



Laboratory Procedure Manual

Analyte: **Vitamin C**

Matrix: **Serum**

Method: **HPLC with an Internal Standard and Electrochemical Detection**

CLIA Account No: 11D0668290

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as performed by: Nutrition Laboratory
Inorganic Toxicology and Nutrition Branch (ITN)
Division of Laboratory Sciences (DLS)
National Center for Environmental Health (NCEH)

contact: Dr. Eric J. Sampson, Director
Division of Laboratory Sciences
(404) 488-4151

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 NHANES 2003-2004 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label (and SI units)
I03_c	LBXVIC	Vitamin C (mg/dL)
	LBDVIC	Vitamin C (mmol/L)

1. SUMMARY OF TEST PRINCIPLE AND CRITICAL RELEVANCE

Serum vitamin C (ascorbic acid) is currently measured by the Centers for Disease Control and Prevention (CDC), Inorganic Toxicology and Nutrition Branch (ITN), Nutrition Laboratory for several studies including the National Eye Institute Age-Related Eye Disease Study (AREDS II), Massachusetts Eye and Ear Infirmary for Age-Related Macular Degeneration Study (AMD) and other domestic intervention trails.

Vitamin C (ascorbic acid) in serum is measured using isocratic high performance liquid chromatography (HPLC) with electrochemical detection at 650 mV¹. One part serum is mixed with four parts 6% MPA to acidify the serum and stabilize ascorbate. The specimen is frozen at -70± X until analysis. After the specimen is thawed at room temperature and centrifuged at 2,500 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). It is re-acidified with 40% MPA to stabilize the ascorbate. The sample is filtered to remove insoluble material. A 10 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. It is adjusted to pH 3.00 ± 0.03 with 10 N sodium hydroxide. Quantitation is by peak area and is based on a standard curve generated using three different concentrations of an external standard (0.025, 0.150, and 0.500 mg/dL).

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.

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 for this analysis include those listed in Section 6. Material safety data sheets (MSDSs) for these chemicals are readily accessible as hard copies in the lab. If needed, MSDS for other chemicals can be viewed at <http://www.ilpi.com/msds/index.html> or at <http://intranet.cdc.gov/ohs>.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

Chromatographic processing and calculation of concentrations for a given run is accomplished through Agilent ChemStation software installed on personal computers attached to the instruments. After a run is complete the Excel export files are copied to the instrument hard drive D:\HPCHEM\1\DATA and to the following network location I:\INSTRUMENT\AGILENT.

The analyst loads the *.xls files from I:\INSTRUMENT\AGILENT into an Access database where the data are checked, corrected if necessary, comments may be added and reports are generated. Replicate data in the Access database are resolved by selecting as ready to report the first replicate value that complies with the following specification:

- The ratio of the lower value to the higher value is greater than or equal to 0.8.

If the replicate values do not agree:

- The sample is reanalyzed, all results are reevaluated and a final value is selected to report.

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After each run is completed (i.e., when all clinical evaluations and analyses from each study set are completed and all replicates are resolved), the ready to report values are generally compiled in a report that is filed by the supervisor for review by the Branch Chief and Division Statistician. Data are transmitted electronically when study is complete or at intervals for large studies. Abnormal values are confirmed, and codes for missing data are entered by the analyst and are transmitted as part of the data file.

Files stored on the network are automatically backed-up nightly by the DLS IT support staff. Backup of the daily data containing all raw data files and result files for each run are the responsibility of Team Leader. Typically these files are backed up once a month or as needed onto a floppy disk or a CD-ROM using a CD writer.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

Specimen handling conditions are outlined in the latest version of the POLICIES AND PROCEDURES MANUAL OF DLS (copies are available in the Nutritional Laboratory and the electronic copy of this file is located at Q:\ITN\Nutrition Laboratory\CLIA). The protocol discusses collection and transport of specimens and the special equipment required. In general, serum should be transported and stored at or below -20°C. Samples thawed and refrozen less than three times are generally not compromised. Vitamin C has its own special processing requirements.

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:

- One part serum with four parts 6% (60.00 g/L) metaphosphoric acid (MPA) in a polypropylene storage vial (e.g., 100 µL of serum is mixed with 400 µL of 6 g/dL MPA).
- The vial contents are then vortexed and immediately frozen, preferably at -7°C.

At least 500 µL of serum prepared as indicated above (with 2000 µL 6 g/dL MPA added) is preferable, but a sample prepared from 100 µL of serum may be analyzed. The appropriate amount of diluted serum is dispensed into a Nalge cryovial or other plastic screw-capped vial labeled with the participant's identification. Specimens collected in the field are stabilized with MPA according to directions given above, frozen, and then shipped on dry ice by overnight mail. Frozen samples are stored at -70°C. 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 at room temperature is no more than 4 hours. Specimens generally arrive frozen. Refrigerated samples may be used provided that they are kept cold and brought promptly (within 2 hours) from the site of collection. Specimens that have been improperly stabilized will give inaccurate test results. Biologic specimen handling conditions are outlined in the DLS protocol for whole blood collection and handling. The protocol describes collection and transport procedures and the special equipment required.

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 3-L beaker, combine 975 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 14.1 g monochloroacetic acid, 0.76 g disodium EDTA, and enough 10 N NaOH (1 mL or less) or phosphoric acid to bring the solution to pH 3.00 ± 0.03 . Add 15 mL methanol. The solution is stable indefinitely at room temperature.

(2) 40 g/dL Metaphosphoric Acid

Dissolve 40.0 g MPA in a 100-mL volumetric flask and dilute to volume with deionized water. The solution is stable for 3-4 weeks at 4°C.

(3) 5 g/dL Metaphosphoric Acid

Dissolve 25.0 g MPA in deionized water in a 500-mL volumetric flask and dilute to volume. The solution is stable for 3-4 weeks at 4°C.

(4) 6 g/dL Metaphosphoric Acid + 0.25 g/dL Dithiothreitol

Dissolve 30.0 g MPA and 1.25 g DTT in deionized water in a 500-mL volumetric flask and dilute to volume. The solution pH is 1.82. The solution is stable for 3-4 weeks at 4°C.

(5) 3.8 g/dL Trisodium Phosphate + 0.25 g/dL Dithiothreitol

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. The solution is stable for 3-4 weeks at 4°C.

(6) 17.53 g/dL Sodium Chloride

Dissolve 17.53 g NaCl in deionized water in a 100-mL volumetric flask and dilute to volume (3M). The solution is used only for storing the reference electrode and is stable for about 3 months at 4°C.

(7) 15 mg/dL 1-Methyl Uric Acid Internal Standard Stock

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. The solution is stable for 3 months at 4°C.

(8) 1.5 mg/dL 1-Methyl Uric Acid Internal Standard Working Solution

Transfer 10 mL of the internal standard stock solution to a 100 mL volumetric flask. Add 0.250 g DTT. Fill to the mark with deionized water. The solution is stable for 3 months at 4°C.

B. Standards Preparation

(1) Stock Standards

Stock #1: Prepare the stock ascorbic acid standard #1 (0.10 g/dL) in duplicate by weighing out 100.00 mg of ascorbic acid in a 100-mL volumetric flask and adding 100 mL of 5.0 g/dL MPA, using a Digiflex; mix well by inversion.

Stock #2: Prepare the stock ascorbic acid standard #2 (2.0 mg/dL) in duplicate by pipeting 2 mL of Stock #1 into a 100-mL volumetric flask and diluting with 100 mL of 5.0 g/dL MPA, using a Digiflex; mix well by inversion

Measurement of stock #2 concentrations: The concentrations of stock #2 solutions are measured using a spectrophotometer (CARY 300) at 245 nm using 5.0 g/dL MPA solution as blank. Select the stock #2 solution that gives the concentration closest to 2.0 mg/dL to make Cal3. Use the molar extinction coefficient (E_c) 567.88 to calculate the concentration.

Stock Standards #1 and #2 are prepared every 90 days.

(2) Calibrators

Cal3 is prepared by measuring 25 mL of the Stock Standard #2 / 75 mL 6.0 g/dL MPA + 0.25 g/dL DTT solution into a 100 mL volumetric flask using Digiflex. Cal1 and Cal2 are made by measuring 0.5 mL Cal3 / 9.5 mL 6.0 g/dL MPA + 0.25 g/dL DTT solution and 3 mL Cal3 / 7 mL 6.0 g/dL MPA + 0.25 g/dL DTT solution, respectively, into 16 X 125 mm screw-top culture tubes using Digiflex. Cap the tubes and mix by inversion. The concentrations of all three calibrator are calculated based on stock #2 Cary reading and should be within 2% of the targeted concentrations (0.025 mg/dL for Cal1, 0.150 mg/dL for Cal2, and 0.500 mg/dL for Cal3). Prepare these calibrators every 90 days and store at -70°C in labeled 0.5 mL cryovials (each containing a 0.4 mL aliquot). The frozen aliquots are stable at -70°C for 90 days. All three calibrators are run on HPLC as samples to confirm their concentrations.

C. Preparation of Quality Control Materials

(1) Bench Quality Control

All serum pools are filtered through sterile gauze before being dispensed. Serum (500 μ L) and 6.0 g/dL MPA (2 mL) are aliquoted into sterile 2-mL Nalge cryovial, sealed, and vortexed. The QC pools are stored at -70°C and are stable for more than 10 years.

Either one of two strategies is used to prepare bench quality control pools:

Strategy I

- Spike serum that has not been MPA-stabilized to achieve low, medium or high levels. The blind quality control pools are prepared by pooling serum from several individuals. Non-MPA-stabilized serum is spiked with appropriate volumes of 1 g/100-mL ascorbic acid solution in deionized water to achieve 0.2 – 2.0 mg/dL final concentration.

Strategy II

- The low quality control pool is prepared by pooling serum from smokers, who have very low vitamin C levels.

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- The medium quality control pool is prepared by pooling serum from people whose vitamin C level is close to the mean levels observed in normal subjects.
- The high QC pool is prepared by pooling serum from volunteers who take doses of >1 g of vitamin C per day for at least 5 days prior to collection.

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

(2) Blind Quality Control

All serum pools are filtered through sterile gauze before being dispensed. Serum (500 µL) and 6.0 g/dL MPA (2-mL) are aliquoted into sterile 2-mL Nalge cryovial, sealed, and vortexed. The blind quality control pools are stored at -70°C and are stable for more than 10 years.

Either one of two strategies is used to prepare blind quality control pools:

Strategy I

- Spike serum that has not been MPA-stabilized to achieve low, medium or high levels. The blind quality control pools are prepared by pooling serum from several individuals. Non-MPA-stabilized serum is spiked with appropriate volumes of 1 g/100-mL ascorbic acid solution in deionized water to achieve 0.2 – 2.0 mg/dL final concentration.

Strategy II

- The low quality control pool is prepared by pooling serum from smokers, who have very low vitamin C levels.
- The high QC pool is prepared by pooling serum from volunteers who take doses of >1 g of vitamin C per day for at least 5 days prior to collection.

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

Full documentation for each lot of bench or blind QC is available on the <Q:\ITN\VITC\DOCUMENTS\QUALITY CONTROL>.

D. Other Materials

- (1) Clear autosampler vials and inserts (KimbleBrand 1.8 mL vials, 12 x 32 mm, clear, w/separately packaged caps from Lab Depot, and 100 :L glass inserts with spring feet from Lab Depot.)
- (2) YMC ODS-A S-3 120 Angstrom, 2.0 mm ID X 15 cm length or equivalent
- (3) 0.45 :m syringe tip PVDF hydrophilic filter (4 mm diameter) (obtained from various sources)
- (4) Plastic tuberculin syringes (obtained from various sources)
- (5) Methanol, HPLC grade, (JT Baker, St Louis, MO) or equivalent
- (6) Monochloroacetic acid, AR crystal, (Mallinckrodt, Paris, KY) or equivalent
- (7) Disodium Ethylenediamine Tetraacetate (EDTA), AR grade (Fisher Scientific, Fairlawn, NJ) or equivalent
- (8) Phosphoric Acid Meta (MPA), AR grade (Mallinckrodt, Paris, KY) or equivalent
- (9) 10 N Sodium Hydroxide (NaOH) (Fisher Scientific, Fairlawn, NJ) or equivalent
- (10) Sodium Phosphate, tribasic (TSP), ACS grade (Mallinckrodt, Paris, KY) or equivalent

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- (11) L-Ascorbic acid, ACS grade (Fisher Scientific, Fairlawn, NJ) or equivalent
- (12) Dithiothreitol (DTT) (Sigma Chemical Co., St. Louis, MO) or equivalent
- (13) Sodium Chloride (NaCl), ACS grade (Fisher Scientific, Inc.) or equivalent
- (14) Nalgene cryovials, (Nalge Company, Rochester, NY) or equivalent
- (15) Vials for calibrators (0.5 mL) – PP (Sarstedt, Inc, Newton, NC)
- (16) PK-4 Polishing Kit (Bioanalytical Systems Inc., West Lafayette, IN)
- (17) pH 1.68 Buffer (VWR, Suwanee, GA) or equivalent
- (18) pH 4.00 Buffer (VWR, Suwanee, GA) or equivalent
- (19) pH 7.00 Buffer (VWR, Suwanee, GA) or equivalent
- (20) pH 10.00 Buffer (VWR, Suwanee, GA) or equivalent
- (21) 4M Potassium chloride (Beckman Coulter, Fullerton, CA) or equivalent
- (22) 200 proof dehydrated ethyl alcohol (Pharmco Products, Brookfield, CT) or equivalent
- (23) 12 x 75 mm disposable glass culture tubes (Corning Glassworks) or equivalent
- (24) 16 x 125 mm disposable glass screw top culture tubes (Corning Glassworks) or equivalent
- (25) 5¾ inch pasteur pipettes (Kimble, Toledo, OH) or equivalent
- (26) Various glass beakers, volumetric flasks, graduated cylinders, and bottles or equivalent

E. Instrumentation

- (1) Agilent High Performance Liquid Chromatography system (Agilent Corporation Wilmington, DE)
 - (a) Agilent 1100 Thermostated autosampler
 - (b) Agilent 1100 Thermostated column compartment
 - (c) Agilent 1100 Quaternary pump
 - (d) Agilent 1100 Degasser
 - (e) HP A/D Converter
 - (f) Agilent 1100 ChemStation data analysis software
- (2) BAS LC-4C Electrochemical Detector with a thin-layer amperometric detector cell (TL5A) (Bioanalytical Systems, Inc., West Lafayette, IN) or equivalent
- (3) Vortex mixer (American Scientific Products, McGaw Park, IL) or equivalent
- (4) Beckman Allegra 6 centrifuge (Beckman Coulter, Fullerton, CA) or equivalent
- (5) Magnetic stirrer (obtained from various vendors) or equivalent
- (6) Digiflex automatic pipette (Titertek, Huntsville, AL) or equivalent
- (7) Beckman pH Meter 390 (Beckman Coulter, Fullerton, CA) or equivalent
- (8) S/P Multi-tube vortexer (Baxter-Scientific Products, Stone Mountain, GA) or equivalent

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- (9) Eppendorf pipettes (Brinkmann Instruments, Inc., Westbury, NY) or equivalent
- (10) Mettler PM400 balance (Mettler-Toledo, Greifensee, Sztitwerland) or equivalent
- (11) Mettler analytical balance, model AG104 (Mettler-Toledo, Greifensee, Switzerland) or equivalent
- (12) CARY 300 Bio UV-Visible Spectrophotometer (Varian Analytical Instruments, Cary, NC) or equivalent

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

Each time an analysis is run, the system is calibrated through the use of three different concentrations of working standards--0.025 mg/dL, 0.150 mg/dL, and 0.500 mg/dL--prepared as described in Section 6. The ChemStation software generates a three-point linear standard calibration curve forced through zero. The system is recalibrated approximately every 80 specimens, depending on the run length. The calibrators are reanalyzed as unknowns at the end of each run and must be within $\pm 15\%$ of their established concentrations, 0.025 mg/dL, 0.150 mg/dL, and 0.500 mg/dL. 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.

Standard curves are replaced in each new run.

For troubleshooting purposes and accuracy verification, NIST SRM 970 is available.

For comparison of instruments, once every six months a set of at least five samples spanning the reportable range of the analyte of interest are run on both instruments. The Pearson correlation coefficient of results should be greater than 0.95 or appropriate remedial action should be taken. In special situations, the laboratory Director may give written approval that the methods are sufficiently similar for the intended use of the data.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Preliminaries for the Agilent 1100 LC

(1) Instrument Preparation

- (a) Turn on the A/D converter.
- (b) Turn on all the Agilent 1100 modules (Degasser, Pump, Autosampler, Column Oven)
- (c) Turn on the BAS electrochemical detector to a positive current of 650 mV with 100 nA full scale.
- (d) Turn on CPU
- (e) Launch ChemStation Software
- (f) Set the refrigeration unit to maintain a temperature of 4°C.
- (g) Set the column heater temperature to 30°C.
- (h) Place mobile phase in the reservoir for the pump (if adding to the existing volume of mobile phase, be sure to mix the old and new mobile phase to avoid major shifts in retention times. Flush system for 20 min.), turn the pump on, and set the flow rate to 0.3 mL/min (ramp flow rate up from 0.1 mL/min to 0.3 mL/min if methanol/water was run through the system last).
- (i) If the HPLC has been up-and-running (not idle for more than 3-4 days), open the valve to take the column out of the flow path and run at 5 mL/min for 3-4 minutes to flush the Degasser with mobile phase. Close the valve and allow the column to equilibrate with the mobile phase under normal flow conditions (0.3 mL/min) for 10 min before starting the analysis.
- (j) Load a method that contains the method parameters shown in FIGURE 1 a-k (APPENDIX).

The calibration table FIGURE 1 i should have all components calibrated on the basis of area, with no weighting, and a linear curve forced through the origin. The retention

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times will vary with the age and cleanliness of the column, flow rate, and other system variables. Retention times need to be determined and may need to be adjusted on a daily basis, and the calibration table should be adjusted when necessary. For each run, the sensitivity of the electrode is checked by determining that the peak area of cal1 (0.025 mg/dL) is at least 0.3 nAmp. The four components present in the chromatogram in order of elution are ascorbic acid, uric acid, dithiothreitol and 1-methyl uric acid. The retention times will generally be 2.0 ± 0.2 min for ascorbic acid and 10.0 ± 0.6 min for 1-methyl uric acid. The exact retention times will depend on the length of tubing, the age of the column, the cleanliness of the column, and the flow rate.

The integration parameters will vary with the column type, column age, electrode age and other factors. The parameters shown in FIGURE 2 may be used as a starting point (APPENDIX). FIGURE 3 shows a typical chromatogram.

- (k) Edit the Sequence Table (FIGURE 4 a-b) making sure all sample identification numbers, method names, and vial numbers, etc are correct. Enter the three calibrators (0.025, 0.150, 0.500 mg/dL) and a reagent blank. The reagent blank contains everything except ascorbic acid. Insert a single set of calibrators and low, medium, and high controls before and after approximately every 80 samples. Reanalyze the calibrators and the reagent blank at the end of the run as unknowns. Enter a multiplier of 5 for all patient samples and bench control specimens on the initial run. Use a dilution factor of 5 and a multiplier of 5 for repeat analysis of specimens that have had a 5-fold dilution with 6.0 g/dL MPA + 0.25 g/dL DTT because the concentration was at least 3.00 mg/dL on the initial analysis. Save the Sequence Table and Method for every run. For NIST SRM 970, enter a multiplier of 1.
- (l) Press the START Button to execute method

B. Sample Preparation

- (1) Thaw prepared samples, calibrators, and QC vials at room temperature for less than 1 hour.
- (2) Hand-shake samples, calibrators and QC (see FIGURE 5). Centrifuge vials for 20 minutes at 3,000 rpm (1,000 x g).
- (3) Decant supernatants into barcode labeled 12 x 75 mm culture tubes.
- (4) Add 50 μ L internal standard (IS) into a separate set of 12 x 75 mm culture tubes.
- (5) Add 100 μ L of supernatant and 300 μ L of TSP/DTT with the Digiflex diluter to the tubes containing IS. Hand-shake the vials and let the solutions stand 30 min at 4°C.
- (6) Add 45 μ L of 40.0 g/dL MPA to each tube with a 10-100 μ L Eppendorf pipettor and hand-shake the vials.
- (7) Draw the sample into a 1-mL tuberculin syringe and filter it through a 0.45- μ m PVDF filter into a sample vial containing an insert.
- (8) Inject 10 μ L for calibrators, QC and patient samples.

C. System Maintenance

- (1) System Shutdown

When instrument will be idle for longer than 4 days, a complete shut-down of the instrument is necessary.

Turn-off the detector (see FIGURE 6 a). Flush the system, including column, with DI for 20 min. Flush the system with a solution of 50% methanol and 50% deionized water for 10 min.

Remove the electrode (see FIGURE 6 b). Polish the working electrode with alumina on the soft polishing pad for at least one minute. Rinse thoroughly with deionized water, followed by methanol, and then allow it to air dry. Place the working electrode in its storage container. See BAS operating manual for explicit directions.

(2) Cleaning the Working Electrode

When peak area of the 0.025 mg/dL cal1 is < 0.3 nAmps, polish the working electrode. Polish the working electrode with alumina on the soft polishing pad for at least one minute. Rinse thoroughly with deionized water, followed by methanol, then allow it to air dry (See operating manual for explicit directions). Reinstall working electrode on the BAS detector.

(3) Changing the Reference Electrode

Rinse the reference electrode with deionized water and store it in a 4-mL vial with a septum and screw cap containing 3 M sodium chloride.

D. Special Method Notes

- (1) Always keep the column at 30°C in the column oven during an analytical run
- (2) Keep the autosampler refrigeration at 4°C
- (3) Allow the BAS detector at least 0.5 hour to stabilize.
- (4) Allow at least 20 min for the column to equilibrate to a new mobile phase at a flow rate of approximately 0.3 mL/min.
- (5) Turn the system completely off only if it will be idle for more than 4 days.
- (6) If the system will be idle more than 1-2 weeks, turn the detector off, wash the column with deionized water for 20 min, and followed by a solution of 50% methanol and 50% deionized water. Before using the system again, re-equilibrate the column with mobile phase.
- (7) Turn the BAS detector to standby whenever the flow is stopped. It is preferable to have at least two reference electrodes, one in use and one equilibrating in 3 mol/L sodium chloride. It is also preferable to have at least two working electrodes, polished and ready to install when needed.
- (8) Plug the BAS detector, A/D converter, and printer into line voltage conditioners to minimize the effects of electrical current fluctuations.
- (9) Monitor instrument for leaks and change filters when necessary because the mobile phase is corrosive and damages the valves. Monitor the inlet and outlet check valves of the pump; log a service call to instrument manufacturer if visible leaks or variable pump pressure indicates a problem.

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Calculations

The analyst reviews all chromatograms to confirm that peaks are correctly identified and integrated. Any manually integrated peaks are identified in the Access database using code 764. All comment codes entered on hard copies of set-up sheet and sequence table during the sample preparation and run period, are transferred to the Access database when data are imported. The analyst is responsible for verifying that the data in the database match the data in the instrument printouts. In ChemStation software, only after all peaks are checked for integration is the report sent to printer as a *.xls file. The ChemStation software performs all calculations. Calibration curves are linear, forced through zero, and calculated on the basis of a single analysis of three different standard concentrations according to the following formula:

$$CONCENTRATION = RF \times (ASCORBIC\ ACID\ PEAK\ AREA / IS\ PEAK\ AREA) \times DILUTION\ FACTOR$$

RF = Response Factor (Amount/Area)

CDC Modifications

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

9. REPORTABLE RANGE OF RESULTS

The reportable range of results for vitamin C is 0.012 mg/dL (limit of detection) to 5.00 mg/dL.

Samples with results <0.02 mg/dL or >2.16 mg/dL are reanalyzed before results are released.

Linearity for this method has been established for the range from 0.012 mg/dL to 3.0 mg/dL.

Samples with vitamin C levels > 3.0 mg/dL are diluted 5-fold with 6.0 g/dL MPA + 0.25 g/dL DTT and reanalyzed and are injected using smaller volumes (8, 5, 2 µL). Injecting un-diluted samples at less than 10 µL tends to give values closer to the original value.

This method has a total coefficient of variation in the range of <10%.

10. QUALITY CONTROL (QC) PROCEDURES

A. Blind Quality Controls

Blind QC specimens are inserted prior to the arrival of the samples in the Inorganic Toxicology and Nutrition Branch. These specimens are prepared at two levels to emulate the patient samples; the labels used are identical to those used for patient samples. The specimen volume must match the patient sample volume. One blind QC specimen randomly selected for concentration (high or low) is included at a randomly selected location in every 20 specimens analyzed.

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:

(1) For 3 QC pools per run:

(a) 1 QC result per pool (accidental loss of the duplicate)

If all 3 QC run results are within $2S_i$ limits, then accept the run.
If 1 of the 3 QC run results is outside a $2S_i$ limit - reject run if:

Extreme Outlier – Run result is beyond the characterization mean $\pm 4S_i$
1 3S Rule - Run result is outside a $3S_i$ limit
2 2S Rule - 2 or more of the 3 run results are outside the same $2S_i$ limit

10 X-bar Rule – Current and previous 9 run results are on same side of the characterization mean
R 4S Rule – Two consecutive standardized* run results differ by more than $4S_i$

Note: Since runs have a single result per pool for 3 pools, comparison of results for the R 4S rule will be with the previous result within the current run or with the last result of the previous run.

(2) For 2 or more QC result per pool

(a) If all 3 QC run means are within $2S_m$ limits and individual results are within $2S_i$ limits, then accept the run.

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

Extreme Outlier – Run mean is beyond the characterization mean $\pm 4S_m$
1 3S Rule - Run mean is outside a $3S_m$ limit
2 2S Rule - 2 or more of the 3 run means are outside the same $2S_m$ limit
10 X-bar Rule – Current and previous 9 run means are on same side of the characterization mean

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

R 4S Rule - 2 or more of the within-run ranges in the same run exceed $4S_w$ (i.e., 95% range limit)

Note: Since runs have multiple results per pool for 3 pools, the R 4S rule is applied within runs only.

Abbreviations:

S_i = Standard deviation of individual results (the limits are not shown on the chart unless run results are actually single measurements).

S_m = Standard deviation of the run means (the limits are shown on the chart).

S_w = Within-run standard deviation (the limits are not shown on the chart).

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

The initial limits are established by analyzing pool material in 20 consecutive runs and then are reevaluated as needed. When necessary, limits are updated to include more runs.

While a study is in progress, electronic copies of the QC results from each run are stored in an Access database. The analyst also keeps a hardcopy of the QC results from each run.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

Check to make sure that the hardware is functioning properly. Make sure the pump is operating at the appropriate pressure with steady delivery. Check the autosampler to make sure the injections are being made as programmed.

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

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Samples that are improperly stabilized or have not been promptly stabilized after collection will give inaccurate results. Little or no supernatant, no precipitate, or sample volumes inconsistent with the other samples are evidence that a sample has not been diluted correctly. When you encounter any of these situations, write "CODE 58" for "MPA apparently not added to serum" or "CODE 26" for an "invalid result", or some other appropriate code (consult the list of comment codes in the Access Database) next to the sample number on the sequence table along with a description of the problem, if needed. Codes generally indicate that the sample or result is unsatisfactory.

13. REFERENCE RANGES (NORMAL VALUES)

The reference range (1-99%) for vitamin C in the U.S. population has been established from the NHANES III data: 0.02 - 2.16 mg/dL.

14. CRITICAL CALL RESULTS ("PANIC VALUES")

Because there is no evidence that excessively high or low serum concentrations of this analyte constitute an immediate threat to the life of a subject, this analyte is not considered critical, and thus there are no procedures for reporting abnormal results on an emergency basis. Specimens with values > 5.00 mg/dL may be due to excessive supplementation, but they may also be the result of incorrect sample preparation. Such results should be followed up with the principal investigator to determine if specimen re-collection is desired or feasible.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens are allowed to reach room temperature during preparation. Once the samples are ready to run, they are placed in the autosampler at 4°C. Return the unused portion of the patient sample to the freezer as soon as possible.

16. ALTERNATE METHODS FOR PERFORMING TEST OF STORING SPECIMENS IF TEST SYSTEM FAILS

Because the analysis of serum for vitamin C is inherently complex and challenging, there are no acceptable alternative methods of analysis in the nutrition laboratory. If the analytical system fails, then we recommend storing the acidified serum at ~ -20°C until the analytical system is restored to functionality.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

A. Quality Control Data

The QC data are reviewed and approved by the supervisor from the Access database, hard copy reports, and from SAS files generated by Team Leader. The QC results from each analysis are stored in SQL Server ITNMultistudy database while the relevant study is in progress and for at least 5 years after its completion.

B. Analytical Results

After a run is complete the Excel export data files are copied to instrument hard drive D:\HPCHEM\1\DATA and to the following network location I:\INSTRUMENT\AGILENT. These files contain all of the results for calibrators, QC pools, and participant samples. The Team Leader gives a SAS report for the study to the supervisor. The team leader maintains monthly SAS QC reports for the assay. The supervisor generates a final report including the patient sample report, SAS bench QC report, blind QC report, comment codes and LOD information. The supervisor keeps a hard copy of the report while the relevant study is in progress and for at least 5 years after its completion.

The analyst loads the *.xls files from I:\INSTRUMENT\AGILENT into an Access database where the data are checked, evaluated, and put into the correct format for generating a report. Replicate data in the Access database are resolved by selecting as ready to report the first replicate value that complies with the following specification: the ratio of the lower value to the higher value is greater than or equal to 0.8. If the replicate values do not agree, the sample is reanalyzed, all results are reevaluated and a final value is selected to report. After each run is completed (i.e., when all clinical evaluations and analyses from each study set are completed and all replicates are resolved) the ready to report values are compiled in a report that is filed by the supervisor for review by the Branch Chief and Division Statistician. Data are transmitted electronically when study is complete. Abnormal values are confirmed, and codes for missing data are entered by the analyst and are transmitted as part of the data file.

Files stored on the network are automatically backed-up nightly by DLS IT support staff. Backup of the daily data containing all raw data files and result files for each run are the responsibility of the Team Leader. Typically these files are backed up once a month or as needed onto a floppy disk or a CD-ROM using a CD writer.

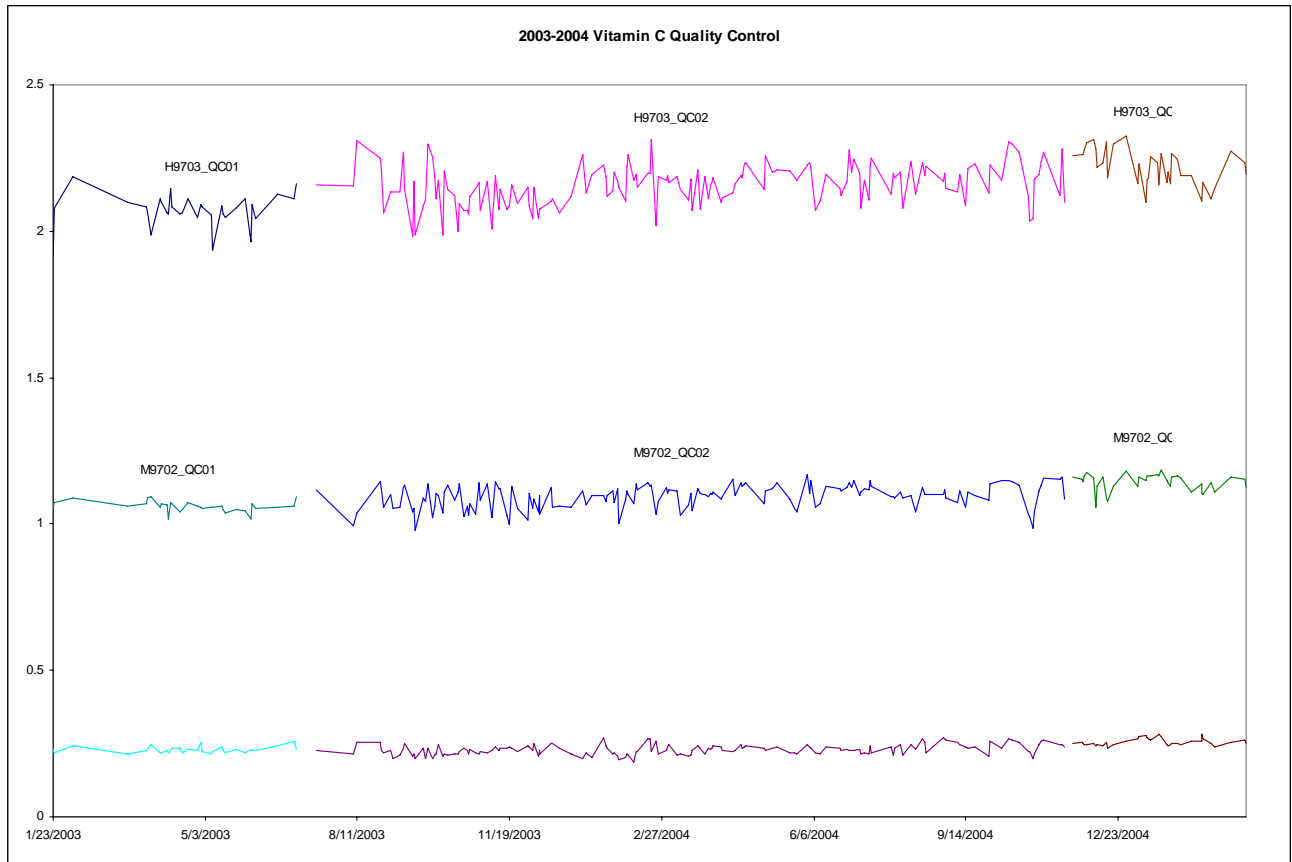
18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

The Microsoft Access database is used to keep records and track specimens for studies related to vitamin C. If vitamin C analyses are used for smaller, non-NHANES studies, records are kept on files in I:\INSTRUMENT\AGILENT on the DLS LAN. Only numerical identifiers should be used (e.g., case ID numbers). All personal identifiers should be available only to the medical supervisor or project coordinator. Residual supernatant from samples may be discarded at the request of the principal investigator. Very little residual material will be available after multiple repeats for NHANES analyses, and these vials may be routinely autoclaved.

19. SUMMARY STATISTICS AND QC GRAPHS

Summary Statistics for Vitamin C by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
L9701_QC01	32	1/23/2003	7/2/2003	0.2289	0.0107	4.7
M9702_QC01	32	1/23/2003	7/2/2003	1.0601	0.0179	1.7
H9703_QC01	32	1/23/2003	7/2/2003	2.0716	0.0576	2.8
L9701_QC02	158	7/15/2003	11/18/2004	0.2295	0.0175	7.6
M9702_QC02	158	7/15/2003	11/18/2004	1.0936	0.0404	3.7
H9703_QC02	158	7/15/2003	11/18/2004	2.1577	0.0699	3.2
L9701_QC03	37	11/23/2004	3/17/2005	0.2562	0.0128	5.0
M9702_QC03	37	11/23/2004	3/17/2005	1.1423	0.0298	2.6
H9703_QC03	37	11/23/2004	3/17/2005	2.2172	0.0626	2.8



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.

ACKNOWLEDGMENTS

We gratefully acknowledge the contribution of Mary Xu who performed the analyses for NHANES using this method.

Figure 1

Method Parameters

a. Setup Pump

Setup Pump : Ralphie

Control

Flow: ml/min

StopTime: min

PostTime: min

Solvents

A: 0.0 %

B: %

C: %

D: %

Pressure Limits

Max: bar

Min: bar

Timetable

Time	%B	%C	%D	Flow	Max. Press.
------	----	----	----	------	-------------

Insert

Append

Cut

Copy

Paste

Display:

OK Cancel Help

Figure 1

Method Parameters

b. Setup Injector

Setup Injector : Maya

Injection

Standard Injection Injection Volume: 10.0 µl

Injection with Needle Wash Wash Via: 91

Use Injector Program Total Lines: 0 Edit ...

Optimization: Overlap Injection Cycle 11.00 min. after Injection

Auxiliary

Draw Speed: 100 µl/min

Eject Speed: 100 µl/min

Draw Position: 5.0 mm

Store Temperature

Time

Stoptime: as Pump 12.50 min

Posttime: Off min

OK Cancel Help

Figure 1

Method Parameters

c. Setup Column Thermostat

Column Thermostat Method : Maya

Temperature

30.0 °C

Not controlled

Time

Stoptime:
as Pump 12.50 min

Posttime:
Off min

Column Switching Valve

Column 1

Temperature (right)

20.0 °C

Not controlled

Same as left

Timetable:

Line	Time	Column	Temp.(left)	Temp.(right)
------	------	--------	-------------	--------------

Table Graphic

Store

Temperature (left)

Temperature (right)

Enable Analysis

With any Temp.

When Temperature is within Setpoint

+/- 0.8 °C

Figure 1

Method Parameters

d. Setup ADC1 ADC: Signals

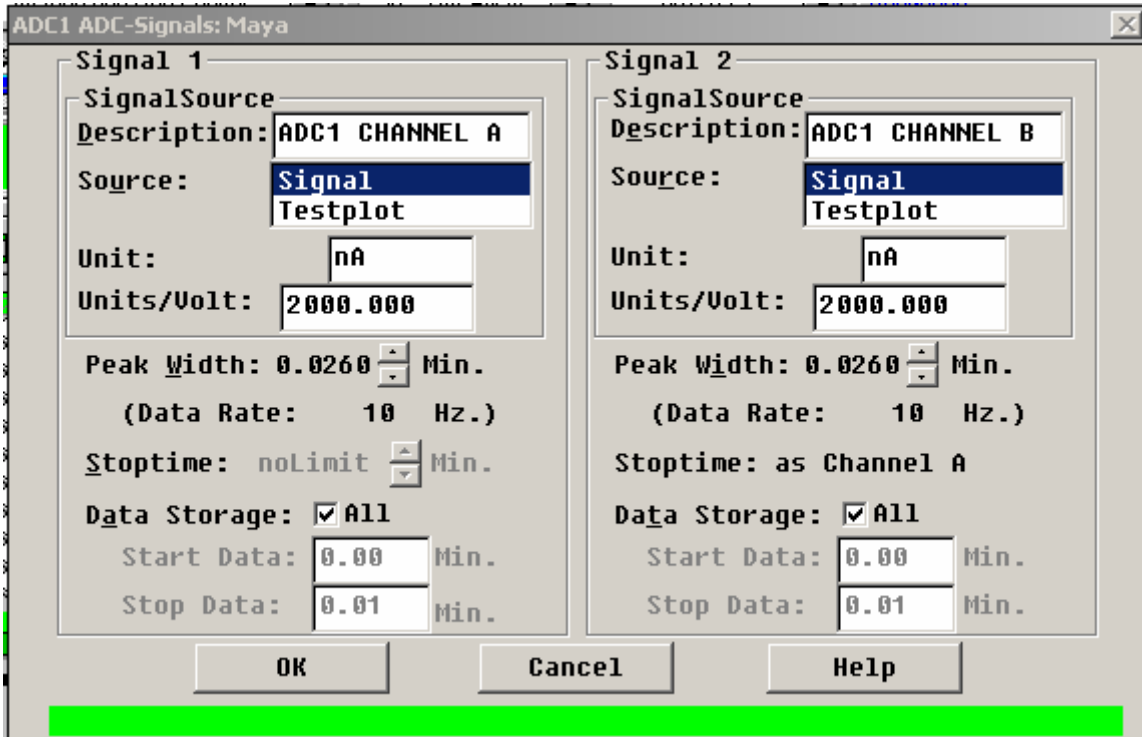


Figure 1

Method Parameters

- e. Setup ADC1 Timed Event Table (External Control)

ADC1 Timed Event Table: Maya

Start Signal Source

35900 Will Start External Devices

External Device Will Start 35900

Timed Event Table

Time	When	Event
Minutes		

Add Delete Replace

OK Cancel Help

Figure 1

Method Parameters

f. Setup Signal Details

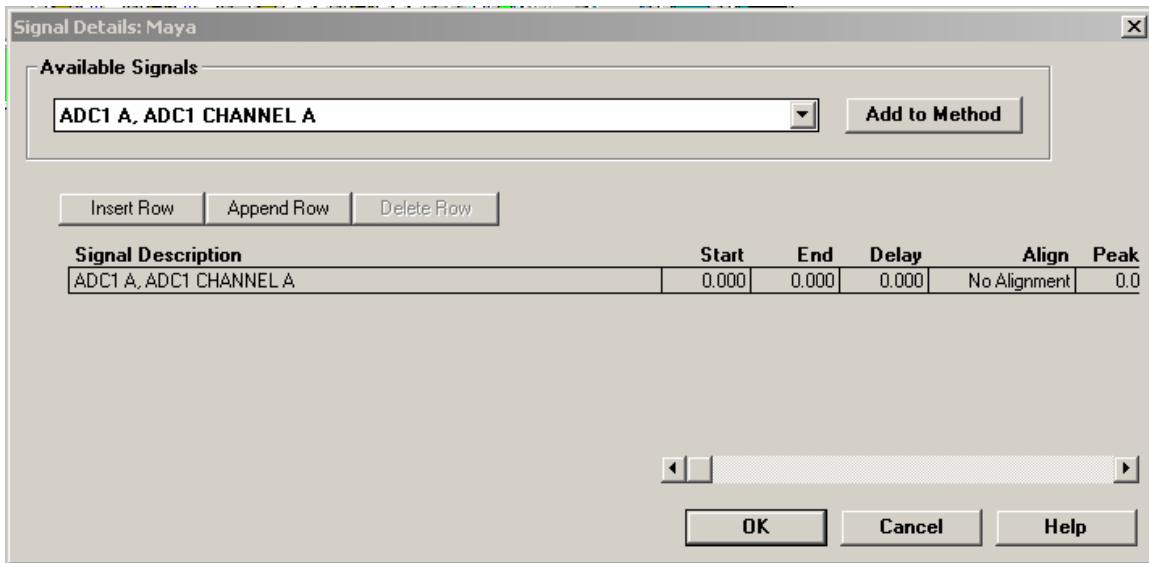


Figure 1

Method Parameters

g. Setup Report Layout

The image shows a software dialog box titled "Specify Report: Maya". It is divided into several sections for configuring report parameters:

- Destination:** Includes checkboxes for "Printer" and "Screen".
- File:** Includes a "File Prefix" field with the text "Report" and a "File Type" section with checkboxes for ".TXT", ".WMF", ".DIF", ".CSV", ".XLS", and ".HTM". The ".XLS" checkbox is checked.
- Quantitative Results:** Includes dropdown menus for "Calculate:" (set to "ISTD"), "Based On:" (set to "Area"), and "Sorted By:" (set to "Signal"). A "Signal Options..." button is also present.
- Style:** Includes a "Report Style:" dropdown set to "Short". It also has checkboxes for "Sample info on each page", "Add Chromatogram Output" (checked), and "Add Summed Peaks Table".
- Report Layout For Uncalibrated Peaks:** Includes radio buttons for "Separately", "With Calibrated Peaks", and "Do Not Report" (selected).
- Chromatogram Output:** Includes radio buttons for "Portrait" (selected), "Landscape", and "Multi-Page (Landscape)". It also has a "Pages" spinner set to "1".
- Size:** A sub-section with "Time:" and "Response:" spinners, both set to values in a "% of Page" box. "Time" is set to 100 and "Response" is set to 34.

At the bottom of the dialog are "OK", "Cancel", and "Help" buttons.

Figure 1

Method Parameters

h. Setup Calibration Settings

Calibration Settings: Maya

Title: **Inorganic Toxicology and Nutrition Ascorbic Acid**

Use Sample Data: **From Data File**

Sample Defaults

Amount: **0.000** I# **1** Compound **1 methyluric** ISTD Amount **1.00000**

Amount Units: **mg/dL**

Multiplier: **1.000**

Dilution: **1.000**

I#	Compound	ISTD Amount
1	1 methyluric	1.00000

Default RT Windows

Reference Peaks	Minutes	+	%
	2.10		20.00
Other Peaks	Minutes	+	%
	0.00		5.00

Default Calibration Curve

Type: **Linear**

Origin: **Include**

Weight: **Linear (Amnt)**

Calculate Uncalibrated Peaks

For Signal: **ADC1 A, ADC1 CHANNEL A**

Using Compound: **None**

With Rsp Factor: **1.000**

Use ISTD: **None**

No

If Peaks Missing

Partial Calibration

Correct All RTs

OK **Cancel** **Help**

Vitamin C in Serum
 NHANES 2003-2004
Appendix (Figures)

Figure 1

Method Parameters

i. Setup Calibration Table

Calibration Table											
Enter		Delete		Insert...		Print		OK		Help	
#	RT	Def	From	To	Signal	Compound	Area	Rsp%	Def	+/-	Pk Usage
1	2.117	<input type="checkbox"/>	2.013	2.621	ADC1 A	ascorbic acid	4.525	100.0	<input checked="" type="checkbox"/>	0.0	Main
							25.114				
							83.375				
2	3.170	<input type="checkbox"/>	2.770	3.550	ADC1 A	uric acid	6.829	100.0	<input checked="" type="checkbox"/>	0.0	Main
							3.179				
							3.108				
3	9.489	<input type="checkbox"/>	9.059	10.814	ADC1 A	dithiothreitol	1144.400	100.0	<input checked="" type="checkbox"/>	0.0	Main
							995.440				
							902.830				
4	10.477	<input type="checkbox"/>	10.077	11.434	ADC1 A	1 methyluric acid	88.135	100.0	<input checked="" type="checkbox"/>	0.0	Main
							84.100				
							82.187				

Figure 1

Method Parameters

j. Setup Instrument Curves

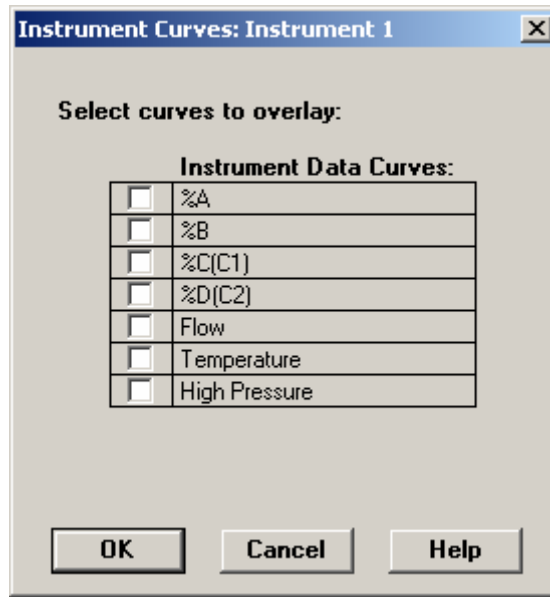
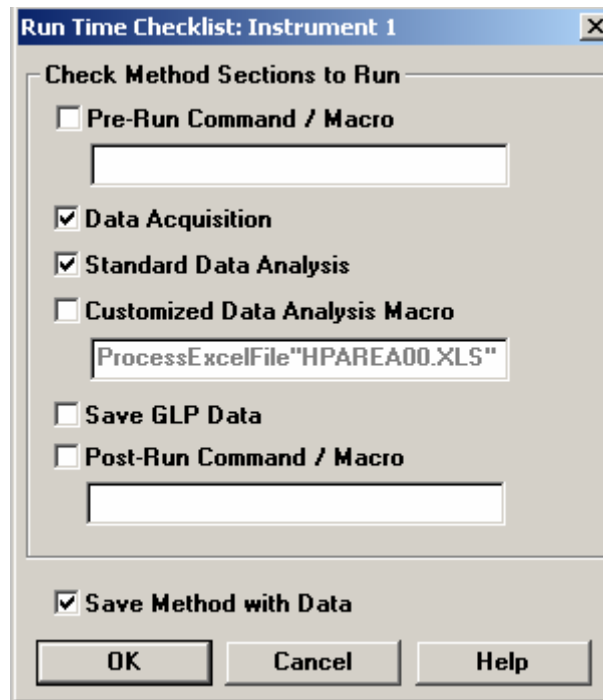


Figure 1

Method Parameters

K. Run Time Checklist



The image shows a dialog box titled "Run Time Checklist: Instrument 1". It contains several checkboxes and text input fields. The "Check Method Sections to Run" section includes: "Pre-Run Command / Macro" (unchecked), "Data Acquisition" (checked), "Standard Data Analysis" (checked), "Customized Data Analysis Macro" (unchecked) with a text field containing "ProcessExcelFile\"HPAREA00.XLS\"", "Save GLP Data" (unchecked), and "Post-Run Command / Macro" (unchecked). Below this is a "Save Method with Data" checkbox (checked). At the bottom are "OK", "Cancel", and "Help" buttons.

Section	Checked	Value / Command
Pre-Run Command / Macro	<input type="checkbox"/>	
Data Acquisition	<input checked="" type="checkbox"/>	
Standard Data Analysis	<input checked="" type="checkbox"/>	
Customized Data Analysis Macro	<input type="checkbox"/>	ProcessExcelFile"HPAREA00.XLS"
Save GLP Data	<input type="checkbox"/>	
Post-Run Command / Macro	<input type="checkbox"/>	
Save Method with Data	<input checked="" type="checkbox"/>	

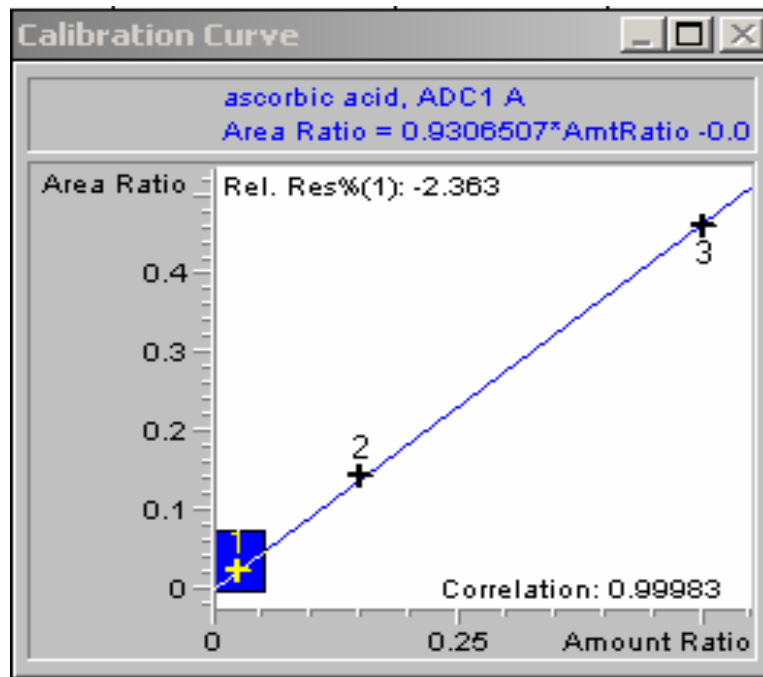
Figure 2

a. Integration Parameters

Time	Integration Events	Value
Initial	Slope Sensitivity	0.5
Initial	Peak Width	0.2
Initial	Area Reject	0.5
Initial	Height Reject	0.5
Initial	Shoulders	OFF
0.100	Integration	OFF
1.800	Integration	ON

Figure 2

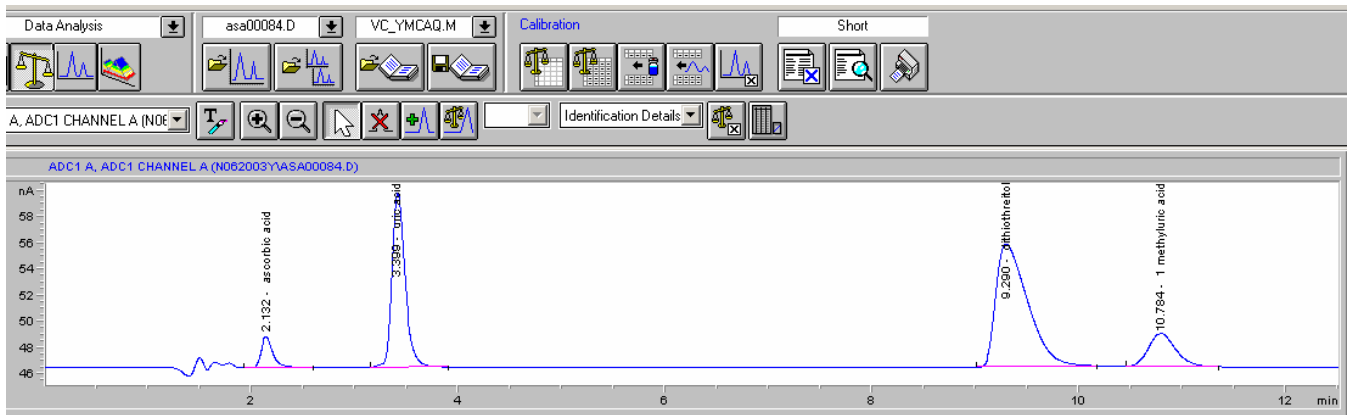
b. Calibration Curve



Vitamin C in Serum
NHANES 2003-2004
Appendix (Figures)

Figure 3

Sample Chromatogram



Vitamin C in Serum
 NHANES 2003-2004
Appendix (Figures)

Figure 4

a. Sample Sequence Table

Sequence Table: Maya

Currently Running
 Line: Method: Location: Inj:

Sample Info for Vial 91:

Line	Location	Sample Name	Method Name	Inj/Location	Sample Type	Cal Level	Update RF	Update RT	Interval	Sa
1	Vial 91	MOBILE PHASE	VC_YMCAQ		1 Sample					
2	Vial 1	blank	VC_YMCAQ		1 Sample					
3	Vial 2	cal1	VC_YMCAQ		1 Calibration	1	Replace	Replace		
4	Vial 3	cal2	VC_YMCAQ		1 Calibration	2	Replace	Replace		
5	Vial 4	cal3	VC_YMCAQ		1 Calibration	3	Replace	Replace		
6	Vial 5	L9701_QC01	VC_YMCAQ		1 Sample					
7	Vial 6	M9702_QC01	VC_YMCAQ		1 Sample					
8	Vial 7	H9703_QC01	VC_YMCAQ		1 Sample					
9	Vial 8	638388079	VC_YMCAQ		1 Sample					
10	Vial 9	835491079	VC_YMCAQ		1 Sample					
11	Vial 10	961633079	VC_YMCAQ		1 Sample					
12	Vial 11	429661079	VC_YMCAQ		1 Sample					
13	Vial 12	817838079	VC_YMCAQ		1 Sample					
14	Vial 13	309063079	VC_YMCAQ		1 Sample					
15	Vial 14	251766079	VC_YMCAQ		1 Sample					
16	Vial 15	121080079	VC_YMCAQ		1 Sample					
17	Vial 16	243071079	VC_YMCAQ		1 Sample					
18	Vial 17	546766079	VC_YMCAQ		1 Sample					
19	Vial 18	163066079	VC_YMCAQ		1 Sample					
20	Vial 19	232289079	VC_YMCAQ		1 Sample					
21	Vial 20	226859079	VC_YMCAQ		1 Sample					

Insert Cut Copy Paste Append Line

Insert Vial Range... Run Sequence Read Bar Code

OK Cancel Help

Sample location (leave empty for a non-injection blank)

Vitamin C in Serum
NHANES 2003-2004
Appendix (Figures)

Figure 4

b. Selecting the internal standard peak

