	19.0	18.45	0.3025	0.3025	680.805
2	17.5	16.0	16.75	0.5625	0.5625	561.125
3	16.0	17.5	16.75	0.5625	0.5625	561.125
4	15.7	17.0	16.35	0.4225	0.4225	534.645
5	15.9	18.0	16.95	1.1025	1.1025	574.605
6	20.8	17.2	19.00	3.24	3.24	722
7	15.8	14.7	15.25	0.3025	0.3025	465.125
8	15.8	15.6	15.70	0.01	0.01	492.98
9	17.7	16.7	17.20	0.25	0.25	591.68
10	15.6	16.5	16.05	0.2025	0.2025	515.205
Grand sum	336.9	Grand mean	16.845			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	13.915	1.3915	1.179618582	7.00
Between Run	24.2145	2.6905	0.805915628	4.78
Total	38.1295		1.428635713	**8.48**

8. trans-lycopene (LYC)

Precision - fill in yellow shaded cells

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	Vitamins A and E and carotenoids in serum
Method #:	4020
Matrix:	Serum
Units:	µg/dL
Analyte:	***trans*-lycopene (LYC)**

Quality material 1 (Low QC:LS10493)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	14.8	15.1	15.0	0.0225	0.0225	447.005
2	15.9	16.2	16.1	0.0225	0.0225	515.205
3	15.3	15.5	15.4	0.01	0.01	474.32
4	14.4	14.6	14.5	0.01	0.01	420.5
5	15.1	15.4	15.3	0.0225	0.0225	465.125
6	14.8	14.5	14.7	0.0225	0.0225	429.245
7	14.8	14.9	14.9	0.0025	0.0025	441.045
8	15.4	15.7	15.6	0.0225	0.0225	483.605
9	17.4	15.6	16.5	0.81	0.81	544.5
10	15.6	15.1	15.4	0.0625	0.0625	471.245
Grand sum	306.1	Grand mean	15.305			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	2.015	0.2015	0.448887514	2.93
Between Run	6.9345	0.7705	0.533385414	3.49
Total	8.9495		0.697137002	**4.55**

Quality material 2 (High QC:HS10492)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	16.1	16.3	16.2	0.01	0.01	524.88
2	18.0	16.4	17.2	0.64	0.64	591.68
3	16.8	17.4	17.1	0.09	0.09	584.82
4	16.1	16.6	16.4	0.0625	0.0625	534.645
5	16.1	16.7	16.4	0.09	0.09	537.92
6	15.2	15.8	15.5	0.09	0.09	480.5
7	17.3	16.1	16.7	0.36	0.36	557.78
8	16.7	16.6	16.7	0.0025	0.0025	554.445
9	19.4	17.6	18.5	0.81	0.81	684.5
10	16.5	16.6	16.6	0.0025	0.0025	547.805
Grand sum	334.3	Grand mean	16.715			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	4.315	0.4315	0.656886596	3.93
Between Run	11.1505	1.238944444	0.635391393	3.80
Total	15.4655		0.913904931	**5.47**

9. retinyl palmitate (RPL)

Precision - fill in yellow shaded cells

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	Vitamins A and E and carotenoids in serum
Method #:	4020
Matrix:	Serum
Units:	µg/dL
Analyte:	**retinyl palmitate (RPL)**

Quality material 1 (Medium QC:MS10491)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	11.90	11.60	11.75	0.0225	0.0225	276.125
2	11.70	11.30	11.50	0.04	0.04	264.5
3	11.80	11.80	11.80	0	0	278.48
4	12.00	11.70	11.85	0.0225	0.0225	280.845
5	11.10	11.30	11.20	0.01	0.01	250.88
6	11.80	11.70	11.75	0.0025	0.0025	276.125
7	11.50	11.90	11.70	0.04	0.04	273.78
8	12.40	11.90	12.15	0.0625	0.0625	295.245
9	12.10	13.30	12.70	0.36	0.36	322.58
10	13.00	11.90	12.45	0.3025	0.3025	310.005
Grand sum	237.7	Grand mean	11.885			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	1.725	0.1725	0.415331193	3.49
Between Run	3.5005	0.388944444	0.328971461	2.77
Total	5.2255		0.529832259	**4.46**

Quality material 2 (High QC:HS17492)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	6.80	6.92	6.86	0.0036	0.0036	94.1192
2	6.18	6.56	6.37	0.0361	0.0361	81.1538
3	7.28	6.61	6.95	0.112225	0.112225	96.46605
4	6.86	7.10	6.98	0.0144	0.0144	97.4408
5	6.90	6.88	6.89	1E-04	0.0001	94.9442
6	6.51	6.76	6.64	0.015625	0.015625	88.04645
7	6.40	6.46	6.43	0.0009	0.0009	82.6898
8	6.14	6.92	6.53	0.1521	0.1521	85.2818
9	6.63	6.11	6.37	0.0676	0.0676	81.1538
10	5.85	6.16	6.01	0.024025	0.024025	72.12005
		2.129				
Grand sum	132.03	**2.157**	6.6015			
		2.188				

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	0.85335	0.085335	0.29212155	4.43
Between Run	1.819905	0.202211667	0.241740219	3.66
Total	2.673255		0.379174542	**5.74**

10. retinyl stearate (RST)

Precision - fill in yellow shaded cells

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: **retinyl stearate (RST)**

Quality material 1 (Medium QC:MS10491)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	4.99	5.30	5.145	0.024025	0.024025	52.94205
2	4.95	4.87	4.910	0.0016	0.0016	48.2162
3	4.88	5.04	4.960	0.0064	0.0064	49.2032
4	4.82	4.80	4.810	1E-04	0.0001	46.2722
5	4.71	4.79	4.750	0.0016	0.0016	45.125
6	4.77	4.44	4.605	0.027225	0.027225	42.41205
7	5.00	4.88	4.940	0.0036	0.0036	48.8072
8	4.88	4.61	4.745	0.018225	0.018225	45.03005
9	4.64	4.89	4.765	0.015625	0.015625	45.41045
10	4.76	4.77	4.765	2.5E-05	2.5E-05	45.41045
Grand sum	96.79	Grand mean	4.8395			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.19685	0.019685	0.140303243	2.90
Between Run	0.413645	0.045960556	0.114620146	2.37
Total	0.610495		0.181170576	**3.74**

Quality material 2 (High QC:HS17492)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	11.9	12.1	12.00	0.01	0.01	288
2	11.6	11.6	11.60	0	0	269.12
3	12.6	11.7	12.15	0.2025	0.2025	295.245
4	12.7	12.7	12.70	0	0	322.58
5	12.1	12.0	12.05	0.0025	0.0025	290.405
6	11.6	12.0	11.80	0.04	0.04	278.48
7	11.3	11.4	11.35	0.0025	0.0025	257.645
8	10.9	12.1	11.50	0.36	0.36	264.5
9	12.1	10.9	11.50	0.36	0.36	264.5
10	10.6	11.3	10.95	0.1225	0.1225	239.805
Grand sum	235.2	Grand mean	11.76			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	2.2	0.22	0.469041576	3.99
Between Run	4.328	0.480888889	0.361170935	3.07
Total	6.528		0.591983483	**5.03**

11. total lycopene (TLY)

Precision - fill in yellow shaded cells

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	Vitamins A and E and carotenoids in serum
Method #:	4020
Matrix:	Serum
Units:	µg/dL
Analyte:	**total lycopene (TLY)**

Quality material 1 (Low QC:LS10493)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	28.0	28.9	28.5	0.2025	0.2025	1618.805
2	30.2	31.4	30.8	0.36	0.36	1897.28
3	30.5	30.8	30.7	0.0225	0.0225	1878.845
4	28.2	28.5	28.4	0.0225	0.0225	1607.445
5	29.5	30.8	30.2	0.4225	0.4225	1818.045
6	27.2	27.6	27.4	0.04	0.04	1501.52
7	28.0	28.6	28.3	0.09	0.09	1601.78
8	29.3	29.7	29.5	0.04	0.04	1740.5
9	34.1	31.5	32.8	1.69	1.69	2151.68
10	29.9	29.7	29.8	0.01	0.01	1776.08
Grand sum	592.4	Grand mean	29.62			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	5.8	0.58	0.761577311	2.57
Between Run	45.092	5.010222222	1.488324935	5.02
Total	50.892		1.67185858	**5.64**

Quality material 2 (High QC:HS10492)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	36.9	37.7	37.3	0.16	0.16	2782.58
2	40.9	38.4	39.7	1.5625	1.5625	3144.245
3	39.1	40.7	39.9	0.64	0.64	3184.02
4	36.7	37.9	37.3	0.36	0.36	2782.58
5	37.9	39.8	38.9	0.9025	0.9025	3018.645
6	33.4	34.3	33.9	0.2025	0.2025	2291.645
7	39.3	36.4	37.9	2.1025	2.1025	2865.245
8	37.6	37.3	37.5	0.0225	0.0225	2805.005
9	44.5	40.7	42.6	3.61	3.61	3629.52
10	38.0	38.1	38.1	0.0025	0.0025	2895.605
Grand sum	765.6	Grand mean	38.28			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	19.13	1.913	1.383112432	3.61
Between Run	91.922	10.21355556	2.037223055	5.32
Total	111.052		2.462372388	**6.43**

12. retinol (VIA)

Precision - fill in yellow shaded cells

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	Vitamins A and E and carotenoids in serum
Method #:	4020
Matrix:	Serum
Units:	µg/dL
Analyte:	**retinol (VIA)**

Quality material 1 (Med QC:MS10491)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	112.0	114.0	113.00	1	1	25538
2	112.0	111.0	111.50	0.25	0.25	24864.5
3	105.0	108.0	106.50	2.25	2.25	22684.5
4	109.0	111.0	110.00	1	1	24200
5	113.0	108.0	110.50	6.25	6.25	24420.5
6	98.8	97.4	98.10	0.49	0.49	19247.22
7	111.0	103.0	107.00	16	16	22898
8	109.0	105.0	107.00	4	4	22898
9	119.0	106.0	112.50	42.25	42.25	25312.5
10	105.0	105.0	105.00	0	0	22050
Grand sum	2162.2	Grand mean	108.11			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	146.98	14.698	3.833797073	3.55
Between Run	357.778	39.75311111	3.539428705	3.27
Total	504.758		5.217811376	**4.83**

Quality material 2 (High QC:HS10492)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	53.0	52.8	52.9	0.01	0.01	5596.82
2	57.5	51.4	54.5	9.3025	9.3025	5929.605
3	49.5	50.4	50.0	0.2025	0.2025	4990.005
4	52.1	52.8	52.5	0.1225	0.1225	5502.005
5	51.1	52.4	51.8	0.4225	0.4225	5356.125
6	60.1	48.4	54.3	34.2225	34.2225	5886.125
7	53.6	49.0	51.3	5.29	5.29	5263.38
8	48.9	48.5	48.7	0.04	0.04	4743.38
9	52.5	50.0	51.3	1.5625	1.5625	5253.125
10	49.4	49.2	49.3	0.01	0.01	4860.98
Grand sum	1032.6	Grand mean	51.63			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	102.37	10.237	3.199531216	6.20
Between Run	68.412	7.601333333	0	0.00
Total	170.782		3.199531216	**6.20**

13. alpha-tocopherol (VIE)

Precision - fill in yellow shaded cells

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	Vitamins A and E and carotenoids in serum
Method #:	4020
Matrix:	Serum
Units:	µg/dL
Analyte:	**Vitamin E (VIE)**

Quality material 1 (Med QC:MS10491)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	2200	2240	2220	400	400	9856800
2	2560	2480	2520	1600	1600	12700800
3	2460	2500	2480	400	400	12300800
4	2320	2290	2305	225	225	10626050
5	2330	2360	2345	225	225	10998050
6	2150	2110	2130	400	400	9073800
7	2430	2300	2365	4225	4225	11186450
8	2320	2170	2245	5625	5625	10080050
9	2270	2260	2265	25	25	10260450
10	2310	2290	2300	100	100	10580000
Grand sum	46350	Grand mean	2317.5			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	26450	2645	51.42956348	2.22
Between Run	247125	27458.33333	111.3852175	4.81
Total	273575		122.6852341	**5.29**

Quality material 2 (High QC:HS10492)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	1470	1460	1465	25	25	4292450
2	1800	1570	1685	13225	13225	5678450
3	1600	1640	1620	400	400	5248800
4	1530	1510	1520	100	100	4620800
5	1550	1570	1560	100	100	4867200
6	1370	1380	1375	25	25	3781250
7	1650	1490	1570	6400	6400	4929800
8	1450	1440	1445	25	25	4176050
9	1570	1440	1505	4225	4225	4530050
10	1500	1480	1490	100	100	4440200
Grand sum	30470	Grand mean	1523.5			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	49250	4925	70.17834424	4.61
Between Run	144005	16000.55556	74.41624673	4.88
Total	193255		102.2877206	**6.71**

Stability

The freeze/thaw, bench-top, processed sample, and long-term stability of the 13 analytes in the low QC and high QC is shown below. The long-term stability of the retinyl esters and most of the carotenoids exceed 15% difference, but the retinyl esters are low level analytes near LOD and the carotenoid pool means from 2010 vs 2017 overlap within 1-2SD.

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions	
Describe condition:	QC vials thawed three times and re-frozen at -80°C (3 freeze-thaw cycles)
Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)	
Describe condition:	QC vials stored on the bench top for 3 hours after removing from -80°C freezer
Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler	
Describe condition:	Processed samples stored at autosampler @ 20°C for 24hr
Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis	
Describe condition:	QC pools prepared stored continuously at -80°C compared to data obtained 9/16/2013

1. alpha-carotene (ALC)

Stability - fill in yellow shaded cells

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: **α-carotene (ALC)**

Quality material 1	Medium QC:MS10491		Low QC:LS10493		Low QC:LS10493		Low QC:LS10493	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	1.45	1.41	10.7	10.9	10.7	10.8	12.5	10.7
Replicate 2	1.32	1.49	11.6	10.7	11.6	11.3	12.2	11.6
Replicate 3	1.41	1.59	11.1	11.1	11.1	10.8		11.1
Mean	1.4	1.5	11.1	10.9	11.1	11.0	12.4	11.1
% difference from initial measurement	--	7.4	--	-2.2	--	-1.5	--	-9.7

Quality material 2	High QC:HS17492		High QC:HS10492		High QC:HS10492		High QC:HS10492	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	1.12	1.18	3.06	2.97	3.06	2.98	3.80	3.06
Replicate 2	1.19	1.17	3.09	2.97	3.09	3.18	3.66	3.09
Replicate 3	1.11	1.20	3.18	2.91	3.18	3.10		3.18
Mean	1.14	1.18	3.11	2.95	3.11	3.08	3.73	3.11
% difference from initial measurement	--	3.9	--	-5.1	--	-0.8	--	-16.7

2. alpha-cryptoxanthin (ARY) – new analyte therefore no long-term stability data available

Stability - fill in yellow shaded cells

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: **α-cryptoxanthin (ARY)**

Quality material 1	Medium QC:MS10491		Low QC:LS10493		Low QC:LS10493		Low QC:LS10493	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	2.61	2.41	2.59	2.58	2.59	2.51		
Replicate 2	2.49	2.51	2.57	2.58	2.57	2.58		
Replicate 3	2.79	2.80	2.49	2.69	2.49	2.32		
Mean	2.63	2.57	2.55	2.61	2.55	2.47	#DIV/0!	#DIV/0!
% difference from initial measurement	--	-2.1	--	2.5	--	-3.3	--	#DIV/0!

Quality material 2	High QC:HS17492		High QC:HS10492		High QC:HS10492		High QC:HS10492	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	0.979	0.855	3.29	3.26	3.29	3.18		
Replicate 2	0.863	0.860	3.36	3.36	3.36	3.26		
Replicate 3	0.849	0.934	3.44	3.20	3.44	3.30		
Mean	0.90	0.88	3.36	3.27	3.36	3.25	#DIV/0!	#DIV/0!
% difference from initial measurement	--	-1.6	--	-2.8	--	-3.4	--	#DIV/0!

3. beta-carotene (BEC)

Stability - fill in yellow shaded cells

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: **β-carotene (BEC)**

Quality material 1	Medium QC:MS10491		Low QC:LS10493		Low QC:LS10493		Low QC:LS10493	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	12.46	11.0	65.4	67.4	65.4	66.9	66.9	65.4
Replicate 2	12.50	12.6	71.8	66.1	71.8	70.0	69.4	71.8
Replicate 3	12.79	13.2	68.2	68.9	68.2	66.6		68.2
Mean	12.6	12.3	68.4	67.5	68.4	67.8	68.2	68.4
% difference from initial measurement	--	-2.4	--	-1.4	--	-0.9	--	0.4

Quality material 2	High QC:HS17492		High QC:HS10492		High QC:HS10492		High QC:HS10492	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	8.41	8.41	43.4	43.3	43.4	43.6	41.4	43.4
Replicate 2	8.64	8.54	45.4	43.0	45.4	44.2	40.3	45.4
Replicate 3	8.45	8.43	45.9	43.1	45.9	44.6		45.9
Mean	8.50	8.46	44.9	43.1	44.9	44.1	40.9	44.9
% difference from initial measurement	--	-0.5	--	-4.0	--	-1.7	--	9.9

4. cis-beta-carotene (CBC)

Stability - fill in yellow shaded cells

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: ***cis*-β-carotene (CBC)**

Quality material 1	Medium QC:MS10491		Low QC:LS10493		Low QC:LS10493		Low QC:LS10493	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	0.603	0.589	3.14	3.12	3.14	3.51	4.26	3.14
Replicate 2	0.617	0.638	3.48	3.26	3.48	3.65	4.16	3.48
Replicate 3	0.548	0.701	3.48	3.20	3.48	3.33		3.48
Mean	0.589	0.643	3.36	3.19	3.36	3.50	4.21	3.36
% difference from initial measurement	--	**9.0**	--	**-5.1**	--	**4.0**	--	**-20.2**

Quality material 2	High QC:HS17492		High QC:HS10492		High QC:HS10492		High QC:HS10492	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	0.464	0.509	1.97	1.96	1.97	2.12	2.91	1.97
Replicate 2	0.495	0.517	2.08	2.09	2.08	2.28	3.20	2.08
Replicate 3	0.436	0.509	2.16	1.92	2.16	2.05		2.16
Mean	0.465	0.512	2.07	1.99	2.07	2.15	3.06	2.07
% difference from initial measurement	--	**10.0**	--	**-3.9**	--	**3.7**	--	**-32.3**

5. beta-cryptoxanthin (CRY)

Stability - fill in yellow shaded cells

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: **β-cryptoxanthin (CRY)**

Quality material 1	Medium QC:MS10491		Low QC:LS10493		Low QC:LS10493		Low QC:LS10493	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	4.18	3.77	6.69	6.97	6.69	6.69	9.31	6.97
Replicate 2	4.16	4.19	7.22	6.89	7.22	7.03	9.59	6.89
Replicate 3	4.44	4.56	6.91	7.07	6.91	6.77		7.07
Mean	4.26	4.18	6.94	6.98	6.94	6.83	9.45	6.98
% difference from initial measurement	--	**-2.0**	--	**0.5**	--	**-1.6**	--	**-26.2**

Quality material 2	High QC:HS17492		High QC:HS10492		High QC:HS10492		High QC:HS10492	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	2.44	2.30	8.69	8.61	8.69	8.68	11.2	8.61
Replicate 2	2.33	2.32	9.09	8.64	9.09	8.82	11.6	8.64
Replicate 3	2.25	2.43	9.24	8.56	9.24	8.90		8.56
Mean	2.34	2.35	9.00	8.60	9.00	8.80	11.4	8.60
% difference from initial measurement	--	**0.3**	--	**-4.4**	--	**-2.3**	--	**-24.6**

6. gamma-tocopherol (GTC)

Stability - fill in yellow shaded cells

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: **γ-tocopherol (GTC)**

Quality material 1	Med QC:MS10491		Med QC:MS10491		Med QC:MS10491		Med QC:MS10491	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	513	527	559	567	559	554	599	559
Replicate 2	511	516	575	560	575	572	603	575
Replicate 3	513	515	558	548	558	558		558
Mean	512	519	564	558	564	562	601	564
% difference from initial measurement	--	1.4	--	-1.0	--	-0.5	--	-6.1

Quality material 2	High QC:HS17492		High QC:HS10492		High QC:HS10492		High QC:HS10492	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	107	111	157	154	157	155	174	157
Replicate 2	111	110	162	155	162	155	176	162
Replicate 3	112	108	163	152	163	156		163
Mean	110	110	161	154	161	155	175	161
% difference from initial measurement	--	-0.4	--	-4.3	--	-3.4	--	-8.3

7. lutein/zeaxanthin (LUZ)

Stability - fill in yellow shaded cells

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: **lutein/zeaxanthin (LUZ)**

Quality material 1	Medium QC:MS10491		Low QC:LS10493		Low QC:LS10493		Low QC:LS10493	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	11.4	12.5	14.3	16.6	14.3	14.5	15.1	14.3
Replicate 2	11.4	11.6	13.7	17.2	13.7	13.3	16.9	13.7
Replicate 3	12.2	12.7	15.5	15.1	15.5	15.3		15.5
Mean	11.7	12.2	14.5	16.3	14.5	14.3	16.0	14.5
% difference from initial measurement	--	4.9	--	12.6	--	-0.9	--	-9.5

Quality material 2	High QC:HS17492		High QC:HS10492		High QC:HS10492		High QC:HS10492	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	4.30	3.83	15.7	14.7	15.7	15.5	17.1	15.7
Replicate 2	3.94	3.84	16.7	16.9	16.7	16.1	16.5	16.7
Replicate 3	4.29	4.16	17.0	15.3	17.0	16.2		17.0
Mean	4.18	3.94	16.5	15.6	16.5	15.9	16.8	16.5
% difference from initial measurement	--	-5.6	--	-5.1	--	-3.1	--	-2.0

8. trans-lycopene (LYC)

Stability - fill in yellow shaded cells

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: ***trans*-lycopene (LYC)**

Quality material 1	Medium QC:MS10491		Low QC:LS10493		Low QC:LS10493		Low QC:LS10493	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	21.0	18.5	14.4	14.8	14.4	15.1	17.0	14.4
Replicate 2	19.9	21.0	15.5	14.4	15.5	15.9	16.4	15.5
Replicate 3	20.4	22.2	14.6	15.1	14.6	14.9		14.6
Mean	20.4	20.6	14.9	14.8	14.9	15.3	16.7	14.9
% difference from initial measurement	--	0.8	--	-0.6	--	2.9	--	-11.0

Quality material 2	High QC:HS17492		High QC:HS10492		High QC:HS10492		High QC:HS10492	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	13.5	13.4	16.1	16.0	16.1	16.7	16.6	16.1
Replicate 2	13.5	13.5	16.4	15.8	16.4	16.7	16.8	16.4
Replicate 3	13.1	13.4	16.6	15.8	16.6	17.1		16.6
Mean	13.4	13.4	16.4	15.9	16.4	16.8	16.7	16.4
% difference from initial measurement	--	0.4	--	-2.9	--	2.8	--	-2.1

9. retinyl palmitate (RPL)

Stability - fill in yellow shaded cells

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: **retinyl palmitate (RPL)**

Quality material 1	Med QC:MS10491		Med QC:MS10491		Med QC:MS10491		Med QC:MS10491	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	11.9	11.3	12.3	10.6	12.3	12.7	12.0	12.3
Replicate 2	11.5	11.8	11.9	11.6	11.9	12.6	12.2	11.9
Replicate 3	11.5	11.5	12.3	12.1	12.3	12.8		12.3
Mean	11.6	11.5	12.2	11.4	12.2	12.7	12.1	12.2
% difference from initial measurement	--	-0.7	--	-6.2	--	4.3	--	0.4

Quality material 2	High QC:HS17492		High QC:HS17492		High QC:HS17492		High QC:HS10492	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	6.29	6.70	6.80	6.50	6.80	6.91	3.04	2.17
Replicate 2	6.61	6.61	6.72	6.69	6.72	6.91	3.10	2.19
Replicate 3	6.28	6.76	6.92	5.83	6.92	7.03		2.17
Mean	6.39	6.69	6.81	6.34	6.81	6.95	3.07	2.17
% difference from initial measurement	--	4.7	--	-6.9	--	2.1	--	-29.1

10. retinyl stearate (RST)

Stability - fill in yellow shaded cells

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: **retinyl stearate (RST)**

Quality material 1	Med QC:MS10491		Med QC:MS10491		Med QC:MS10491		Med QC:MS10491	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	4.64	4.41	4.82	4.22	4.82	4.94	4.46	4.82
Replicate 2	4.44	4.67	4.56	4.56	4.56	4.70	4.35	4.56
Replicate 3	4.57	4.33	4.81	4.85	4.81	4.85		4.81
Mean	4.55	4.47	4.73	4.55	4.73	4.83	4.40	4.73
% difference from initial measurement	--	-1.8	--	-3.8	--	2.2	--	7.3

Quality material 2	High QC:HS17492		High QC:HS17492		High QC:HS17492		High QC:HS10492	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	11.1	11.9	11.9	11.6	11.9	12.5	0.651	0.608
Replicate 2	11.7	11.8	11.9	11.8	11.9	12.4	0.759	0.638
Replicate 3	11.1	12.1	12.1	10.3	12.1	12.7		0.492
Mean	11.3	11.9	12.0	11.2	12.0	12.6	0.705	0.579
% difference from initial measurement	--	5.5	--	-6.2	--	5.1	--	-17.8

11. total lycopene (TLY)

Stability - fill in yellow shaded cells

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: **total lycopene (TLY)**

Quality material 1	Medium QC:MS10491		Low QC:LS10493		Low QC:LS10493		Low QC:LS10493	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	37.6	33.7	28.2	28.7	28.2	29.4	35.1	28.2
Replicate 2	35.9	38.2	29.9	27.9	29.9	31.3	33.8	29.9
Replicate 3	37.8	40.4	28.5	29.3	28.5	28.8		28.5
Mean	37.1	37.4	28.9	28.6	28.9	29.8	34.5	28.9
% difference from initial measurement	--	1.0	--	-0.7	--	3.3	--	-16.2

Quality material 2	High QC:HS17492		High QC:HS10492		High QC:HS10492		High QC:HS10492	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	24.3	24.4	36.7	36.4	36.7	37.6	39.9	36.7
Replicate 2	24.6	24.5	37.4	36.2	37.4	38.9	39.7	37.4
Replicate 3	23.9	24.4	37.9	36.1	37.9	39.3		37.9
Mean	24.2	24.5	37.3	36.3	37.3	38.6	39.8	37.3
% difference from initial measurement	--	0.9	--	-2.9	--	3.5	--	-6.2

12. retinol (VIA)

Stability - fill in yellow shaded cells

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: **retinol (VIA)**

Quality material 1	Med QC:MS10491		Med QC:MS10491		Med QC:MS10491		Med QC:MS10491	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	98.8	115	109	134	109	107	109	109
Replicate 2	97.5	98.4	124	114	124	122	109	124
Replicate 3	97.4	98.2	111	108	111	109		111
Mean	97.9	104	114.5	119.0	114.5	112.9	109	114
% difference from initial measurement	--	6.0	--	3.9	--	-1.4	--	4.9

Quality material 2	High QC:HS17492		High QC:HS10492		High QC:HS10492		High QC:HS10492	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	30.8	30.5	52.1	51.2	52.1	51.2	50.2	52.1
Replicate 2	31.4	30.5	52.4	55.9	52.4	51.8	49.7	52.4
Replicate 3	30.8	30.5	52.8	52.8	52.8	51.8		52.8
Mean	31.0	30.5	52.4	53.3	52.4	51.6	50.0	52.4
% difference from initial measurement	--	-1.5	--	1.6	--	-1.6	--	5.0

13. alpha-tocopherol (VIE)

Stability - fill in yellow shaded cells

Method name: Vitamins A and E and carotenoids in serum
Method #: 4020
Matrix: Serum
Units: µg/dL
Analyte: **Vitamin E (VIE)**

Quality material 1	Med QC:MS10491		Med QC:MS10491		Med QC:MS10491		Med QC:MS10491	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	2114	2078	2317	2189	2317	2308	2127	2317
Replicate 2	2112	2130	2294	2244	2294	2295	2135	2294
Replicate 3	2123	2135	2287	2252	2287	2318		2287
Mean	2116	2114	2299	2228	2299	2307	2131	2299
% difference from initial measurement	--	-0.1	--	-3.1	--	0.4	--	7.9

Quality material 2	High QC:HS17492		High QC:HS10492		High QC:HS10492		High QC:HS10492	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	676	699	1528	1484	1528	1511	1468	1528
Replicate 2	703	695	1504	1457	1504	1520	1461	1504
Replicate 3	690	683	1514	1456	1514	1523		1514
Mean	690	692	1515	1466	1515	1518	1464	1515
% difference from initial measurement	--	0.4	--	-3.3	--	0.2	--	3.5

LOD, specificity and fit for intended use

The LOD, specificity, and fit for intended use of the 13 analytes is summarized here:

LOD, specificity and fit for intended use - fill in yellow shaded cells

Method name:	Vitamins A and E and carotenoids in serum
Method #:	4020
Matrix:	Serum
Units:	µg/dL

Analytes	Limit of Detection (LOD)	Interferences successfully checked in at least 50 human samples	Accuracy, precision, LOD, specificity and stability meet performance specifications for intended use
α-carotene (ALC)	0.7	yes	yes
α-crypotoxanthin (ARY)	0.2	yes	yes
β-carotene (BEC)	0.8	yes	yes
cis -β-carotene (CBC)	0.7	yes	yes
β-crypotoxanthin (CRY)	0.9	yes	yes
γ-tocopherol (GTC)	11	yes	yes
lutein/zeaxanthin (LUZ)	2.4	yes	yes
trans -lycopene (LYC)	0.8	yes	yes
retinyl palmitate (RPL)	1.3	yes	yes
retinyl stearate (RST)	0.7	yes	yes
total lycopene (TLY)	1	yes	yes
retinol (VIA)	1	yes	yes
α-tocopherol (VIE)	40	yes	yes

Appendix B: Ruggedness Testing

Detailed information can be found at:

\\cdc.gov\project\CCEHIP_NCEH_DLS_NBB_LABS\AECarDocuments\Freeze Thaw and Ruggedness Testing\AECAR Ruggedness

1. **Ice bath temperature**
   a. **Principle:** To compare the effect of using higher temperature ice bath before hexane transfer.
   b. **Proposal:** Three QC pools were prepared using ice bath temperatures of 0°C, -20°C, and -35°C compared to the SOP results using a -70°C bath.
   c. **Findings:** Percent difference between the concentrations and peak height (peak area for TLY) compared to results obtained at -70°C.

Analyte	Concentration % difference			Signal % difference		
	0°C	-20°C	-35°C	0°C	-20°C	-35°C
ALC	-0.04%	4%	2%	-31%	-9%	4%
ARY	9%	12%	-20%	-28%	28%	19%
BEC	2%	1%	3%	-30%	-11%	5%
CBC	2%	7%	12%	-30%	-5%	14%
CRY	-0.03%	2%	5%	-31%	-10%	7%
GTC	0.4%	-1%	-1%	-30%	-11%	6%
LUZ	3%	4%	5%	-29%	-9%	7%
LYC	2%	2%	3%	-30%	-11%	4%
RPL	-3%	-4%	-1%	-32%	-14%	7%
RST*	-5%	-11%	-2%	-32%	-13%	5%
TLY	0.8%	0.02%	0.9%	-31%	27%	26%
VIA	0.03%	-1%	-2%	-30%	-11%	5%
VIE	0.4%	-1%	-0.2%	-30%	-11%	7%

* Low level analyte, only one pool had detectable RST (retinyl stearate)

   d. **Summary:** Bath temperatures significantly affected peak height for all analytes. Lower bath temperatures produced stronger signals. A temperature of at least -35°C appears to be adequate, but the aqueous phase must be frozen solid to avoid transfer of water to the organic phase. Thus, ≤ -70°C is preferred.

2. **Preservative/salt composition**

   a. **Principle:** To compare different preservative/salt compositions to the method standard of 10%:10% ascorbic acid:sodium chloride solution.

   b. **Proposal:** Alter the preservative and/or salt composition to 10% sodium chloride only, 10% ascorbic acid only, and 10%:20% ascorbic acid:sodium chloride. Each solution was tested on 4 QC pools.

   c. **Findings:** Average percent difference between the concentrations using different preservative and/or salt compositions compared to the concentrations of the 10%:10% ascorbic acid:sodium chloride solution.

Analyte	10% sodium chloride	10% ascorbic acid	10%:20% ascorbic acid:sodium chloride
ALC	-1%	-15%	-1%
ARY	4%	-7%	-2%
BEC	0.3%	-12%	-2%
CBC	2%	-13%	-1%
CRY	0.3%	-9%	-2%
GTC	0.02%	1%	-0.2%
LUZ	4%	5%	-3%
LYC	-0.2%	-12%	-1%
RPL	3%	-8%	7%
RST	1%	-11%	3%
TLY	2%	-12%	-1%
VIA	5%	3%	0.3%
VIE	-2%	-2%	0.2%
Overall average (SD)	-1% (2%)	-7% (7%)	0.2% (3%)

   d. **Summary:** The 10% ascorbic acid only solution had >10% difference on 6/12 analytes and was on average (SD) -7% (7%). The addition of salt to the serum denatures protein and provides assistance in extraction. The 10%:20% ascorbic acid:sodium chloride solution yielded similar results to the standard 10%:10% ascorbic acid:sodium chloride solution. Therefore the additional salt isn't necessary and the 10%:10% ascorbic acid:sodium chloride solution will be used.

3. **Hexane pour-off**

   a. **Principle:** To compare the effects of losing hexane during the pour-off step. After the hexane extraction step, the aqueous layer is frozen in an ethanol:dry ice bath and the hexane layer is poured-off into a new culture tube for the dry-down and reconstitution steps.

   b. **Proposal:** Split the hexane pour-off from 3 QC pools into two fractions to simulate a spill during the step.

   c. **Findings:** Average percent difference from 3 QC pools comparing the split fractions of hexane pour-off compared to the standard 100% fraction collection.

Percent difference of each analyte (concentrations) or internal standard (peak height)			
Analyte	Split #1	Split #2	Average split
ALC	7%	-2%	2%
ARY	0.1%	-4%	-2%
BEC	1%	-0.3%	0.4%
CBC	4%	8%	6%
CRY	-1%	-2%	-1%
GTC	1%	2%	1%
LUZ	1%	2%	1%
LYC	1%	-1%	0.2%
RPL	-2%	-6%	-4%
RST	12%	10%	11%
TLY	4%	2%	3%
VIA	1%	2%	1%
VIE	1%	1%	1%
C45 (@450nm)	-51%	-52%	-51%
RBA (@325nm)	-50%	-49%	-49%
RBE (@300nm)	-50%	-49%	-49%

   d. **Summary:** Although the internal standard recovery was on average (SD) -50% (1%) for the split fractions, the concentrations of each analyte were still comparable with an overall average (SD) of 2% (4%).

4. **Post dry-down phase**

   a. **Principle:** Following the addition of hexane for extraction, the hexane must be evaporated in order to concentrate the analyte for re-suspension with soluble solvents for the assay. The process of removing the hexane results in an empty tube with a residual 'button' that must be reconstituted. The residue is either completely dry or a bit moist. This test evaluates the resulting effects of the state of the residue.

   b. **Proposal:** To continue to run the Speedvac beyond the time necessary to dry the sample resulting in a dry button or stop the Speedvac before the samples are fully dry resulting in a wet button. The times vary depending upon the number of samples within a run so the evaluation is visual not based upon a specific Speedvac operation time.

   c. **Findings:** Average percent difference of the 3 pools with a wet button or dry button compared to the proper dry time.

	Percent difference in concentrations (test/SOP)		signal peak height ratio (test/SOP)	
Analyte	Wet button	Dry button	Wet button	Dry button
ALC	4%	2%	1.06	0.930
ARY	6%	3%	1.07	0.938
BEC	1%	4%	1.03	0.943
CBC	5%	12%	1.06	1.08
CRY	4%	4%	1.05	0.940
GTC	-1%	0.5%	1.06	0.970
LUZ	2%	3%	1.04	0.937
LYC	2%	3%	1.03	0.933
RPL	-5%	-5%	1.01	0.923
RST	-3%	-3%	0.960	1.07
TLY	1%	3%	1.02	0.933
VIA	-2%	-2%	1.05	0.950
VIE	-0.4%	0.2%	1.05	0.967
average	**1%**	**2%**	**1.04**	**0.96**
SD	**3%**	**4%**	**0.03**	**0.05**

   * Low level analyte, only one pool had detectable RST (retinyl stearate)

   d. **Summary:** There is minimal difference overall between the wet button and dry button. The wet button produces somewhat higher signal than the dry button on average (SD) 1.04 (0.03) and 0.96 (0.05), respectively.

5. **Assessing column performance**

   a. **Principle:** Some columns do not separate the various carotenoids and/or give poor peak resolution.

   b. **Proposal:** To test multiple C18 columns from the same manufacturer but different serial numbers to assess the peak shape and separation.

   c. **Findings:** Although several analytes showed poor peak resolution, we will only show the data for total lycopene (TLY) since that peak is integrated by area which includes the trans- and cis- lycopene isomers. TLY best illustrates the need for good peak resolution.

Figure 1. chromatography at 450nm on a column with poor TLY peak resolution

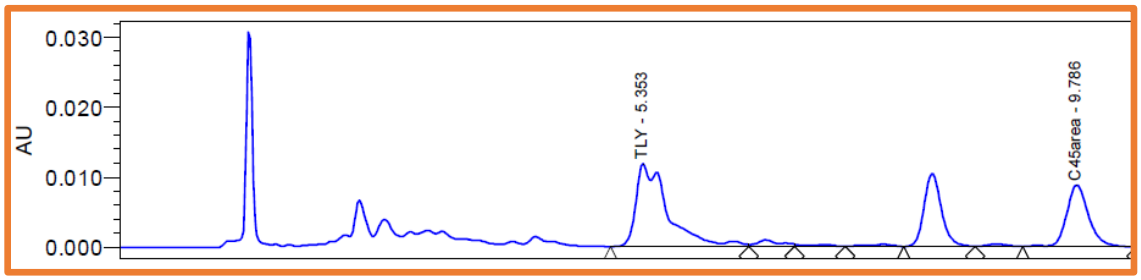


Figure 2. chromatography at 450nm on a column with good TLY peak resolution

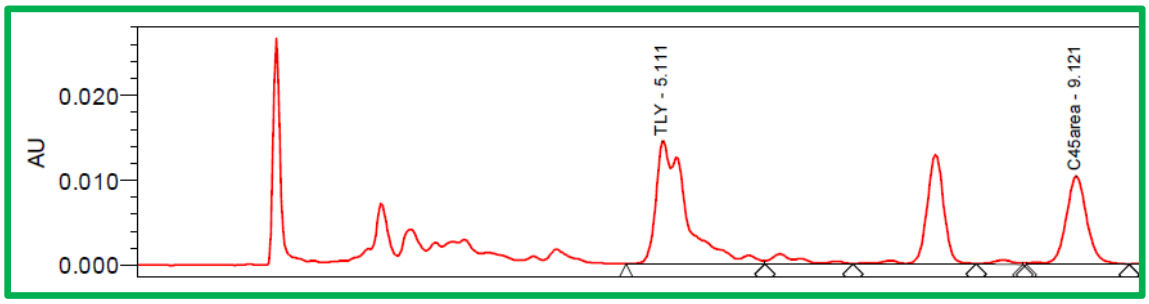


Table 1. Comparison of TLY values from the chromatography shown in figures 1 and 2

	SRM target (ug/dL)	SRM measured (ug/dL)	% diff
Figure 1	86	69.5	-19%
Figure 2	86	80.5	-6%

   d. **Summary:** Total lycopene was lower by 13% in the chromatography with poor resolution. Therefore assessing multiple columns with various SRM and quality control materials is essential to obtaining accurate and consistent results.