Laboratory Procedure Manual

Analyte: **Vitamin C (Ascorbic Acid)**

Matrix: **Serum**

Method: **Liquid Chromatography Electrochemical Detection**

Method No: 4031.01

Revised: 6/2020

as performed by: Nutritional Biomarkers Branch (NBB)
Division of Laboratory Sciences (DLS)
National Center for Environmental Health (NCEH)

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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
VIC_J	LBXVIC	Vitamin C (mg/dL)
	LBDVICSI	Vitamin C (umol/L)

1. Summary of Clinical Relevance and Principle

A. Clinical Relevance

Ascorbic acid deficiency causes scurvy; however, scurvy is uncommon in the United States. Ascorbic acid has very low toxicity. Adverse effects reported from excess vitamin C consumption include diarrhea and kidney stones.

B. Test Principle

Vitamin C (ascorbic acid) in serum is measured using isocratic ultra-high performance liquid chromatography (UPLC) with electrochemical detection at 450 mV (range 200 nA). One part serum is mixed with four parts 6% metaphosphoric acid (MPA) to acidify the serum and stabilize ascorbate. The specimen is frozen at -70 °C until analysis. After the specimen is thawed at room temperature and centrifuged at 3,000 rpm, the supernatant is decanted. This supernatant is mixed with a solution containing trisodium phosphate and dithiothreitol (to reduce dehydroascorbate to ascorbate) and an internal standard (1-methyl uric acid) to reduce dehydroascorbate to ascorbate. It is re-acidified with 40% MPA to stabilize the ascorbate. The sample is filtered to remove insoluble material. A 4 µL aliquot is injected onto a C-18 reversed-phase column and eluted with a mobile phase containing 14.1 g/L monochloroacetic acid, 0.76 g/L disodium ethylenediamine tetraacetate, 1% (by volume) 10 N sodium hydroxide, and 1.5% (by volume) methanol, adjusted to pH 3.00 ± 0.03 with 10 N sodium hydroxide. Quantitation is accomplished by comparing the peak area of vitamin C in the unknown with the peak area of a known amount in a calibrator solution. Calculations are corrected based on the peak area of the internal standard in the unknown compared with the peak area of the internal standard in the calibrator solution.

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 (SDSs) for these chemicals are readily accessible as hard copies in the lab. If needed, SDSs 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 electrochemical detector 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 **4031_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) Specimens for vitamin C analysis must be prepared from fresh (not frozen) serum harvested from blood collected in a red-top, blue-top or serum-separator vacutainer tubes collected using standard venipuncture procedures. Serum is immediately mixed as follows:

(1) One part serum with four parts 6% MPA in water in a polypropylene storage vial (e.g. 100 µL of serum is mixed with 400 µL of 6% MPA solution) labeled with the specimen ID

(2) The vial contents are then vortexed and immediately frozen, preferably at -70 °C

(B) A 0.5 mL sample of 1 part serum and 4 parts 6% MPA solution is required to allow for repeat analyses; a volume of 100 µL is required per analysis.

(C) Specimens collected in the field are frozen and then shipped on dry ice by overnight carrier. Frozen samples are stored at -70 °C.

(D) 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.

(E) Specimens that have been improperly stabilized with 6% MPA solution will give inaccurate test results.

(F) Samples are stable for at least 5 years at -70 °C and may withstand at least three freeze/thaw cycles if the total time thawed is no more than 4 hours.

(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 Nalgene 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

> *Prepare all reagents using deionized water with resistance of at least 15 megaohms. Store all reagents (except mobile phase) at 4 °C, bringing them to room temperature before use.*

1) Mobile Phase

In a 1-L beaker, combine 950 mL deionized water (with a resistance of at least 15 megaohms) and 10 mL 10 N NaOH while stirring on a magnetic stir plate. Then add 0.76 g disodium EDTA and continue stirring until dissolved. Then add 14.2 g monochloroacetic acid and continue stirring until dissolved. Verify pH is 3.00 ± 0.03; adjust using 1mL or less of 10N NaOH or HCl. Then add 15 mL methanol and 25 mL deionized water. The solution is stable indefinitely at room temperature.

2) 5 g/dL Metaphosphoric Acid (MPA) Solution (only used in Round Robin and Calibration)

Dissolve 30.0 g MPA solid in deionized water in a 500 mL volumetric flask and dilute to volume. The solution is stable for 3-4 weeks at 4°C. Note that the solution is 1.8% MPA (weight/volume).

3) Assay Blank

6 g/dL Metaphosphoric Acid + 0.25 g/dL Dithiothreitol (DTT) Solution (used in routine assay as a blank or as a diluent for high vitamin C samples)

Dissolve 30.0 g MPA solid and 1.25 g DTT in deionized water in a 500 mL volumetric flask and dilute to volume. The solution pH is 1.82. Note that the solution is 2.1% MPA weight/volume. Aliquots of 25 mL in 50 mL tubes are stored at -70 °C. The solution is stable for 3-4 weeks at 4 °C once thawed.

4) Assay Reagent #1

3.8 g/dL Trisodium Phosphate (TSP) + 0.25 g/dL Dithiothreitol Solution (used in routine assay)

Dissolve 19.0 g TSP (0.1 M) and 1.25 g DTT with deionized water in a 500 mL volumetric flask and dilute to volume. Aliquots of 25 mL in 50 mL tubes are stored at -70 °C; one aliquot is enough for ~120 samples. The solution is stable for 3-4 weeks at 4 °C once thawed.

5) Assay Reagent #2

40 g/dL Metaphosphoric Acid (MPA solution)

Dissolve 40.0 g MPA solid in a 100 mL volumetric flask and dilute to volume with deionized water. Note that the solution is 14% MPA (weight/volume). Aliquots of 2 mL in cryovials are kept at -70 °C. Use repeater pipette to add 45 uL into sample tube; one aliquot is enough for ~ 35 samples. The solution is stable for 3-4 weeks at 4 °C once thawed.

6) Stock IS Solution

15 mg/dL 1-Methyl Uric Acid Internal Stock Solution

Weigh out 15 mg of 1-methyl uric acid and transfer to a 100 mL volumetric flask. Add 5 drops of concentrated ammonium hydroxide to help it go into solution. Fill to the mark with deionized water. Keep 2 mL aliquots in cryovials at -70°C. The solution is stable for 3 months at 4°C once thawed. (1/10 dilution needs to be prepared before each experiment.)

7) Stock DTT Solution for IS

0.075 g/mL Dithiothreitol Stock Solution

Weigh out 3.75 g DTT and transfer to 50 mL volumetric flask. Fill to the mark with deionized water. Keep 2 mL aliquots in cryovials at -70 °C.

8) Working IS Solution

1.5 mg/dL 1-Methyl Uric Acid Internal Standard Working Solution (used in routine assay)

In a 100 mL volumetric flask, add 10 mL of (6) 15 mg/dL 1-methyl uric acid IS stock solution then add 3.3 mL of (7) 0.075 g/mL DTT stock solution and fill to the mark with deionized water. The solution is stable for 3 months at 4 °C.

B. Standards Preparation

Stock standards and calibrators should be prepared on the same day.

1) Stock Standards

a) Stock #1 (AA stock I):

Prepare the stock ascorbic acid standard #1 (0.10 g/dL) in duplicate (AA stock 1_1 and AA stock 1_2) by weighing out 100.00 mg of ascorbic acid and dissolve in 5 g/dL MPA solution in a small beaker. Transfer the solution into a 100 mL volumetric flask and rinse the beaker several times with 5 g/dL MPA solution and pour into the flask. Fill to the mark with 5 g/dL MPA solution. Mix well by inversion. Discard remainder after calibrators have been completed and verified.

b) Stock #2 (AA stock II):

Prepare the stock ascorbic acid standard #2 (2.0 mg/dL) in duplicate by pipetting 2 mL of AA stock 1_1 into 100 mL volumetric flask and 2 mL AA stock 1_2 into 100 mL volumetric flask and diluting to the mark with 5 g/dL MPA solution. Mix well by inversion. Discard remainder after calibrators have been completed and verified.

c) Measurement of AA stock II_1 and AA stock II_2 concentrations:

The concentrations of AA stock II_1 and AA stock II_2 solutions are measured in triplicate using a spectrophotometer (CARY 300) at 244 nm using 5 g/dL MPA solution as blank. Be sure to go to ZERO (5 g/dL MPA solution) between each reading. Use the average of the two closest readings to calculate the concentration. Use the molar extinction coefficient (Ec) 546.13 to calculate the concentration. Calculation: [(avg two closest readings)*1000]/546.13. Select the stock #2 solution that gives the concentration closest to 2.0 mg/dL to make Cal5.

Stock Standards #1 and #2 are prepared when fresh calibrators are prepared.

2) Calibrators

Prepare in order shown in table. Calculate actual concentrations based on value obtained from Cary reading of the stock #2 solution.

Calibrator Level	Target Concentration (mg/dL)	AA stock used	AA stock volume used	Volume 6 g/dL MPA + 0.25 g/dL DTT solution used
5	0.5	AA stock II	50 mL	150 mL
4	0.3	AA stock II	15 mL	85 mL
3	0.15	AA calibrator 5	30 mL	70 mL
2	0.075	AA calibrator 5	15 mL	85 mL
1	0.025	AA calibrator 5	5 mL	95 mL

Prepare these calibrators as needed and store at -70 °C in labeled 0.5 mL narrow volume cryovials (each containing a 0.4 mL aliquot). The frozen aliquots are stable at -70 °C for at least 4 years. Calibrators 1-5 are used as routine assay. Calibrators 1-5 are run on UPLC as unknowns to confirm their concentrations prior to use as calibrators.

C. Preparation of Quality Control Materials

1) Bench Quality Controls

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 Nalgene cryovial, sealed, and vortexed. The QC pools are stored at -70 °C and are stable for more than 10 years. Vitamin C in serum is not stable, therefore fresh serum should be used. Screen serum blood bank donors for endogenous vitamin C. When necessary spike serum to achieve low, medium or high levels with appropriate volumes of 1 g/100-mL ascorbic acid solution in deionized water to achieve ~0.2 – 2.0 mg/dL final concentration(s). Limits for all pools are established by analyzing duplicates for at least 20 runs.

2) Blind Quality Controls

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 Nalgene cryovial, sealed, and vortexed. The Blind QC pools are stored at -70 °C and are stable for more than 10 years. Vitamin C in serum is not stable, therefore fresh serum should be used. Screen serum blood bank donors for endogenous vitamin C. When necessary spike serum to achieve low, medium or high levels with appropriate volumes of 1 g/100-mL ascorbic acid solution in deionized water to achieve ~0.2 – 2.0 mg/dL final concentration(s). 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) LCMS certified clear glass 12 x 32 mm screw neck max recovery vial, with cap and preslit PTFE/Silicone Septa, 2 mL volume (Waters 600000670CV)

b) Acquity UPLC HSS T3 column 1.8 um x 2.1 mm x 150 mm (Waters 186003540)

c) 0.45 µm syringe tip PVDF hydrophilic filter (4 mm diameter) (Millipore)

d) Plastic tuberculin syringes (obtained from various sources)

e) 2 mL polypropylene cryovials (Thermo Scientific)

f) 2 mL externally threaded cryovials with orange caps (Corning)

g) 0.5mL skirted microcentrifuge tubes with caps with o’rings for calibrators – PP (Fisher)

h) 12 x 75 mm disposable glass culture tubes (Fisher)

i) 5¾ inch pasteur pipettes (Fisher)

j) Combitip Plus (0.5 mL) for Eppendorf repeater pipette (Eppendorf)

k) Combitip Plus (5.0 mL) for Eppendorf repeater pipette (Eppendorf)

l) Yellow tips (2-200 uL) for Eppendorf pipette (Eppendorf)

m) Various glass beakers, graduated cylinders and glass bottles, class A glassware

(2) Chemicals

a) Methanol, ACS/HPLC grade (Burdick & Jackson AH230)

b) Monochloroacetic acid, ACS grade (Fisher A176)

c) Disodium Ethylenediamine Tetraacetate (EDTA), ACS grade (Fisher S311)

d) Metaphosphoric acid (MPA), ACS grade (Fisher A280; Usually 34-37% pure MPA by weight.

e) 10 N Sodium Hydroxide (NaOH) (Fisher SS255)

f) Trisodium Phosphate (TSP), ACS grade (Fisher S377)

g) L-Ascorbic acid, ACS grade (Fisher A61)

h) 1-methyl uric acid (Sigma-Aldrich M6885)

i) Dithiothreitol (DTT) (Acros 32719-0100)

j) 6N hydrochloric acid solution (Ricca 3750-32)

k) pH 1.00 Buffer(Fisher SB140)

l) pH 4.00 Buffer (Fisher SB101)

m) pH 7.00 Buffer (Fisher SB107)

n) pH 10.00 Buffer (Fisher SB115)

o) Saturated potassium chloride for filling pH electrode

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, electrochemical detector) 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 Acquity H-Class ultra-performance liquid chromatography (UPLC) System (Waters Milford, MA)

a) Waters H-Class Sample Manager – Flow Through Needle (SM-FTN)

b) Waters H-Class Quaternary Solvent Manager (QSM)

c) Waters H-Class Column heater

d) Waters 2465 Electrochemical Detector (ECD)

e) Waters e-Satin data converter

f) Waters Empower data analysis software

(2) Single tube vortex mixer

(3) Eppendorf Centrifuge 5810R

(4) Magnetic stirrer

(5) Fisher XL 150 pH meter

(6) Eppendorf single channel pipettes

(7) Eppendorf repeater pipettes

(8) Mettler ML203T analytical balance

(9) Cary 300 Bio UV-visible spectrophotometer (Varian instruments)

7. Calibration and Calibration Verification Procedures

A. Method Calibration

Three calibration standards plus a blank are prepared along with the samples. The concentrations of the calibrators are multiplied by factor 5 to calculate the final concentration of ascorbic acid in serum because of the dilution of serum with MPA as described in Section 1.

The calibration curve is injected at the beginning of the analytical run. Using the Empower software a five-point linear standard calibration curve is generated. Each chromatographic peak is reviewed for correct delineation of the baseline and proper retention. Vitamin C concentrations in unknown samples are calculated from the calibration curve. The calibrators are reanalyzed as unknowns at the end of each run and must be within ± 15% of their established concentrations.

Calibration verification is conducted at least twice a year. For details, see **4031_SOP Calibration Verification Vitamin C**.

This method uses water as the matrix for the calibrators. Matrix based calibration was tested by comparing the average slope of three 10-point calibration curves prepared using metaphoric acid stabilized serum with three 10-point water-based calibration curves. A ≤ 3% difference in the average calibration curve slope was observed between serum and water-based calibrations for vitamin C.

Dilution linearity was assessed by diluting the high quality control pool with the assay blank (6 g/dL Metaphosphoric Acid + 0.25 g/dL Dithiothreitol Solution) to read off the standard curve. Three dilutions were prepared in duplicate (dilution factors: 1.25, 2, and 4). The vitamin C results were on average (SD) 96% (2%) of the undiluted value. For the routine method, vitamin C results high than the calibration curve (2.5 mg/dL) will be diluted with the assay blank to read within the calibration curve.

For troubleshooting and accuracy verification, NIST SRM 970 (Level I and II) is available.

External proficiency testing programs currently do not exist for vitamin C. An in-house proficiency testing program has been developed and is conducted at least twice a year. For details, see **4031_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**.

Method comparison results are presented in **Appendix C**.

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 needed using calibrated weights.

D. Cary UV/vis spectrophotometer

Calibration verification is performed three times per year by participation in the CAP instrumentation survey.

E. pH meter

Calibration verification is performed by the analyst prior to use as needed using commercial calibration solutions.

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 prior to centrifugation. Visually check each sample for unusual specimen color or debris/precipitate.

(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) Prepare barcode labels for the unknowns and label the supernatant orange cap cryovials.

(5) Set-up and label one 12 x 75 mm culture tube and one HPLC vial per unknown/quality control/calibrator/blank.

(6) A typical run consists of 5 calibrators, a blank, 3 bench QC samples (first set), 40 patient samples, 2 blind QC samples, 3 bench QC samples (second set).

B. Sample Preparation

(1) Thaw unknowns, quality controls, and standards at room temperature for less than 1 hour.

(2) Hand-mix unknowns, quality controls, and standards via inversion.

(3) Centrifuge unknowns and quality controls for 20 minutes at 3000 rpm at room temperature.

(4) Decant supernatants into barcode labeled 2 mL orange cap cryovials.

(5) Add 50 µL internal standard into labeled 12 x 75 mm culture tubes.

(6) Aliquot 100 µL supernatant, standard or blank directly into the internal standard in each 12 x 75 mm culture tube.

(7) Add 300 µL of Assay Reagent #1 (3.8 g/dL TSP + 0.25 g/dL DTT Solution) into each 12 x 75 mm culture tube.

(8) Vortex gently for 5-10 seconds.

(9) Incubate 12 x 75 mm culture tubes at 4 °C for 30 minutes.

(10) Add 45uL of Assay Reagent #2 (40 g/dL MPA Solution) into each 12 x 75 mm culture tube.

(11) Vortex gently for 5-10 seconds.

(12) Draw the sample into a 1 mL tuberculin syringe and filter through a 0.45 µm PVDF filter into a labeled HPLC vial.

(13) Place HPLC vials in tray, then into refrigerated autosampler (~4 °C).

C. UPLC-ECD Instrument Preparation

(1) LC Preparation

a) Refill solvents and/or mobile phase

b) Pump: Prime solvent and seal wash lines: 4mL/min for 5 minutes

c) Autosampler: Prime wash solvent (15 seconds) and purge solvent (5 cycles)

d) Start equilibrating the system as follows:

1. Line B (80:20 water:ethanol): 0.1mL/min until pressure stabilizes (delta psi <100)

2. Line D (mobile phase): 0.1mL/min until pressure stabilizes (delta psi <100); once the pressure is stable increase by 0.1mL/min until the run flow rate of 0.4mL/min is reached (make sure pressure is stabilizing between flow rate increases).

(2) ECD settings (DC STAT)

a) Range: 200nA

b) Filt = 0.1s

c) Ec = 0.45V

D. 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 4031_SOP Processing and reporting a run.

a) Reviewing the chromatography

1. When the run is finished acquiring the data, the data is reviewed in Empower. Chromatograms for vitamin C, uric acid, 1-methyl uric acid (internal standard) and dithiothreitol are checked for retention times, peak shapes, peak separation, intensity and/or potential interferences. Note: Uric acid and dithiothreitol are not quantified.

b) Quantitation and integration of the completed data file

1. Generate results using auto integration.

2. Review integrations and make any necessary integration corrections either using the manual or auto integration option. Auto integration is preferred over manual integration.

3. Print the results for each analyte as a PDF to allow future review and documentation (routine procedure) or print hardcopies (exception).

4. Save the results in an ASCII file to import into the LIMS database.

5. Import the results file into the LIMS database for further data review.

E. Calculations

The Empower software performs all calculations. Calibration curves are linear and are based on the single analysis of five (Cal 1 – Cal 5) standard concentrations. Vitamin C concentrations in unknown samples are calculated using the regression parameters. Calculations are based on the single analysis of five (Cal 1 – Cal 5) standard concentrations according to the following formula: Concentration = (Response factor) (amount/area ratio) x (peak area ratio) x (dilution factor).

Response Factor – amount/ascorbic acid peak area

Peak area ratio – Ascorbic acid peak area/internal standard peak area

F. System Maintenance (other than daily maintenance)

Waters UPLC-ECD – 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

The analysis is based on the analytical method described by McCoy et. al. **[1]**.

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

The reportable range of results for vitamin C is 0.03 mg/dL (limit of detection) to 2.5 mg/dL. Samples with vitamin C results < 0.2 mg/dL (11.4 µmol/L) are reanalyzed for confirmation before results are released. Samples with vitamin C results >2.5 mg/dL are diluted with assay blank (6 g/dL Metaphosphoric Acid + 0.25 g/dL Dithiothreitol Solution) and reanalyzed for confirmation before results are released. There is no known maximum acceptable dilution. When possible, avoid small volume pipetting and minimize use of serial dilutions when generating diluted samples.

10. Quality Control (QC) Procedures

A. Blind Quality Controls

Blind QC specimens can be inserted into the mix of patient specimens. These QC specimens are often prepared at two levels that would be encountered in patient samples; the labels used are identical to those used for patient samples. One blind QC specimen randomly selected for concentration is included at a randomly selected location in every 20 specimens analyzed. Alternatively, open label blind QC specimens can be used where the analyst knows that the sample is a blind QC, but does not know what pool the sample is from. Open label blind QCs are only used if one can choose from at least 6 different pools and the analyte concentrations are similar to those found in patient samples.

B. Bench Quality Controls

Bench QC specimens are prepared from three serum pools, which represent low, intermediate, and high levels of vitamin C 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 the pools are checked after each run. The system is declared "in control" if the results pass the following tests:

Multi-rule quality control system: quality control rules for three QC pools per run

(1) If all three QC run means are within 2 S_m limits and individual results are within 2 S_i limits, accept the run

(2) If 1 of the 3 QC run means is outside a 2 S_m limit – reject run if:

a) 1 3S Rule—Run mean is outside a 3 S_m limit or

b) 2 2S Rule—Two or more of the three run means are outside the same 2 S_m limit or

c) 10 X-bar Rule—Current and previous nine run means are on the same side of the characterization mean

(3) If one of the six QC individual results is outside a 2 S_i limit – reject run if:

a) Outlier – One individual result is beyond the characterization mean ± 4 S_i or

b) R 4S Rule—Two or more of the within-run ranges in the same run exceed 4 S_w (i.e. 95 percent range limit)

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 **[2]**. 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., analyst forgot or under-pipetted the internal standard or re-acidify after reaction step; or any sample preparation problems, e.g. vial spilled during vortexing.

(B) Check the calibration of the pipettes.

(C) Check to make sure that the hardware is functioning properly. Make sure the ECD parameters are set correctly. The ECD is not controlled by the software but directly on the instrument panel. 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.

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

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

12. Limitations of Method; Interfering Substances and Conditions

(A) 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 by inversion before centrifuging to obtain the supernatant.

(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) This method has also undergone a series of in-house ruggedness testing experiments designed to assess how much method accuracy changes when certain experimental parameters are varied. A total of five parameters judged to most likely affect the accuracy of the method have been identified and tested. Testing generally consisted of performing replicate measurements on a test specimen with the selected parameter set at a value substantially lower and higher than that specified in this method while holding all other experimental variables constant. **The ruggedness testing findings for this method are presented in Appendix B**. Please refer to Chapter 21 of the *DLS Policies and Procedures Manual* for further information on ruggedness testing.

13. Reference Ranges (Normal Values)

The reference range (2.5 – 97.5%) for vitamin C in the U.S. population established from the NHANES 2003-2006 data is 0.11 – 2.04 mg/dL.

14. Critical Call Results (“Panic Values”)

Any NHANES samples with serum vitamin C levels < 0.2 mg/dL (11.4 µmol/L) are considered to require follow-up. 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 °C) as soon as possible. Once the samples are ready to run, they are placed in the autosampler at 4 °C.

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

Because the analysis of Vitamin C is inherently complex and challenging, there are no acceptable alternative methods of analysis in the nutrition laboratory. If the analytical system fails, we recommend storing the acidified serum at ≤ -70 °C 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.

We recommend that records, including related QA\QC data, be maintained for 10 years after completion of studies. Only numerical identifiers should be used (e.g., Sample ID); all personal identifiers should be available only to the medical supervisor or project coordinator. Residual serum or plasma from these analyses for non-NHANES studies may be discarded at the request of the principal investigator, or may be transferred to the CDC CASPIR facility for use by other investigators with the permission of the principal investigator. Very little residual material will be available after NHANES analyses are completed, and these vials may be routinely autoclaved.

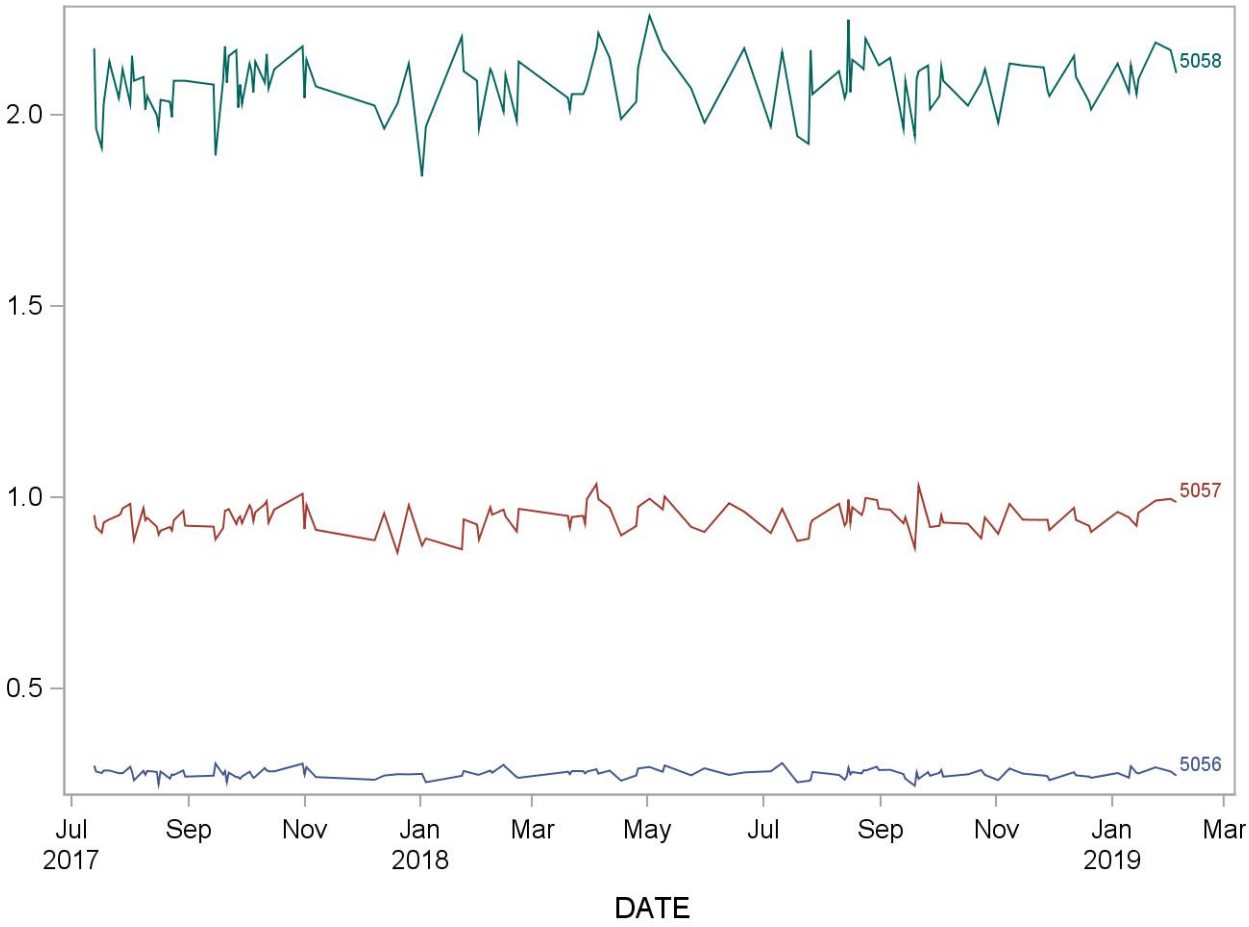
The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the specimens are stored in a freezer at -70 °C. The specimen ID is read by a barcode reader attached to the computer used to prepare the electronic specimen table for the analytical system. When the analyses are completed, the export file containing the electronic copy of the results is loaded in the LIMS database, and the analytical results are linked to the LIMS database by ID number. The analyst is responsible for keeping records of specimens prepared incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies. In general, these are documented using codes in the LIMS.

19. Summary Statistics and QC Graph

See following page.

2017-2018 Summary Statistics and QC Chart for Vitamin C (mg/dL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
5056	128	13JUL17	04FEB19	0.2785	0.0114	4.1
5057	128	13JUL17	04FEB19	0.9456	0.0341	3.6
5058	128	13JUL17	04FEB19	2.0814	0.0743	3.6



20. 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) McCoy LF, Bowen MB, Xu M, Chen H, Schleicher RL. Improved HPLC Assay for Measuring Serum Vitamin C with 1-Methyluric Acid Used as an Electrochemically Active Internal Standard. Clin Chem. 2005;51:1062-4.

(2) Caudill SP, Schleicher RL, Pirkle JL. 2008. Multi-rule quality control for the age-related eye disease study. Stat Med. 27:4094-4106.

Acknowledgements

We gratefully acknowledge the contribution of Sweta Patel who performed the validation of this method.

Appendix A: Method Performance Documentation

Accuracy using Spike Recovery

Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at LLOQ where can be 80-120%

Method name:	Vitamin C in serum
Method #:	4031
Matrix:	serum
Units:	mg/dL
Analyte:	**VIC**

		Sample 1 (Low QC)					Sample 2 (Medium 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	0.304	0.310	0.301		0	0.969	0.972	0.947		100.2	2.1
	2		0.290	0.300				0.944	0.913				
	3		0.290	0.313				0.984	0.900				
Sample + Spike 1	1	0.126	0.421	0.439	0.432	103.6	0.158	1.16	1.08	1.10	98.9		
	2		0.427	0.442				1.09	1.07				
	3		0.437	0.424				1.13	1.09				
Sample + Spike 2	1	0.756	1.09	1.09	1.07	101.3	0.945	1.98	1.80	1.88	98.8		
	2		1.12	1.05				1.92	1.77				
	3		1.01	1.04				1.98	1.83				
Sample + Spike 3	1	2.52	2.89	2.85	2.85	101.0	1.26	2.30	2.06	2.18	97.7		
	2		2.89	2.84				2.30	2.10				
	3		2.89	2.72				2.23	2.08				

Accuracy compared to Reference Material

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

Method name:	Vitamin C in serum
Method #:	4031
Matrix:	serum
Units:	umol/L
Reference material:	**NIST SRM 970**
Analyte:	**VIC**

			Measured concentration								
Reference material	Replicate	Nominal value	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	Difference from nominal value (%)
Level 1	1	8.41	8.71	9.14	7.81	8.68	8.78	8.65	0.38	4.41	2.9
	2		8.69	8.88	8.61	8.99	8.23				
Level 2	1	28.05	29.0	29.0	26.0	30.3	28.5	28.6	1.83	6.38	2.0
	2		30.0	29.0	25.7	31.3	27.2				
Level 3	1	not available									
	2										

Precision

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

Method name:	Vitamin C in serum
Method #:	4031
Matrix:	serum
Units:	mg/dL
Analyte:	VIC

Quality material 1 (Low QC)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	0.290	0.290	0.29	0	0	0.1682
2	0.309	0.326	0.32	7.225E-05	7.225E-05	0.2016125
3	0.336	0.311	0.32	0.00015625	0.00015625	0.2093045
4	0.273	0.292	0.28	9.025E-05	9.025E-05	0.1596125
5	0.282	0.309	0.30	0.00018225	0.00018225	0.1746405
6	0.282	0.275	0.28	0.00001225	1.225E-05	0.1551245
7	0.293	0.276	0.28	7.225E-05	7.225E-05	0.1618805
8	0.301	0.281	0.29	1E-04	0.0001	0.169362
9	0.304	0.259	0.28	0.00050625	0.00050625	0.1584845
10	0.294	0.282	0.29	3.6E-05	3.6E-05	0.165888
Grand sum	5.865	Grand mean	0.29325			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.0024555	0.00024555	0.015670035	5.34
Between Run	0.00419825	0.000466472	0.010510048	3.58
Total	0.00665375		0.018868257	**6.43**

Quality material 2 (High QC)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	2.13	2.08	2.10	0.00042025	0.00042025	8.8578405
2	2.20	2.10	2.15	0.0025	0.0025	9.270818
3	2.25	2.20	2.22	0.000484	0.000484	9.892352
4	2.18	2.11	2.14	0.001444	0.001444	9.193472
5	2.12	1.87	1.99	0.014641	0.014641	7.952072
6	2.18	2.11	2.14	0.001444	0.001444	9.184898
7	2.08	2.12	2.10	0.000361	0.000361	8.811602
8	2.01	1.98	1.99	0.00030625	0.00030625	7.9401125
9	2.06	2.07	2.07	4.9E-05	4.9E-05	8.52845
10	2.10	2.10	2.10	4E-06	4E-06	8.828402
Grand sum	42.04	Grand mean	2.102			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	0.043307	0.0043307	0.065808054	3.13
Between Run	0.091939	0.010215444	0.054243638	2.58
Total	0.135246		0.085282309	**4.06**

Stability

The initial measurement can be from the same day for all stability experiments.

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions

Describe condition: 2016 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: 2016 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 (2016 QC pools) stored at -80°C for 24hr; Ruggedness testing showed samples left in the autosampler aren't stable (Appendix B)

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 in 2004 stored continuously at -80°C compared to data obtained 8/11/2011

All stability sample results should be within ±15% of nominal concentration

Method name:	Vitamin C in serum
Method #:	4031
Matrix:	serum
Units:	mg/dL
Analyte:	**VIC**

Quality material 1 (Low QC)

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	0.314	0.309	0.314	0.292	0.295	0.291	0.229	0.237
Replicate 2	0.296	0.303	0.296	0.301	0.309	0.298	0.223	0.222
Replicate 3	0.288	0.307	0.288	0.305	0.294	0.294	0.223	0.232
Mean	0.299	0.306	0.299	0.299	0.299	0.294	0.225	0.230
% difference from initial measurement	--	**2.3**	--	**0.00**	--	**-1.7**	--	**2.3**

Quality material 2 (Medium QC)

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	0.964	0.941	0.964	0.932	0.972	0.984	0.748	0.710
Replicate 2	0.956	0.926	0.956	0.936	0.963	0.959	0.762	0.733
Replicate 3	0.858	0.927	0.858	0.917	0.904	0.932	0.757	0.759
Mean	0.926	0.931	0.926	0.928	0.946	0.958	0.756	0.734
% difference from initial measurement	--	**0.6**	--	**0.3**	--	**1.3**	--	**-2.9**

LOD, specificity and fit for intended use

Method name:	Vitamin C in serum
Method #:	4031
Matrix:	serum
Units:	mg/dL
Analyte:	**VIC**

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
Vitamin C	0.0311	yes	yes

Appendix B: Ruggedness Testing

Detailed information can be found at:

\\cdc.gov\project\CCEHIP_NCEH_DLS_NBB_LABS\CLIA\Method Validation and Verification\Test Method Verification\In House Assays\Vitamin C - 4031\Ruggedness

A. Purity of water used during sample preparation

(1) Principle: To determine if the purity of water has an impact on the final vitamin C concentration.

(2) Proposal: To use DI water or high purity water (>15 megaohms) to prepare solutions used during sample preparation.

(3) Findings: Comparison of average (SD) vitamin C concentrations for 51 specimens.

High Purity Water	In-house DI water
1.05 (0.48)	1.12 (0.58)

(4) Summary: Based on a paired t-test ($p > 0.05$), the difference between the vitamin C results using the two waters is not significant. However, it is known that metal and other contaminants can oxidize vitamin C, so high purity water is preferred.

B. Incubation Time

(1) Principle: The method states 30 minutes incubation time at 4°C. Occasionally it might be necessary to either pull the samples out early or leave the samples incubating, i.e. fire alarm/drill, etc. This experiment will determine how critical the incubation time is for the reaction to complete.

(2) Proposal: Incubate QC pools for 15, 30, or 45 minutes at 4 °C.

(3) Findings: Comparison of vitamin C concentrations and percent difference after 15 or 45 minutes incubation compared to the standard 30 minute incubation.

		30 minutes	15 minutes		45 minutes	
Replicate	QC Pool ID	Vitamin C (mg/dL)	Vitamin C (mg/dL)	% diff	Vitamin C (mg/dL)	% diff
1	LS16480_4031	0.280	0.283	1%	0.286	2%
2	LS16480_4031	0.284	0.276	-3%	0.291	2%
1	MS16481_4031	0.91	0.892	-2%	0.925	2%
2	MS16481_4031	0.91	0.929	2%	0.912	0%
1	HS16482_4031	2.06	2.063	0%	2.07	0%
2	HS16482_4031	2.13	2.086	-2%	2.09	-2%

(4) Summary: The samples incubated for 15 minutes and 45 minutes were on average (SD) -1% (2%) and 1% (2%) compared to the standard 30 minute incubation. Incubation time of 30 minutes is not critical to complete the reaction.

C. Delayed Injection

(1) Principle: Occasionally a run gets interrupted while being analyzed on the instrument. This experiment is to determine vitamin C stability in processed samples in the autosampler at 4 °C for up to 48 hours.

(2) Proposal: Reinject prepared samples stored at 4 °C at 24 hrs and 48 hrs storage to determine if there are effects on vitamin C concentration.

(3) Findings: Comparison of vitamin C concentrations and percent difference at initial, 24 hr and 48 hr storage time.

Table 1: Storage at 4 °C

Replicate	Sample Name	Initial injection (mg/dL)	Injection after 24 hrs (mg/dL)	24 hr % diff	Injection after 48 hrs (mg/dL)	48 hr % diff
1	LS16480_4031	0.350	0.346	-1%	0.283	-24%
2	LS16480_4031	0.335	0.297	-13%	0.300	-12%
1	MS16481_4031	1.054	0.856	-23%	0.752	-40%
2	MS16481_4031	0.968	0.714	-36%	0.818	-18%
1	HS16482_4031	2.304	1.72	-34%	1.70	-36%
2	HS16482_4031	2.166	1.50	-44%	1.86	-17%

(4) Summary: The samples stored in the autosampler at 4 °C and reinjected 24 and 48 hours later were on average (SD) -25% (16%) and -24% (11%) compared to the initial injection. If the prepared samples cannot be run the same day, then they should be stored at -80 °C. See **Appendix A** Method Performance for data.

D. Mobile Phase pH

(1) Principle: The method specifies the mobile pH should be 3.00 ± 0.03.

(2) Proposal: Test the effect on vitamin C concentrations in 3 QC pools using mobile phase at pH 2.9 and pH 3.1.

(3) Findings: Comparison of vitamin C concentrations and percent difference at mobile phase at optimal pH 3.0 and testing pH 2.9 and pH 3.1.

		pH 3.0	pH 2.9			pH 3.1		
Replicate	Sample Name	VIC (mg/dL)	VIC (mg/dL)	% diff	avg % diff	VIC (mg/dL)	% diff	avg % diff
1	LS16480_4031	0.254	0.360	29%		0.295	14%	
2	LS16480_4031	0.231	0.345	33%	31%	0.318	27%	21%
1	MS16481_4031	1.02	0.944	-8%		0.978	-4%	
2	MS16481_4031	0.828	0.589	-41%	-24%	0.996	17%	6%
1	HS16482_4031	2.10	1.59	-32%		2.15	2%	
2	HS16482_4031	2.07	1.35	-53%	-42%	2.17	5%	4%

(4) Summary: The samples run with mobile phase pH 2.9 were on average (SD) -12% (36%) and at pH 3.1 10% (11%) compared to the optimal pH 3.0. In addition to concentration differences the retention time for the internal standard (1-methyl uric acid) decreases by ~6% with mobile phase pH

2.9 and remains constant with pH 3.1 compared to pH 3.0. The vitamin C retention time remains constant at the 3 mobile phase pH levels.

E. Centrifugation Temperature

(1) Principle: To determine if centrifugation of the unknowns and QC pools should be done at 4 °C or room temperature

(2) Proposal: Centrifuge 3 QC pools at 4 °C or room temperature.

(3) Findings: Comparison of vitamin C concentrations and percent difference after centrifugation at 4 °C or room temperature.

Sample Name	Refrigerated 4°C Mean (SD) n = 6 (mg/dL)	Room Temperature Mean (SD) n = 6 (mg/dL)
Low QC	0.213 (0.02)	0.211 (0.02)
Medium QC	1.08 (0.04)	1.06 (0.06)
High QC	2.24 (0.04)	2.25 (0.04)

(4) Summary: The vitamin C concentrations from the samples centrifuged at 4 °C were on average (SD) 1% (1%) higher than those centrifuged at room temperature. This concentration difference is negligible; therefore, the samples will be centrifuged at room temperature.

Appendix C: Method Comparison: #4008 vs #4031

A method comparison consisting of 149 patient samples was completed. All comparisons are new (y) vs old (x). The correlation between the new and old method is very good (r = 0.98). Bias based on relative Bland-Altman and weighted Deming is significant but small (<10%). The samples analyzed with the new assay are >10 years old and were stored in 12x75 glass culture tubes closed with a plastic snap cap, which may be suboptimal for long-term storage. 10% of the samples analyzed (15/149) were >20% different from original analysis. The 15 samples come from 8 of 29 original analysis runs and 10 of those samples come from 3 of 8 original runs; else there is just one sample per run with >20% difference. As vitamin C is inherently unstable, we expected some overall degradation (~5-10%), but instead found >20% degradation in 10% of the samples and these were somewhat confined to 3 old runs from 2005 & 2007. It is uncertain why these particular samples seem to be more degraded than the majority of samples.

	Pearson correlation			**Bland Altman relative difference plot (%)**			**Weighted Deming regression**					
Analyte	r	95% LCL	95% UCL	Bias	95% LCL	95% UCL	Slope	95% LCL	95% UCL	Intercept	95% LCL	95% UCL
VIC	0.98	0.97	0.98	-9.85	-11.7	-8.0	0.948	0.917	0.979	-0.027	-0.048	-0.0064

After removing the 15 outliers with >20% difference, the relative Bland-Altman bias was slightly reduced but still significant (-7.3%). Based on the excellent performance with the NIST SRM material, the lower results obtained in this method comparison are likely due to specimen instability during long-term storage.

	Pearson correlation			**Bland Altman relative difference plot (%)**			**Weighted Deming regression**					
Analyte	r	95% LCL	95% UCL	Bias	95% LCL	95% UCL	Slope	95% LCL	95% UCL	Intercept	95% LCL	95% UCL
VIC	0.98	0.98	0.99	-7.27	-8.59	-5.94	0.952	0.932	0.971	-0.0166	-0.0286	-0.0047