# Laboratory Procedure Manual

*Analyte:* **Fat Soluble Micronutrients (Vitamins A, E and Carotenoids) – UV-visible Detection**

*Matrix:* **Serum**

*Method:* **High Performance Liquid Chromatography (Isocratic HPLC)**

*Method No.:*

*Revised:*

*as performed by:* *Inorganic Toxicology and Nutrition Branch*
*Division of Laboratory Sciences*
*National Center for Environmental Health, CDC*

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**Important Information for Users**

The Centers for Disease Control and Prevention (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:

| Lab Number | Analyte | SAS Label |
|---|---|---|
| VITAEC_D | LBXBEC | *trans*-β-carotene (ug/dL) |
| | LBXCBC | *cis*-β-carotene (ug/dL) |
| | LBXCRY | β-cryptoxanthin (ug/dL) |
| | LBXGTC | Gamma tocopherol (µg/dL) |
| | LBXLUZ | Combined Lutein/zeaxanthin (ug/dL) |
| | LBXLYC | *trans*-lycopene (ug/dL) |
| | LBXRPL | Retinyl palmitate (µg/dL) |
| | LBXRST | Retinyl stearate (µg/dL) |
| | LBXTLY | Total lycopene (*cis* and *trans*-; µg/dL) |
| | LBXVIA | Retinol (µg/dL) |
| | LBXVIE | α-Tocopherol (µg/dL) |

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

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

## 2. SAFETY PRECAUTIONS

All serum samples received for analysis must be considered potentially positive for infectious agents including HIV and hepatitis B viruses. Universal Precautions must be observed. Wear gloves, lab coat, and protective eyewear while handling all human blood products. [The hepatitis B vaccine series is recommended for all analysts working with blood, serum, or plasma.] Sample handling should be performed in a biological safety cabinet, if available. Disposable plastic, glass, latex and paper items (pipette tips, autosampler inserts, gloves, etc.) that contact serum are to be placed in biohazard autoclave bags. These bags should be kept in appropriate containers until sealed and autoclaved. Periodically, wipe down all work surfaces with 10% bleach solution or an appropriate disinfectant solution such as ALL SAFE! (Momar, Inc., Atlanta, GA) when work is finished. Discard absorbent material used to cover work surfaces as often as needed. Organic solvents must be handled in a well-ventilated area, preferably in a chemical fume hood.

Reagents and solvents used in this study 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 either <<http://www.ilpi.com/msds/index.html>> or <<http://intranet.cdc.gov/ohs>>.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

A. Management and calculation of the chromatography data are accomplished through Waters Millennium 32 software installed on the personal computer connected via IEEE cable to the Alliance system. After a sample set ('run') is complete, the calculated amount data are exported to a floppy disk and to the network (I:\Instruments\Alliance) using a Millennium export method. The format is a single *.txt file. The *.txt is imported and transferred into a Microsoft Access 2000 database (I:\NHANESMain\NBBMultiStudy.mdb). The bench QC data are reviewed by the analyst, along with other features associated with assay calibration and extraction recovery. Values that are outside of Critical Limits are scheduled for repeat analysis (with or without dilution). If the QC pools are within 2SD of the characterization mean, the data are ready to be submitted to the team leader. The team leader reviews the data in the Access Database on the NCEH/DLS Local Area Network (LAN) and prepares SAS data reports for each study or study segment. Either the supervisor, team leader, or analyst reviews replicate data and selects the appropriate final results. After the results are checked and integration or other corrections are made by the analyst, the supervisor approves the final values for release pending review and approval of the division statistician, Branch Chief and Division Director. The data are copied to a *.txt or *.xls file for release to the investigator. For long term studies, data are

transmitted electronically during the course of the study. Abnormal values are confirmed, and codes for missing data are entered by the analyst and are transmitted as part of the data file.

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

C. Documentation for data system maintenance is contained in logs kept within the laboratory.

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

A. For best results, a fasting sample should be obtained and care should be taken to avoid exposure of the serum to sunlight or other sources of full spectrum radiation.

B. Specimens for fat soluble vitamin analysis may be fresh or frozen. Serum should be harvested from blood collected in redtop or royal bluetop Vacutainer brand tubes by standard venipuncture procedures.

C. A 0.5-mL sample of serum is preferable, but a sample volume of 100 µL may be analyzed.

D. The appropriate amount of serum is dispensed into a Nalge cryovial or other plastic screw-capped vial labeled with the participant's ID.

E. Specimens collected in the field are frozen, and then shipped on dry ice by overnight mail. Frozen serum is stored at -70°C. Retinol and α-tocopherol are stable for at least 5 years at -70°C. The carotenoids are stable for 2 years at -70°C. The stability of the retinyl esters has not been determined. Sample quality may degrade with successive freeze-thaw cycles.

F. Specimens generally arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site of collection.

G. Specimens which have been through more than five freeze-thaw cycles, which have been refrigerated for more than 24 hours, or which have undergone hemolysis may give inaccurate results for one or more of the primary analytes (i.e., retinol, α-tocopherol, or β-carotene). The retinyl ester concentration of non-fasting serum is generally non-informative.

H. Specimen handling conditions are outlined in 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 Lab\CLIA). The document outlines the protocol for sample collection, transport of specimens, specimen storage, and the additional equipment required. In general, serum should be transported and stored at no more than - 20°C. Generally, samples thawed and refrozen less than five times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of serum should be transferred into a sterile Nalge cryovial labeled with the participant's ID.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

6. PREPARATION OF REAGENTS, CALIBRATORS, CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

A. Reagent Preparation

1. Mobile Phase. Ethanol (200 proof) and acetonitrile (HPLC grade) are filtered separately using 0.45

um pore size membranes (Cat. no. HVHP047; Millipore, Medford, MA). Triethylamine (TEA, 'Baker' grade) is added to each prior to filtering at 32 drops per liter using a glass Pasteur pipette to make 0.1% TEA solutions. The components are degassed and automated solvent blending takes place in the Alliance 2695 HPLC system.

2. 10% Ascorbic Acid and 10% NaCl. 0.5 g L-Ascorbic Acid (ACS Certified) and 0.5 g NaCl (Analytical Grade) are dissolved in 5.000 mL deionized water in a 12 x 75 mm test tube. The ascorbic acid is relatively labile but normally can be used for 2 weeks or until there is a noticeable yellow color to the solution. Always cap the tube (with a disposable plastic cap) and store at room temperature in yellow light. If there is significant headspace in the tube, flush with nitrogen or argon before storage.

B. Standards Preparation

1. Purified Stock Solutions. Stock solutions of all standards except retinyl butyrate, zeaxanthin, and C45 are prepared using the following procedure:

A small amount of a standard is dissolved in chloroform. Using an autoinjector, 2-5 µL of the chloroform solution is injected on to a Burdick and Jackson OD5 octadecylsilane 15 cm x 4.6 mm, 5 µm particle size column and eluted with a 50:50 ethanol: acetonitrile mobile phase containing 0.1% TEA. The central third of the peak ("middle cut") is collected. Pooled middle cuts from several injections are diluted with mobile phase while maintaining minimal headspace. The absorbance of the solution is measured using a UV visible spectrophotometer. The concentrations are calculated based on the NHANES lab extinction coefficients (EC) listed in Table 1 (for SRM and NIST Round Robin exercises, NIST EC are used for retinol and retinyl palmitate). The resulting pooled solutions are diluted with mobile phase (50:50 ethanol:acetonitrile with 0.1% TEA ) to approximately the concentrations shown in Table 1. Final working solution is aliquoted into prelabeled amber vials (National Scientific Co. part# C4012-2 12x32 mm). Lycopene standard is aliquoted into 500 µL vials (Sarstedt part# 72.730.009 cap# 65.716).

A small amount of retinyl butyrate is dissolved in ethanol. Using an autoinjector, several aliquots of 15-20 µL of the ethanol solution are injected on to a Luna C18 25 cm x 10.00 mm, 5 µm particle size column and eluted with ethanol containing 0.1%L TEA. The top three quarter of the peaks are collected. The absorbance of the pooled solution is measured with a spectrophotometer. The solution is diluted with 100% ethanol containing 0.1% TEA to obtain a solution with an absorbance of approximately 0.250 AU for retinyl butyrate at 325 nm.

Nonapreno-β-carotene (C45) is synthesized under contract to CDC. A small amount of a powdered C45 is dissolved in chloroform. Using an autoinjector, multiple aliquots of 30-40 µL of the chloroform solution is injected on to a Luna C18 250 mm × 10.00 mm, 5 µm particle size column and eluted with 100% ethanol containing 200 µL TEA per liter. The absorbance of the solution is measured with a spectrophotometer, and the solution diluted with 100% ethanol containing 0.1% TEA to obtain a solution with an absorbance of approximately 0.320 AU for C45 at 482 nm. Equal parts of C45 and retinyl butyrate are mixed to prepare an internal standard. The internal standard solution is stable for at least 24 weeks when blanketed with nitrogen or argon. The internal standard mixture is aliquoted into 20 mL glass vials with Teflon lined caps (KIMBLE Glass Inc., Vineland, NJ. part # 74500-20) with minimal headspace. They are stored at -70 µC freezer.

Zeaxanthin (Extrasynthese) is diluted at 1 mg in 1.0 mL ethanol. Using an autoinjector, multiple 25 µL aliquots of the ethanol solution is injected on to an YMC C30 Carotenoid 15 cm × 4.6 mm column (cat# CT99S051546WT) and eluted with ethanol: acetonitrile (50:50) containing 0.1% TEA. The top third of the zeaxanthin peaks are collected. The absorbance of the pooled middle cuts from several injections is measured with a spectrophotometer. The solution is diluted with ethanol to obtain a zeaxanthin solution with an absorbance of approximately 0.160 AU at 454 nm.

**Table 1. Extinction Coefficients Used to Calculate Concentrations of Standard Stock Solutions (NIST in ethanol unless otherwise indicated; CDC in either 50%:50% ethanol: acetonitrile w/ 0.1%TEA or 100% ethanol).**

| Analyte | Extinction Coefficient* (dL/g·cm) | NHANES Wavelength nm (NIST nm) | Target Concentration of Stock Solutions |
|---|---|---|---|
| Retinol | 1850 CDC (1843 NIST) | 325 (325) | 50 µg/dL |
| Retinyl Palmitate | 1850 CDC (975 NIST) | 325 (325) | 20 µg/dL** |
| Retinyl Stearate | 1850 CDC | 325 | 20 µg/dL** |
| α-Tocopherol | 91.4 CDC (91.4 NIST) | 299 (298) | 900 µg/dL |
| γ-Tocopherol | 75.8 CDC (75.8 NIST) | 293 (292) | 2110 µg/dL |
| Zeaxanthin | 2540 CDC (2540 NIST) | 454 (450) | 63 µg/dL |
| β-Cryptoxanthin | 2370 CDC (2356 NIST) | 454 (452) | 57 µg/dL |
| Lycopene (NIST in hexane) | 3450 CDC (3450 NIST) | 474 (472) | 24 µg/dL |
| α-Carotene (NIST in hexane) | 2725 CDC (2800 NIST) | 447 (444) | 36 µg/dL |
| β-Carotene | 2560 CDC (2560 NIST) | 454 (450) | 23 µg/dL |

*$A^{1\%}_{1cm}$ is defined as the theoretical absorbance of a 1% solution (1g/100 mL) in a cell of 1-cm path length.
** Concentrations of retinyl esters are reported as retinol equivalents by the CDC lab.

2. Mixed Standards (Working Solutions)

A retinyl ester standard is prepared by mixing equal volumes of retinyl palmitate and retinyl stearate stock solutions using one volumetric pipette and rinsing the pipette with 50:50 ethanol: acetonitrile between each standard.

The vitamin E standard is prepared by mixing equal parts of α-tocopherol and γ-tocopherol using one volumetric pipette and rinsing the pipette with 50:50 ethanol: acetonitrile between each standard.

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

All working (mixed) standard solutions are stored in 1.8 mL aliquot in amber glass vials (National Scientific Co. part# C4012-2 12 × 32 mm) at –70°C and are stable for at least 24 weeks.

Retinyl stearate is synthesized using the following procedure:

To 1.0 g of retinol, add 10 mL of pyridine and 2.85 mL of anhydride in a round bottom flask. The reaction mixture is stirred overnight at room temperature (25 °C). When the reaction is complete, neutralize the retinyl ester and extract it from the pyridine solution by adding 100 mL of methylene chloride and 15 mL of dilute (5M) hydrochloric acid. Add 50 mL of 1M sodium hydroxide to neutralize any acid. Solvent layer is evaporated using Rotovap at 45°C. The residue is chromatographed on a column of alumina grade III (or silica woelm 32–63) and the ester is isolated as a colorless material with greenish-yellow fluorescence.

Retinyl butyrate is synthesized using the following procedure:

To 1.0 g of retinol, add 4 mL of triethylamine (TEA), 40 mL of hexane, and 0.6 mL of butyric anhydride in a round bottom flask. The reaction mixture is stirred for 3.5 hours at 60°C. Hexane and TEA are evaporated using Rotovap at 45°C. Retinyl butyrate is isolated on the same column used to isolate retinyl stearate.

C. Preparation of Bench Quality Control (QC) Material

All serum pools are filtered through sterile gauze. One half gram of L-ascorbic acid per liter of serum is added and mixed overnight in a –4°C walk-in refrigerator. The serum is filtered through sterile gauze again prior to aliquoting into sterile 5-mL vials. The vials are blanketed with nitrogen or argon, and sealed. The QC pools are stored at –70°C for up to six years.

The low QC pool is prepared by selecting and blending sera that contain low levels of all analytes.

The medium QC pool is prepared by pooling sera that contain most of the analytes at levels close to the mean levels observed in normal subjects (NHANES III data).

The high QC pool is prepared by pooling sera that contain higher than normal levels of most analytes. Spiking is generally successful only for retinol. People who eat very large quantities of fresh fruits and vegetables and have high serum lipid concentrations are most likely to have high concentrations of fat-soluble micronutrients. In some instances dog serum, which typically has a high retinyl ester concentration, is added to the high pool. Other types of subjects useful for blending into the high pool are Type 2 diabetics who, in the absence of good glycemic control, may have high concentrations of lutein/zeaxanthin, β-cryptoxanthin, and γ-tocopherol. Sera from individuals taking vitamin supplements are also used.

When preparing pools, filters that are hydrophilic should be used to minimize loss of the carotenoids and vitamin E during filtration. If the pool levels are unacceptably low and spiking needs to be done, it is essential that the spiked, pooled sera be mixed overnight before the final filtration step.

D. Instrumentation

1. Waters HPLC system (Waters Chromatography Division, Milford, MA)
   a. Model 2695 Alliance system
   b. Model 2996 or 996 photodiode array detector
   c. Compaq computer with the following specifications: Microsoft Windows 2000 Professional operating system, 1000 MHz, 260 MB RAM, 18 GB hard drive.
2. Cera column cooler 250 (Cera, Inc. Baldwin Park, CA) or equivalent.
3. Rack-type vortex mixer (American Scientific Products, McGaw Park, IL) or equivalent.
4. Cary 3E spectrophotometer (Varian Instruments, Palo Alto, CA) or equivalent.
5. Speedvac SC200 and SC210A Systems (Savant Instrument Co., Farmingdale, NY) or equivalent.
6. Precision Model VP-190 Direct Drive Vacuum Pump (Precision Scientific Inc., Chicago, IL) or equivalent.
7. Refrigerated vapor trap, model RVT-4104 (Savant Instrument Co., Farmingdale, NY) or equivalent.
8. Magnetic stirrer (American Scientific Products) or equivalent.
9. Digiflex Automatic Diluter/Dispenser, with 200-μL sampling and 2.0mL dispensing syringes (Titertek Instruments,Inc.,Huntsville, AL) or equivalent.
10. Gilson Microman positive displacement Pipettes (Gilson, Villiers-le, France) or equivalent. Ranin Pipetman pipette (Ranin, Woburn, MA) or equivalent.

E. Materials

1. 15 cm x 4.6 mm Phenomenex Ultracarb 3 octadecylsilane, 3 µm particle size column (Phenomenex, Torrance, CA )
2. 15 cm x 4.6 mm Burdick and Jackson OD5 octadecylsilane 5 µm particle size column (Burdick and Jackson Laboratories, Muskegan, MI)
3. 15 cm x 4.6 mm YMC C30 Carotenoid column (YMC, Wilmington, NC)
4. 25 cm x 10 mm Phenomenx 5 µ micron C18 Luna column (Phenomenex, Torrance, CA)
5. Hexane UV (Burdick and Jackson Laboratories, Muskegan, MI) or equivalent.
6. Acetonitrile HPLC grade (Burdick and Jackson Laboratories, Muskegan, MI) or equivalent.
7. Ethanol, absolute (U.S.P.), stored in glass, (Pharmco Products, Brookfield, CT) or equivalent
8. Chloroform, spectrophotometric grade (Mallinckrodt, St. Louis, MO) or equivalent.
9. Triethylamine, 'Baker' grade (Fisher Scientific, Inc., Fairlawn, NJ) or equivalent
10. Retinol (Sigma Chemical Co., St. Louis, MO) or equivalent
11. Retinyl Palmitate (Sigma Chemical Co., St. Louis, MO) or equivalent
12. β-Tocopherol (Eastman Chemical Co, Kingsport, TN) or equivalent.
13. γ-Tocopherol (Sigma Chemical Co., St. Louis, MO) or equivalent
14. Zeaxanthin (Extrasynthese S.A., Impasse Jacquard, B.P.62, 69730 Genay, France, Phone (33)78982034, FAX (33)7898l946, Telex 306231F) or equivalent
15. β-Cryptoxanthin (Extrasynthese) or equivalent
16. Lycopene (Sigma Chemical Co., St. Louis, MO) or equivalent
17. β-Carotene (Sigma Chemical Co., St. Louis, MO) or equivalent
18. α-Carotene (Sigma Chemical Co., St. Louis, MO) or equivalent
19. L-Ascorbic acid, ACS grade (Fisher Scientific, Inc., Fairlawn, NJ) or equivalent
20. Stearic anhydride (Sigma Chemical Co., St. Louis, MO) or equivalent
21. Butyric anhydride (Sigma Chemical Co., St. Louis, MO) or equivalent
22. Triethylamine (Fisher Scientific, Inc., Fairlawn, NJ) or equivalent
23. Alumina, Grade III (obtained from various sources) or equivalent
24. Methanol, HPLC grade, (Fisher Scientific, Inc., Fairlawn, NJ) or equivalent
25. Argon, Ultrapure (Air Products, Inc., Chamblee, GA) or equivalent
26. Nitrogen (Air Products, Inc., Chamblee, GA) or equivalent
27. 13 × 100 mm disposable glass culture tubes (Corning Glassworks, Corning, NY) or equivalent
28. Pipette tips for SMI Digitron pipette (American SMI, Emeryville, CA) or equivalent
29. Plastic tuberculin syringes (obtained from various sources) or equivalent
30. Serum extract filters, 0.45 µm pore size (cat. no. SJHV004NS, Millipore Corp., Bedford, MA) or equivalent
31. Solvent filters, 0.45 µm pore size (cat. no. HVHP047, Millipore Corp., Medford, MA) or equivalent
32. 12 x 75 mm disposable glass culture tubes (Corning Glassworks, corning, NY.) or equivalent
33. Autosampler vials and inserts (Wheaton Science Products, 12 x31 mm wide mouth vials (cat. no. TCW224626), 250 µL polypropylene inserts (cat. no. TCW225259), and screw caps with teflon/silicone septa (cat. no. TCW242762, Millville, NJ) or equivalent
34. Actinic glassware (obtained from various sources) or equivalent
35. Nunc vials (obtained from various sources) or equivalent
36. Waters Guard-Pak Module (cat. no. WAT 88141) with Guard Pak filters (cat. no. WAT032472) or equivalent

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

Before each run, duplicate sets of calibrators are prepared by combining 200 µL of the internal standard and 200 µL of working standard solutions using a positive displacement pipette. Thus, each calibrator contains half as much of each component as the working solutions (Table 2).

**Table 2. Concentrations of assay standards**

| Analyte | Stock Concentration (purified unmixed) | Working Concentration (aliquoted frozen) | Final Concentration (plus internal standard) |
|---|---|---|---|
| Retinol | 50 µg/dL ± 10% | 50 µg/dL± 10% | 25 µg/dL ± 10% |
| Retinyl Palmitate | 20 µg/dL ± 10% | 10 µg/dL ± 10% | 5 µg/dL ± 10% |
| Retinyl Stearate | 20 µg/dL ± 10% | 10 µg/dL ± 10% | 5 µg/dL ± 10% |
| α-Tocopherol | 2110 µg/dL ± 10% | 1055 µg/dL ± 10% | 527.5 µg/dL ± 10% |
| α-Tocopherol | 900 µg/dL± 10% | 450 µg/dL ± 10% | 227 µg/dL ± 10% |
| Zeaxanthin | 63 µg/dL ± 10% | 21 µg/dL ± 10% | 10.5 µg/dL ± 10% |
| β-Cryptoxanthin | 57 µg/dL ± 10% | 19 µg/dL ± 10% | 9.5 µg/dL ± 10% |
| β-Carotene | 36 µg/dL ± 10% | 12 µg/dL ± 10% | 6 µg/dL ± 10% |
| Lycopene | 24 µg/dL ± 10% | 24 µg/dL ± 10% | 12 µg/dL ± 10% |
| β-Carotene | 23 µg/dL ± 10% | 23 µg/dL ± 10% | 11.5 µg/dL ± 10% |

The standards are read as calibrators at the beginning of each run. These values are used to generate a one point, single concentration, linear, forced through zero, standard curve for each analyte. The calibrators are injected again at the end of the run and treated as unknowns by the processing software. As unknowns, their values must agree within 20% of the calibrator values.

The Millennium 32 software performs all calculations. Calibration curves are linear, forced through zero, and based on single injection analysis of a single standard concentration. For each analyte not present in a standard solution, linkage to an appropriate standard is made and used to calculate concentrations.

The CDC laboratory participates in a proficiency testing program for retinol, retinyl palmitate, $\alpha$- and $\gamma$-tocopherol, zeaxanthin, $\beta$-cryptoxanthin, lycopene, and $\alpha$- and $\beta$-carotene sponsored by the National Institute of Standards and Technology (NIST, Gaithersburg, MD). Twice a year, Round Robin materials are sent by NIST to assess laboratory performance. At the same time, certified reference materials (SRM) for retinol, $\alpha$-tocopherol and $\beta$-carotene, currently NIST SRM 968c (certificate of values kept by analyst), are analyzed to determine the agreement between results obtained with the CDC laboratory method and the certified values.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Preliminaries

1. Build Sample Sets using Millennium 32 Run Samples as described in Section 8.C. Participant ID numbers may be scanned into the computer if they are bar-coded.

   Transfer the calibrators to autosampler vials and place them in the Alliance (20°C) as in Table 3.

**Table 3. Calibrators used in vitamin A/E/carotenoid assay contain the multiple components**

| Calibrators | Vial Position/Name |
|---|---|
| α-Carotene<br>Zeaxanthin β-Cryptoxanthin<br>Retinyl Butyrate<br>C45 | 97 /<br>Carotenoids/Xanthophylls<br>mixed |
| β-Carotene Retinyl Butyrate<br>C45 | 98 / β-Carotene |
| Lycopene<br>Retinyl Butyrate C45 | 99 / Lycopene |
| Retinol<br>Retinyl Butyrate C45 | 100 / Vitamin A |
| γ-Tocopherol<br>α-Tocopherol<br>Retinyl Butyrate<br>C45 | 101 / Vitamin E |
| Retinyl Palmitate<br>Retinyl Stearate<br>Retinyl Butyrate<br>C45 | 102 / Retinyl Esters |

2. Allow frozen serum, quality control serum, working standards and the internal standard to reach ambient temperature but not to exceed 27°C.
3. Prepare the mobile phase by adding 32 drops of TEA per liter of each solution, and by individually filtering the two components (acetonitrile and ethanol). Always prepare fresh mobile phase. Retention times shift when mobile phase picks up water from the room air.
4. Turn on instrument as described in the instrument set- up section (Section 8.C.) of this document.

B. Sample Preparation

1. Prepare one set of labeled 12 x 75 mm glass tubes and one set of labeled 13 x 100 mm glass tubes for the calibrators, pools and unknowns.
2. Prepare a shallow (2–3'' deep) ethanol and dry ice bath for freezing samples.
3. With an Eppendorf repeating pipette or equivalent, dispense 10 µL of 10% L-Ascorbic Acid/10% NaCl solution into the bottom of each 13 x 100 mm glass tube.
4. Using a micropipette, add 100 µL of serum to each tube containing the 10% ascorbic acid/10% NaCl solution. Cover the tubes with heavy duty aluminum foil and vortex for 60 sec.
5. With the Digiflex Dilutor, or equivalent, add 100 µL of the internal standard solution to each tube.
6. Cover the tubes with foil and vortex the mixture for 60 seconds, being careful not to allow the

liquids to touch the foil during mixing.

7. With the Digiflex Dilutor, add 1.000 mL of hexane. Cover the tubes with foil and vortex for 60 seconds, being careful not to allow the liquids to touch the foil during mixing. Repeat this mixing cycle (60 sec) six times for a total of 6 min of extraction with hexane. For good recovery, it is important to mix as vigorously as possible without touching the foil (liquid should reach up to two-thirds of the tubes while shaking).
8. Allow the samples to rest for 10 min in order for aqueous and organic phases to separate.
9. Carefully transfer the rack of tubes to the dry ice/ethanol bath. Allow the aqueous phase to freeze (≥ 25 min). Pour the hexane (upper) layers into the second set of labeled 12 x 75 mm tubes and evaporate the hexane in the Speedvac (without heat) according to the number of tubes (9 minutes for 40–60 tubes, 10 minutes for 60–100 tubes, and 12 minutes for >100 tubes). Each Speedvac has slight differences in dry times which the analyst has documented. Make sure not to over-dry the extracts.
10. Add 50 µL of ethanol to the tubes containing the dried extracts.
11. Vortex the tubes for 2 cycles of 60 seconds each.
12. Add 50 µL of acetonitrile to each dissolved extract and vortex the tubes for 2 cycles of 60 seconds each.
13. Draw each extract into a 1.0 mL tuberculin syringe taking care to leave an air space between the plunger tip and the solution, place a 0.45 µ pore-size syringe filter on the loaded syringe, and filter the extract into an autosampler vial.
14. Cap the vials and place them in the Alliance carousels at 20°C.

C. Instrument Preparation

1. Load fresh mobile phase into reservoirs. Top off methanol in seal and needle wash reservoirs.
2. Turn on the Waters Alliance 2695. Set the refrigeration unit to maintain a temperature of 20°C in the autosampler compartment. Perform a wet prime for 5 min at flow rate of 5 ml/min and a purge cycle at sample loop volume 6.0 mL.
3. Turn on the Waters 2996 or 996 detector
4. Turn on the Cera column cooler 250 set at 25 °C.
5. Turn on the computer. Log onto Millennium 32. Open the Project folder. Open Run Samples. Load the Instrument Method. Collect baseline absorbance data at normal operating flow (1.0 mL/min) and equilibrate with mobile phase (50% ethanol: 50% acetonitrile) for at least 1 hour.

6. Load a method that contains the following parameters:

Acquisition Parameters for 2695 and 996:

Run time: Typically 13.0 min-depending upon column
Pump pressure limits: 20–3000 psi
Pump flow ramp: 3 min
Degas mode: continuous
PDA sampling frequency: 1.0 points per sec
PDA resolution bandwidth: 4.8 nm
PDA wavelengths: 270–480 nm
Data rate: 10.56 KB/min

**Table 4. Waters 2695 isocratic conditions**

| Time | Flow | % ethanol | % acetonitrile | Curve |
|---|---|---|---|---|
| | 1.0 | 50 | 50 | |
| 16.0 | 1.0 | 50 | 50 | 6 |
| 20.0 | 0.0 | 50 | 50 | 1 |

**Table 5. Component Table Information**

| Component Name | Retention time | Window | Channel | Quantitate | Internal Standard |
|---|---|---|---|---|---|
| Lutein/Zeaxanthin | 2.46 | 0.15 | 450 | Yes | C45 |
| RB300 | 2.87 | 0.20 | 300 | Int | |
| RB325 | 2.87 | 0.20 | 325 | Int | |
| α-Cryptoxanthin | 4.05 | 0.20 | 450 | No | C45 |
| γ-Tocopherol | 4.18 | 0.20 | 300 | Yes | RB300 |
| β-Cryptoxanthin | 4.28 | 0.20 | 450 | Yes | C45 |
| α-Tocopherol | 4.52 | 0.20 | 300 | Yes | RB300 |
| trans-Lycopene | 5.40 | 0.50 | 450 | Yes | C45 |
| α-Carotene | 7.76 | 0.20 | 450 | Yes | C45 |
| β-Carotene | 8.22 | 0.20 | 450 | Yes | C45 |
| Retinyl palmitate | 8.50 | 0.20 | 325 | Yes | RB325 |
| cis-β-Carotene | 8.86 | 0.20 | 450 | Yes | C45 |
| C45 | 9.67 | 0.50 | 450 | Int | |
| Retinyl stearate | 11.21 | 0.50 | 325 | Yes | RB325 |

Integration Parameters

The integration parameters will vary with lamp age, column characteristics and age, and other factors. In the component table (Table 5), all components are calibrated on the basis of height or area using a linear curve forced through the origin. The parameters in Table 5 are acceptable for use as a starting point from which to optimize the conditions. The retention times will vary with age of the column, from column to column, and from instrument to instrument so those in the table should be taken only as a guide. Actual retention times for a given column/instrument combination should be determined individually, monitored on a regular basis, and the component table updated when necessary. Enter a manual response factor for cis-β-carotene that is equal to the calculated response factor for β-carotene under the same chromatographic conditions. Similarly, enter a response factor for α-cryptoxanthin that is equal to the calculated response factor for β-cryptoxanthin. NB: α-cryptoxanthin is tentatively identified as such and will not be reported until a positive identification is made.

7. Edit the Sample Queue so that all of the samples are correctly identified and the appropriate vial numbers entered. Edit the calibrator concentrations in the Amount Table. The concentrations for the internal standards in all calibrator vials are entered as 1.00.
8. Save the Sample Queue and the Sample Set Method.

   Start the run.

D. Maintenance

1. Speedvac. The trap temperature is maintained between –110°C to –97°C and is checked daily before turning on the vacuum pump. The vapor trap of the Speedvac is emptied at least weekly when operating at full capacity. The vacuum pump oil is changed at least annually or when the quality of the vacuum deteriorates or the oil becomes turbid.
2. HPLC System. Precolumn Guard-Pak inserts are changed monthly. Waters-specified preventive maintenance on the HPLC system is performed annually.

E. Calculations

The Millennium 32 software performs all calculations. Calibration curves are linear, forced through zero, and based on single injection analysis of a single standard concentration. For each analyte not present in a standard solution, linkage to an appropriate standard is made and used to calculate concentrations.

The concentration of the components of the mixed standards is equal to the concentration of the purified stock divided by the number of components of the solution, excluding the internal standards.

Example:

In a mixed standard containing zeaxanthin, β-cryptoxanthin, and α-carotene (3 components), if the concentration of the zeaxanthin in the purified stock solution is 64 μg/dL, then the concentration of zeaxanthin in the mixed standard (working concentration) is 64 μg/dL divided by 3, or 21.3 μg/dL. This concentration is entered in the amount table for zeaxanthin in RunSamples even though the actual concentration of zeaxanthin in the mixed standard is half of this after addition of the internal standards.

The following codes and integration parameters (called Metric in the Access database; refers to the manner in which the baseline is drawn) are used for fat-soluble micronutrients in the Access database:

Final Quantitation Parameters:

| Analyte | Code | Metric |
|---|---|---|
| β-carotene | ALC | Height-forced |
| β-carotene | BEC | Height-forced |
| cis-β-carotene | CBC | Height-forced |
| β-cryptoxanthin | CRY | Height-forced |
| α-tocopherol | GTC | Height |
| lutein+zeaxanthin | LUZ | Height-forced |
| trans-lycopene | LYC | Height |
| retinylpalmitate | RPL | Height |
| retinylstearate | RST | Height |
| *Cis*- and *trans*-lycopene | TLY | Area |
| retinol | VIA | Height |
| α-tocopherol | VIE | Height |

F. CDC Modifications

This method is a modification of a method described by Sowell et al. (1).

9. REPORTABLE RANGE OF RESULTS

This method is linear for the carotenoids in the range 1–150 μg/dL, for retinol and the retinyl esters in the range 1–150 μg/dL, and for α-tocopherol in the range 100–6000 μg/dL. The CVs for vitamins A and E and beta-carotene are generally less than 5%. The CVs for the minor carotenoids are generally less than 20%. Analysis is repeated on any sample for which either vitamin A, vitamin E, or β-carotene are outside of the normal range (Table 6).

**Table 6. Updated Ranges for 98-99% of US population (NHANES III), μg/dL**

| | |
|---|---|
| VITAMIN A (4-9 yr) | 19-63 |
| VITAMIN A (10-19 yr) | 24-84 |
| VITAMIN A (20-49 yr) | 25-103 |
| VITAMIN A (50+ yr) | 24-128 |
| VITAMIN E (4-9 yr) | 504-1479 |
| VITAMIN E (10+ yr) | 512-2875 |
| B-CAROTENE | 2-90 |
| LUTEIN/ZEAXANTHIN | 6-59 |
| β-CRYPTOXANTHIN | 1-33 |
| LYCOPENE | 3-56 |
| A-CAROTENE | 1-21 |
| RETINYL ESTERS | 1-18 |

10. QUALITY CONTROL (QC) PROCEDURES

A. Blind controls

For most studies blind controls are inserted prior to the arrival of the samples in the Inorganic Toxicology Nutrition Branch. Blind controls are prepared at two levels to emulate patient samples, including the use of labels identical to those used in the study.

B. Bench controls

Three serum pools are normally used as bench controls. These controls represent high, medium, and low levels of the analytes in serum. Duplicates of these pools are extracted exactly like patient samples and analyzed as part of each run. The run is considered “out of control” based on the quality control scheme detailed below. The initial limits are established from the results of analyzing pool material in twenty consecutive runs and then are updated as needed.

Quality Control Scheme for HPLC Fat-Soluble Vitamin Analysis

The HPLC fat-soluble vitamins assay measures vitamins A and E and carotenoids. Each of these classes of nutrients is

measured at a different wavelength. While there are some common characteristics, each class of analytes has distinct chemical properties and physiological functions. The following five analytes: retinol, α-tocopherol, lutein/zeaxanthin, lycopene, and β-carotene are generally present in significant amounts in most sera. These analytes are either required nutrients or have been associated with health effects in epidemiological studies. Much less is known about health effects associated with the other analytes. Most methodological problems with an analysis affect all analytes in a class in a similar manner, though not necessarily all classes of analytes.

Standard Shewhart QC charts are maintained for this internal QC specimen. A separate QC chart is to be maintained for each QC material used for bench QC specimen. Standard criteria for analyte rejection based on statistical probabilities are used to declare an analyte either in-control or out-of-control. These rules are:

Analytical run with 3 QC results:

1. if all three QC results are within 2s limits then accept the analyte

2. if one of three QC results is outside the 2s limits then apply rules below and reject if any condition is met:
   a. 13s - any of the three QC results are outside a 3s limit
   b. 22s - 2 of the 3 QC results in the run are outside 2s limit (same side of mean)
   c. R4s sequential - previous QC result was outside 2s limit on the opposite side of the mean
   d) 10x sequential - previous 9 QC results were on same side of mean

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

If the blind QC is out of control, all or part of the run is declared out of control.

If the recovery of the internal standards from any sample is above or below three standard deviations from the mean recovery for the run, the sample is reanalyzed.

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

A. Chromatography - Check the chromatography for faulty integration. Correct the integration if indicated. This is an uncommon event for controls, unless the column is new and the retention times/windows are not yet stable.

B. Comment Codes - Check sample preparation notes for comment codes indicating a spill or some other lab error involving the QC samples

C. Pipetting - If analytes are uniformly high or low in the QC, check fluid dispensing devices (Digiflex, pipettes) for accuracy. If not accurate, arrange for recalibration.

D. Extraction - Look for extraction problems by assessing the recovery of the internal standards in the calibrators, QC samples and unknowns. Isolated poor recovery suggests a sample spill. Poor recovery for the entire run suggests analyte degradation. Carotenoids are sensitive to air, light and heat. Was the room too warm? Were the samples at room temperature for longer than necessary? Were the samples dried in SpeedVac too long? Were the samples exposed to full-spectrum light for longer than necessary? Was the ascorbic acid antioxidant prepared fresh and added to the serum samples according to directions? Check the temperature log for the room and the current temperature. If the room was too warm when the samples were processed and are still warm, call the building manager.

E. Calibration - Check the lot of calibrator used to quantitate the analyte that failed QC. Is it more than 6 months old? Check the chart showing peak height or area for that calibrator on the current column. Is there a trend showing decreasing peak height? You might need to schedule calibrator purification. Alternatively, the detector lamp energy may be inadequate. Run lamp diagnostics. Replace lamp about every 6 months. Check the column history for the number of injections run on that column. Is it above average? Are the smaller peaks no longer recognized? If so, you probably need a new column.

F. Hardware - Check to make sure that the HPLC system hardware is functioning properly. Make sure the pump is operating at the appropriate pressure with steady delivery. Check the autosampler and the run sheet to make sure the injections are being made as programmed. Look at vial volumes and puncture marks. Make sure that the run sheet reflects the actual vial sequence by cross-checking sample numbers. Check the temperature of the autosampler. If you suspect that the injection volumes are too variable (check the internal standard peak height or area in the calibrators), you should run a test of the reproducibility of the injection volume using a standard. Check the Instrument Malfunction Log for past hardware/software problems. Check the Delta Pressure Log. When the ripple pressure (delta) exceeds 2.5% of the baseline system pressure, the check valves in the pump probably need to be replaced.

G. Freezer Log - Check the freezer logs for stable temperatures for assay materials requiring –70°C storage. Obtain NIST SRM material if you suspect that your QC pools or calibrators may have degraded (or after a significant change in the system).

H. Miscellaneous - Many obscure problems can be corrected by re-booting the HPLC/detector system. Every time the system is re-booted, diagnostics checks are automatically made. The system should be re-booted at least once a week.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

A. Phytofluene, a UV absorbing carotenoid found in tomatoes, co-elutes with retinyl palmitate and has been found in some sera. The 300 nm chromatogram of sera, which appear to have elevated retinyl palmitate without elevation of the other retinyl esters, should be examined to determine if the peak maximum is at the same retention time. The palmitate value is unreliable in these samples.

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

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

D. The following substitutions may be made for the specified instrumentation:

1. Instead of drying the hexane extracts with a Speedvac system, the samples may be dried under a stream of nitrogen without heating.

2. Instead of the Waters 2695, two Waters 730 data modules may be used by setting the detector to 0.01 AUFS at 450 nm, 0.05 AUFS at 325 nm, and 0.02 AUFS at 300 nm and connecting channel 1 to pen 1 and channel 2 to pen 2 of one data module and channel 3 to pen 1 of the second data module. When this is done it is necessary to manually measure the peak heights and calculate the concentrations and it is not possible to measure normal levels of retinyl esters.

E. All of the HPLC equipment is attached to line conditioners to minimize the effects of fluctuations of electrical current.

13. REFERENCE RANGES (NORMAL VALUES)

See Table 6. These values are based on the 1-99 percentile ranges for 8,284 specimens analyzed for NHANES III.

14. CRITICAL CALL RESULTS (PANIC VALUES)

Any sample with a vitamin A result that is <10 μg/dL or greater than the 99.5% age-specific limit (from NHANES III), or any vitamin A/retinyl ester profile that suggests hypervitaminosis A with hepatotoxicity, i.e. fasting serum with retinol elevated for age/sex and total retinyl esters > 40% of serum retinoid, is repeated and reported to the responsible party for the study (Project Officer) as soon as possible.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens are allowed to reach room temperature during preparation. Once the samples are ready to run, the prepared samples are placed in the Alliance at 20°C. The unused portion of the patient specimen is

returned to the freezer ASAP.

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

Since the analysis of serum for fat-soluble vitamins is inherently complex and challenging, there are no acceptable alternative methods of analysis in the NHANES laboratory. If the analytical system fails, then storage at $\leq$ –20°C of the extracted specimens is recommended until the analytical system is restored to functionality.

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

For studies other than NHANES, where the survey physician is notified by Westat, the collaborating agency with access to patient identifiers or the responsible medical officer is notified by FAX or e-mail of any Vitamin A result that is < 10 μg/dL, or of any Vitamin A/Retinyl Ester profile that suggests hypervitaminosis A as determined by the supervisor. Test results that are not abnormal are reported to the collaborating agency at intervals and by a method determined by the study coordinator.

Data are transmitted via the Director of the Division of Laboratory Sciences, NCEH, CDC after review by the Section Supervisor, Branch Chief, and a CDC statistician.

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

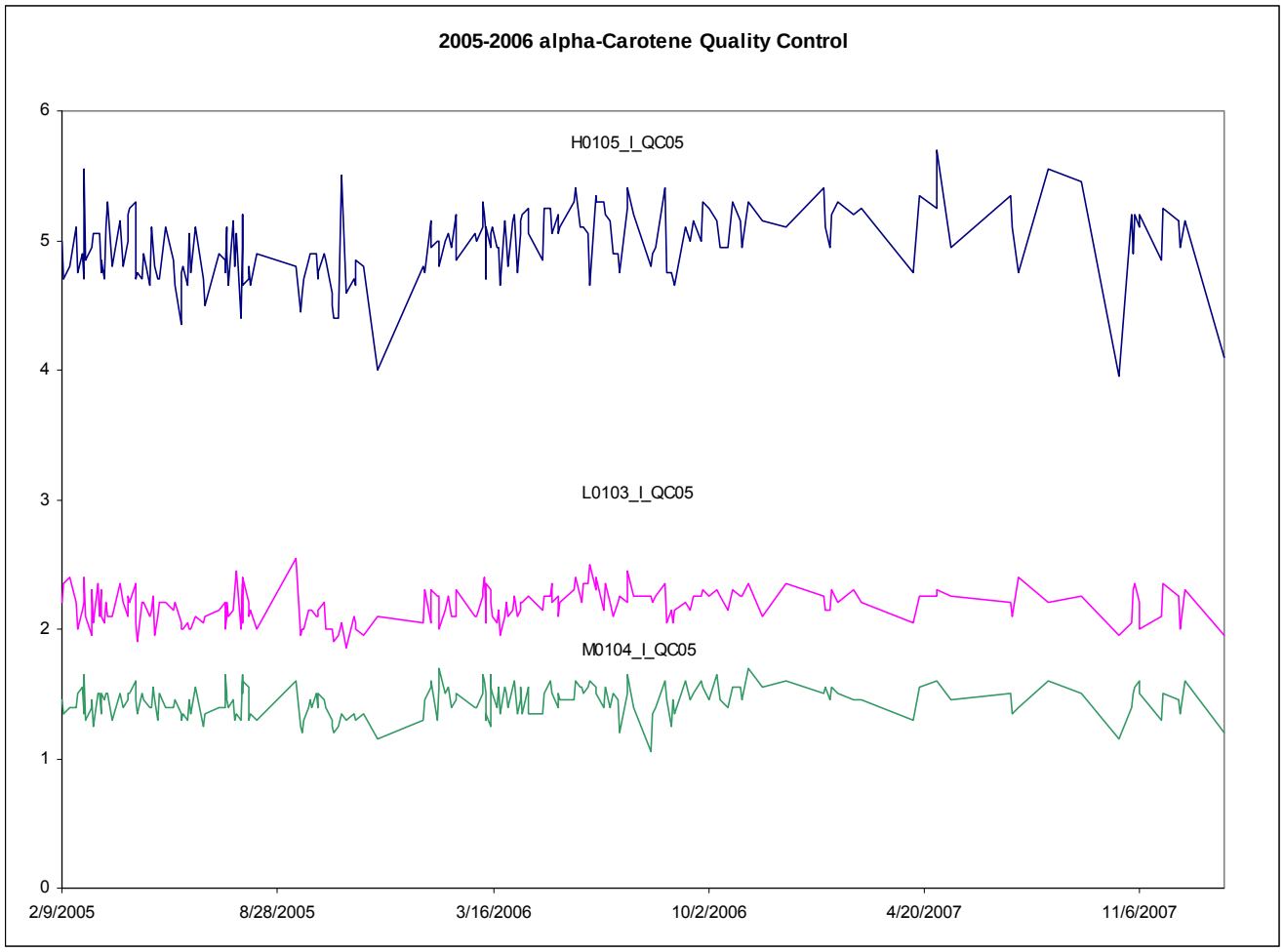
The Microsoft Access database is used to keep records and track specimens for NHANES 1999+. If serum vitamin A analyses are used for smaller, non-NHANES studies, records are kept on files in Q:\ITN\Nutrition Lab on the DLS LAN. We recommend that records, including related QA/QC data, be maintained for 10 years after completion of the study. 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 serum 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. Very little residual material will be available after NHANES analyses are completed, and these vials may be routinely autoclaved.

19. SUMMARY STATISTICS AND GRAPHS

A. α-Carotene

**Summary Statistics for alpha-Carotene by Lot**

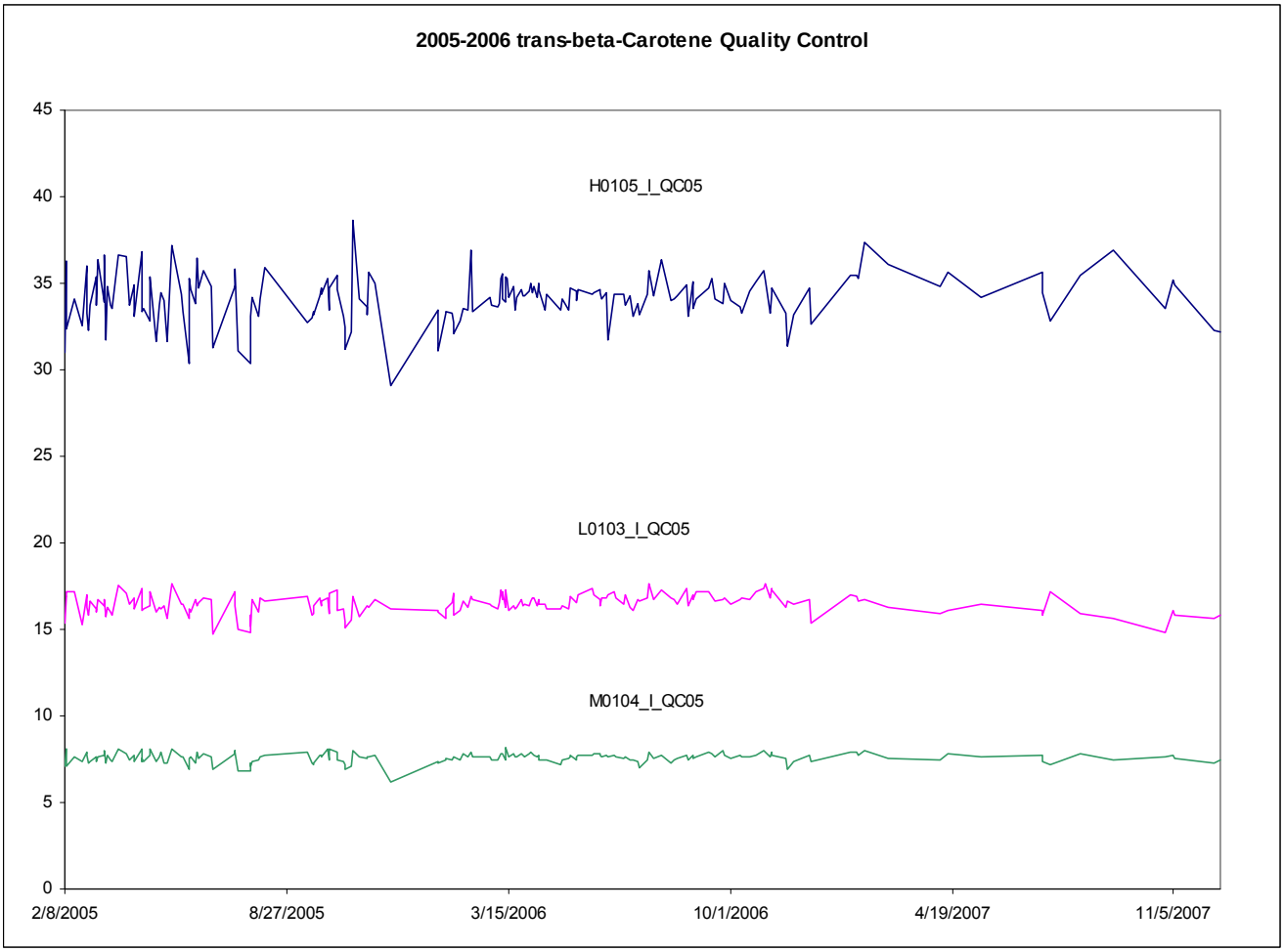
| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| M0104_I_QC05 | 205 | 2/9/2005 | 1/23/2008 | 1.45 | 0.12 | 8.0 |
| L0103_I_QC05 | 205 | 2/9/2005 | 1/23/2008 | 2.18 | 0.13 | 5.9 |
| H0105_I_QC05 | 205 | 2/9/2005 | 1/23/2008 | 4.96 | 0.27 | 5.4 |



B. *trans*-β-carotene

**Summary Statistics for trans-beta-Carotene by Lot**

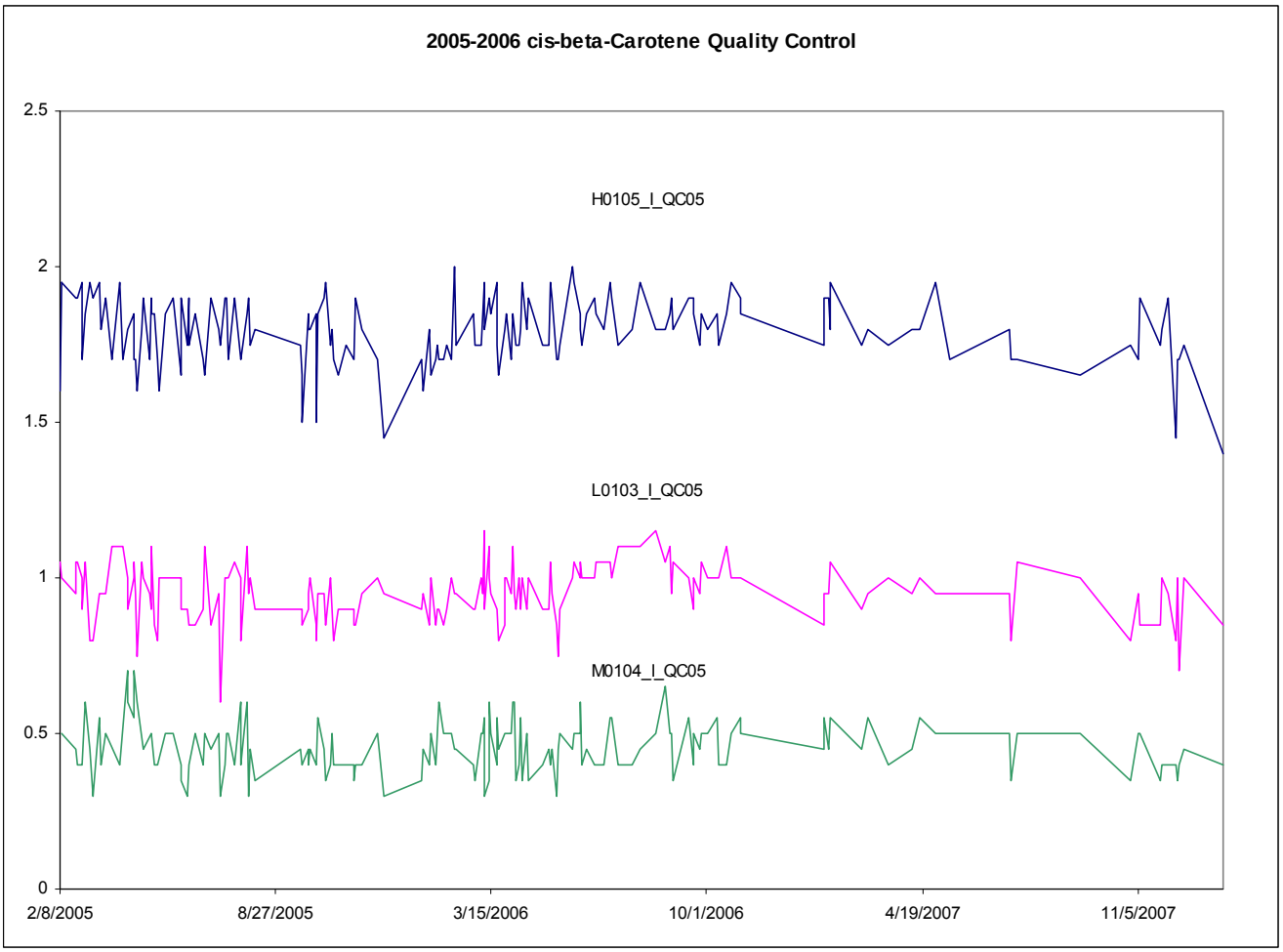
| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| M0104_I_QC05 | 191 | 2/8/2005 | 12/17/2007 | 7.59 | 0.28 | 3.7 |
| L0103_I_QC05 | 191 | 2/8/2005 | 12/17/2007 | 16.47 | 0.56 | 3.4 |
| H0105_I_QC05 | 191 | 2/8/2005 | 12/17/2007 | 34.13 | 1.39 | 4.1 |



C. *cis*-β-carotene

**Summary Statistics for cis-beta-Carotene by Lot**

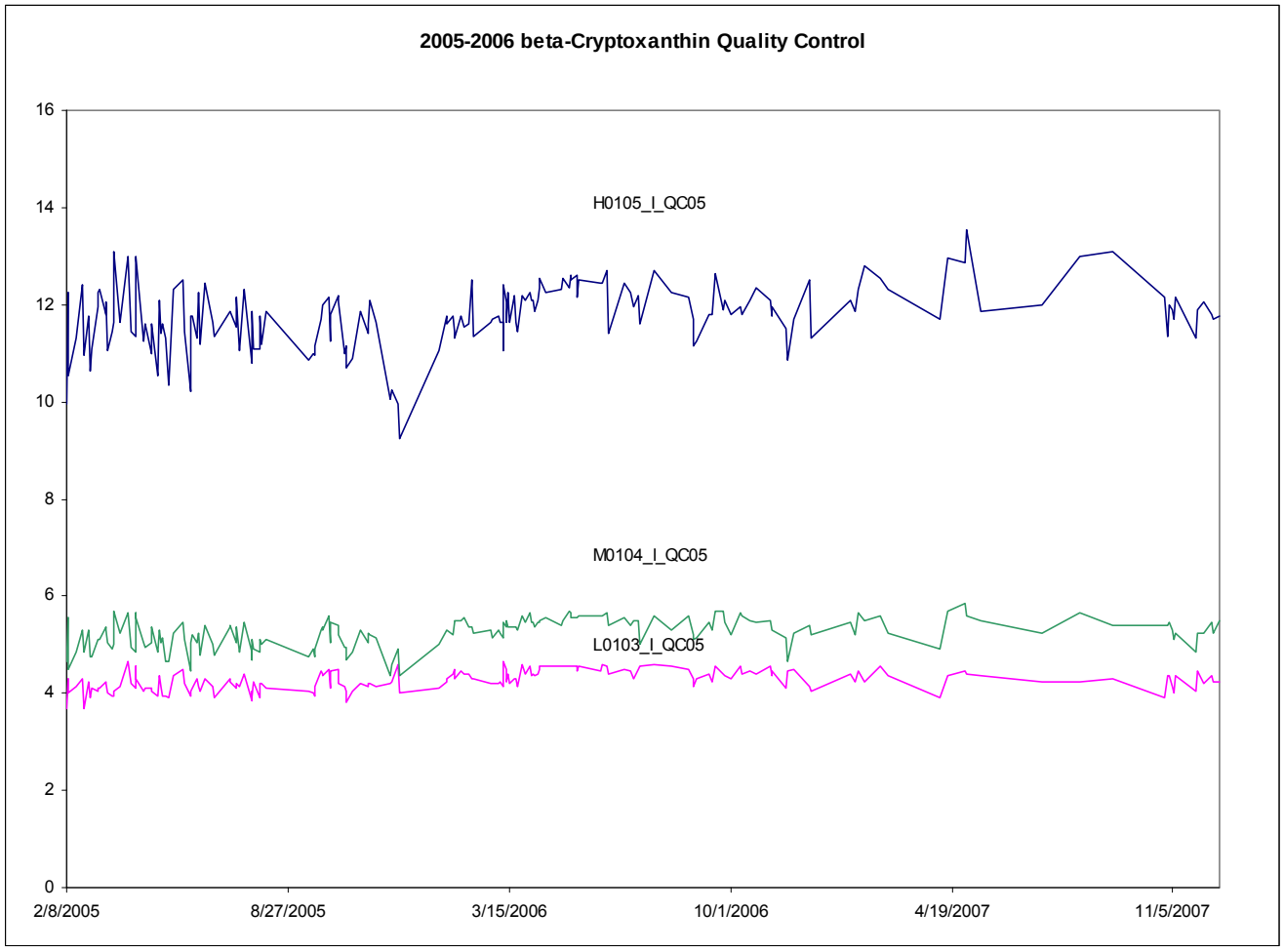
| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| L0103_I_QC05 | 180 | 2/8/2005 | 1/23/2008 | 0.95 | 0.09 | 9.4 |
| H0105_I_QC05 | 180 | 2/8/2005 | 1/23/2008 | 1.80 | 0.11 | 6.0 |
| M0104_I_QC05 | 168 | 2/9/2005 | 1/23/2008 | 0.46 | 0.08 | 17.6 |



D. Beta-cryptoxanthin

**Summary Statistics for beta-Cryptoxanthin by Lot**

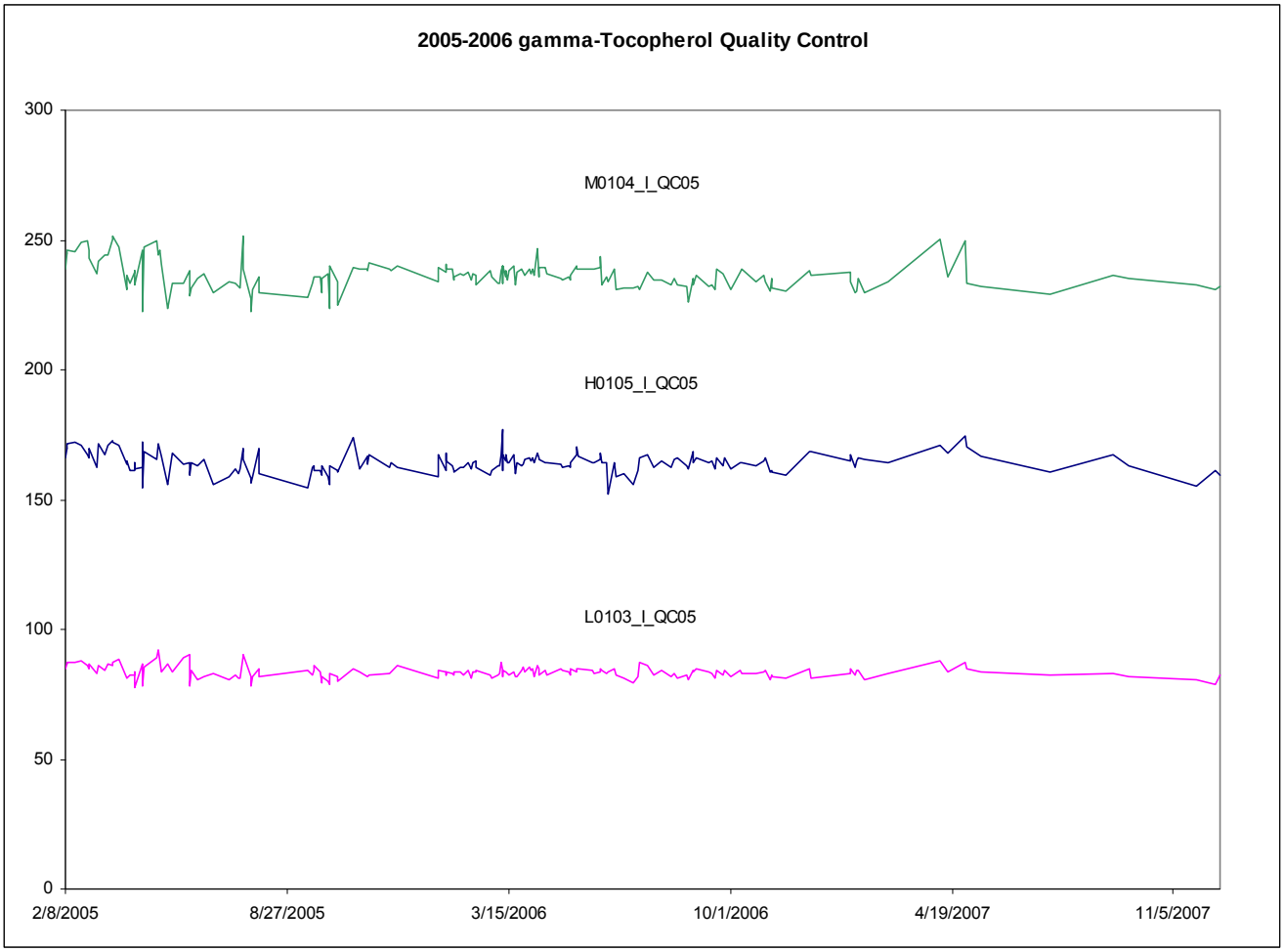
| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| L0103_I_QC05 | 187 | 2/8/2005 | 12/17/2007 | 4.27 | 0.20 | 4.8 |
| M0104_I_QC05 | 187 | 2/8/2005 | 12/17/2007 | 5.24 | 0.30 | 5.7 |
| H0105_I_QC05 | 187 | 2/8/2005 | 12/17/2007 | 11.77 | 0.65 | 5.5 |



E. Gamma tocopherol

**Summary Statistics for gamma-Tocopherol by Lot**

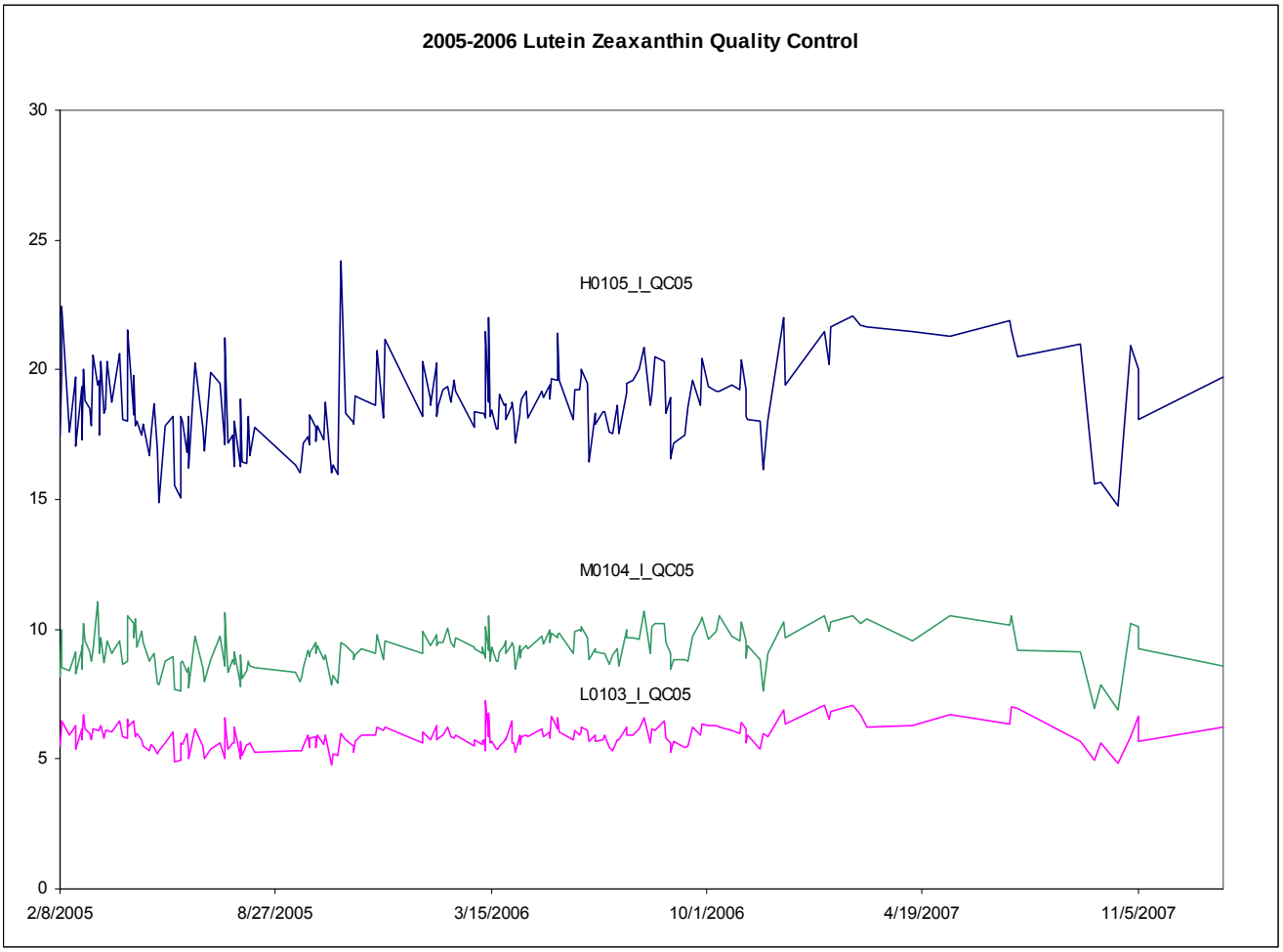
| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| L0103_I_QC05 | 180 | 2/8/2005 | 12/17/2007 | 83.56 | 2.39 | 2.9 |
| H0105_I_QC05 | 180 | 2/8/2005 | 12/17/2007 | 164.22 | 4.08 | 2.5 |
| M0104_I_QC05 | 180 | 2/8/2005 | 12/17/2007 | 236.4 | 5.54 | 2.3 |



F. Lutein-Zeaxanthin

**Summary Statistics for Lutein Zeaxanthin by Lot**

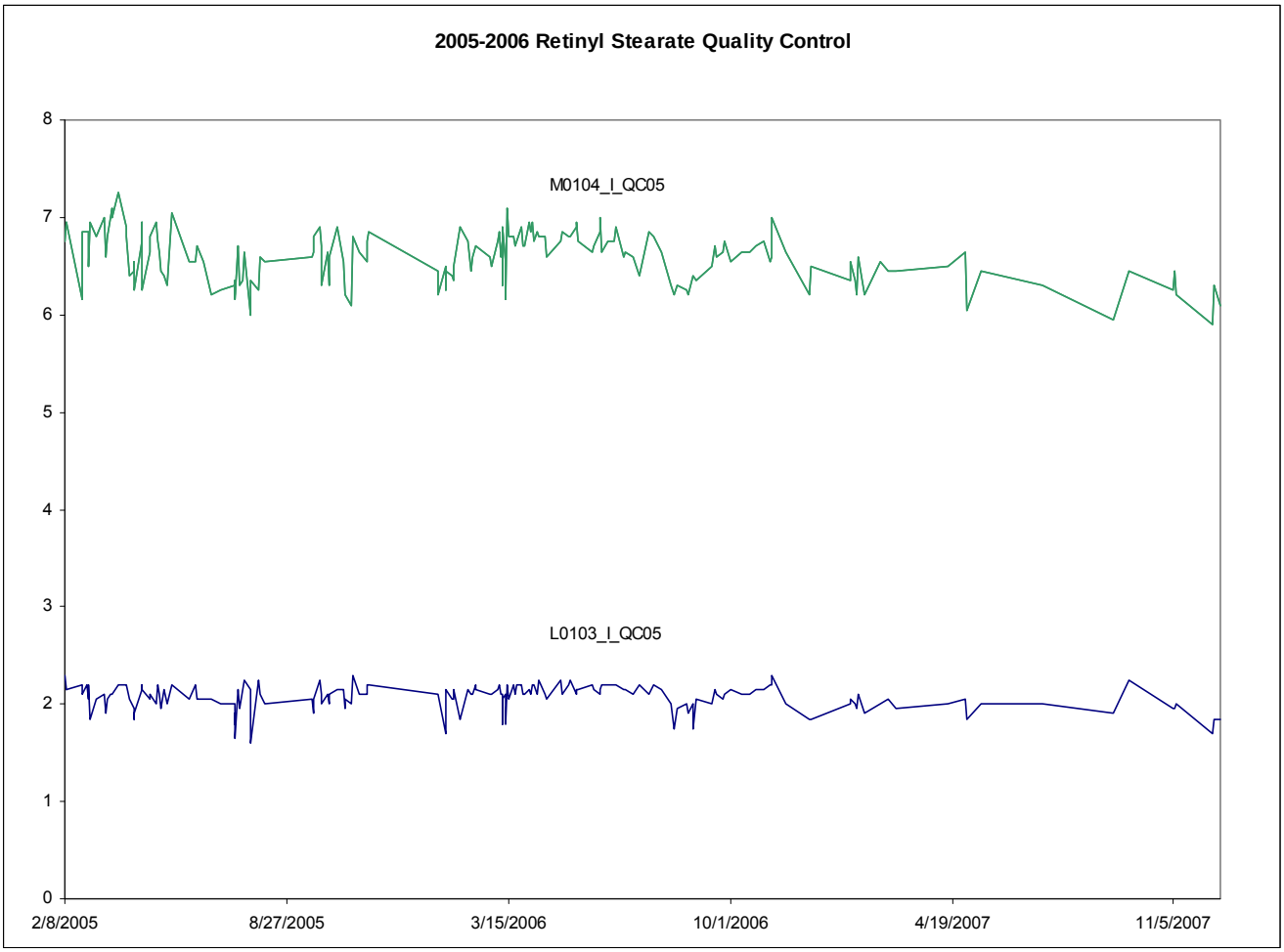
| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| L0103_I_QC05 | 205 | 2/8/2005 | 1/23/2008 | 5.88 | 0.46 | 7.9 |
| M0104_I_QC05 | 205 | 2/8/2005 | 1/23/2008 | 9.23 | 0.73 | 7.9 |
| H0105_I_QC05 | 205 | 2/8/2005 | 1/23/2008 | 18.67 | 1.57 | 8.4 |



G. Retinyl stearate

**Summary Statistics for Retinyl Stearate by Lot**

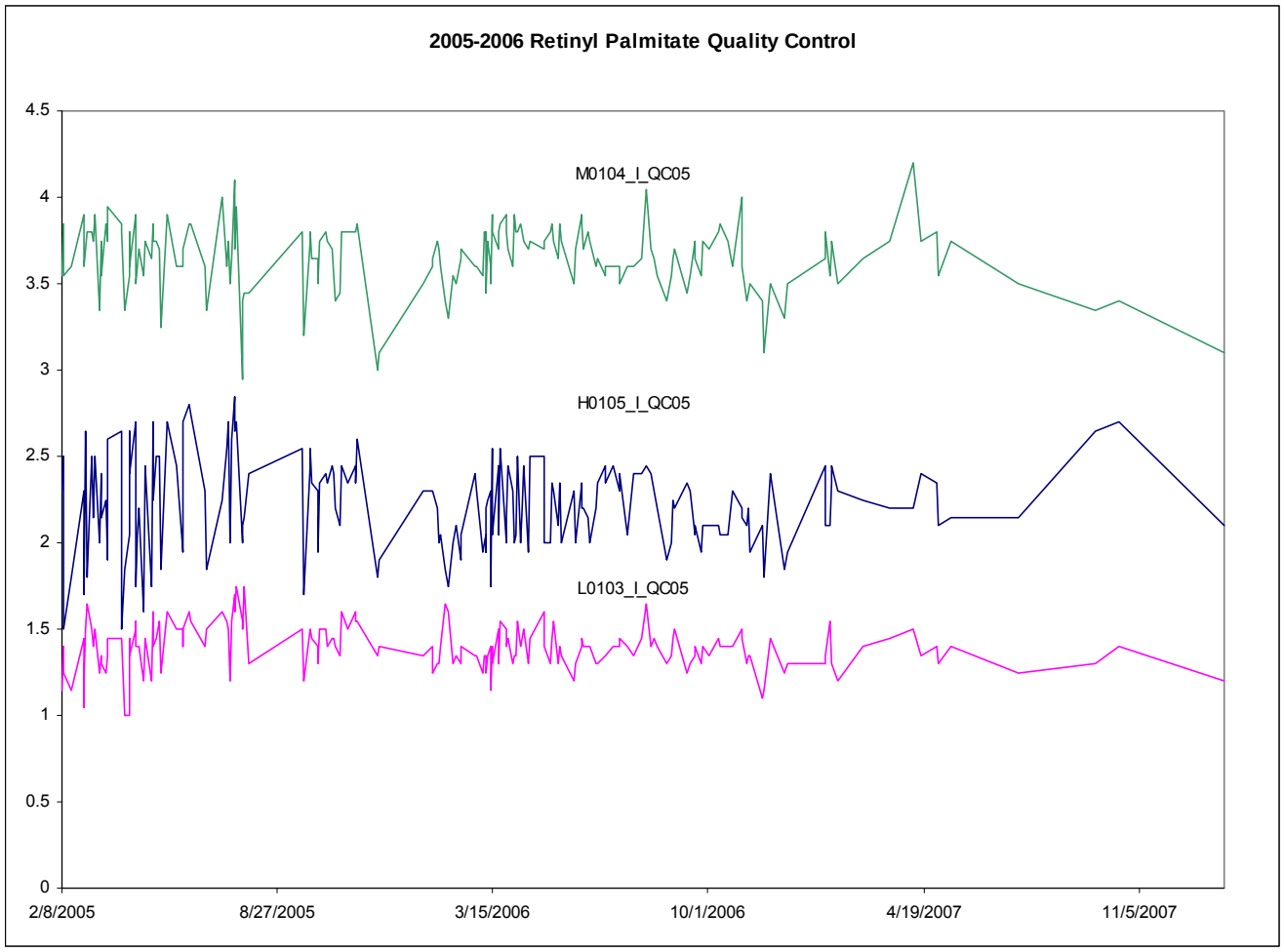
| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| L0103_I_QC05 | 180 | 2/8/2005 | 12/17/2007 | 2.07 | 0.13 | 6.3 |
| M0104_I_QC05 | 180 | 2/8/2005 | 12/17/2007 | 6.59 | 0.26 | 4.0 |



H. Retinyl palmitate

**Summary Statistics for Retinyl Palmitate by Lot**

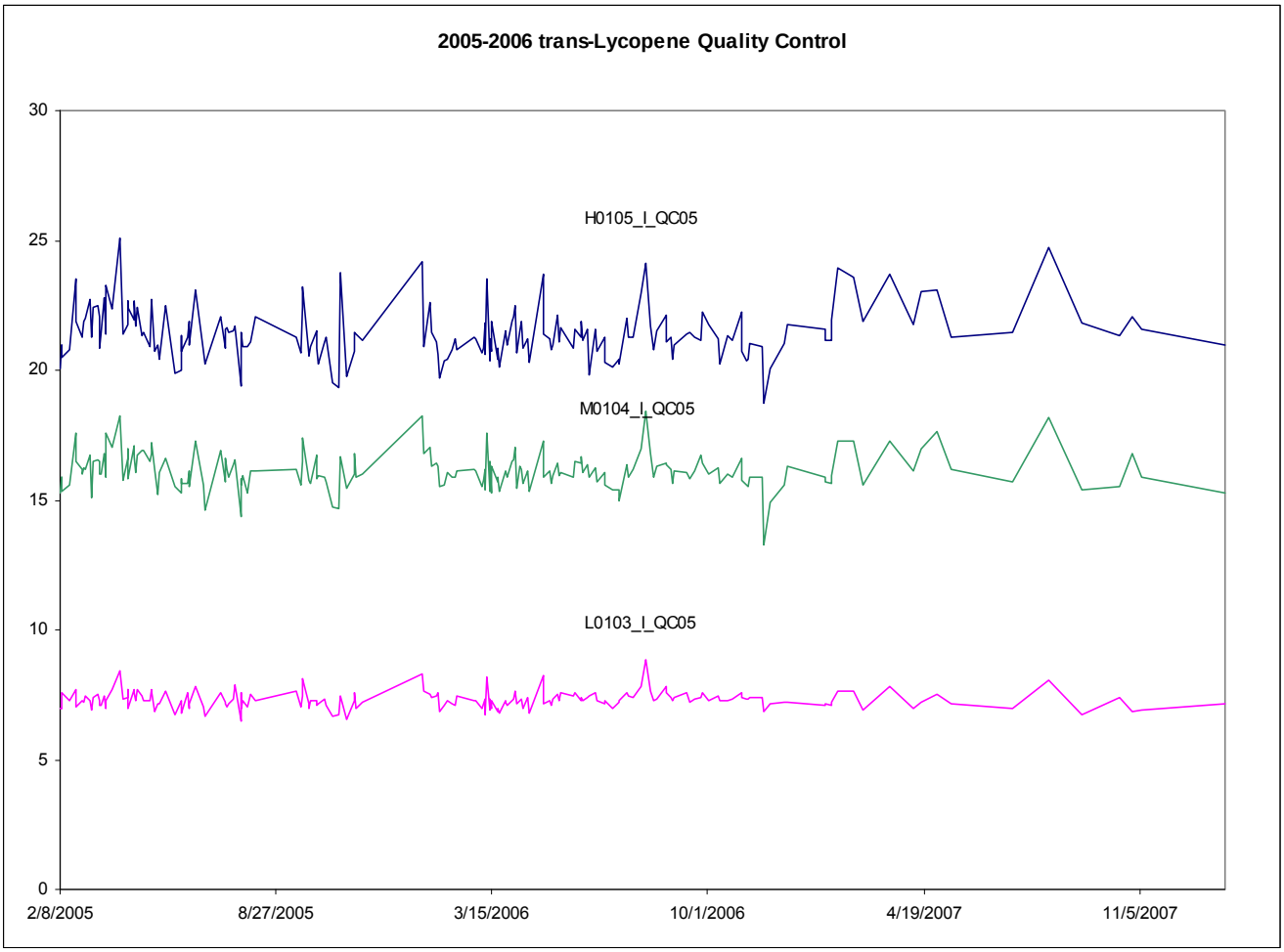
| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| L0103_I_QC05 | 189 | 2/8/2005 | 1/23/2008 | 1.40 | 0.13 | 9.2 |
| H0105_I_QC05 | 189 | 2/8/2005 | 1/23/2008 | 2.23 | 0.27 | 12.0 |
| M0104_I_QC05 | 189 | 2/8/2005 | 1/23/2008 | 3.66 | 0.19 | 5.3 |



I. trans-Lycopene

**Summary Statistics for trans-Lycopene by Lot**

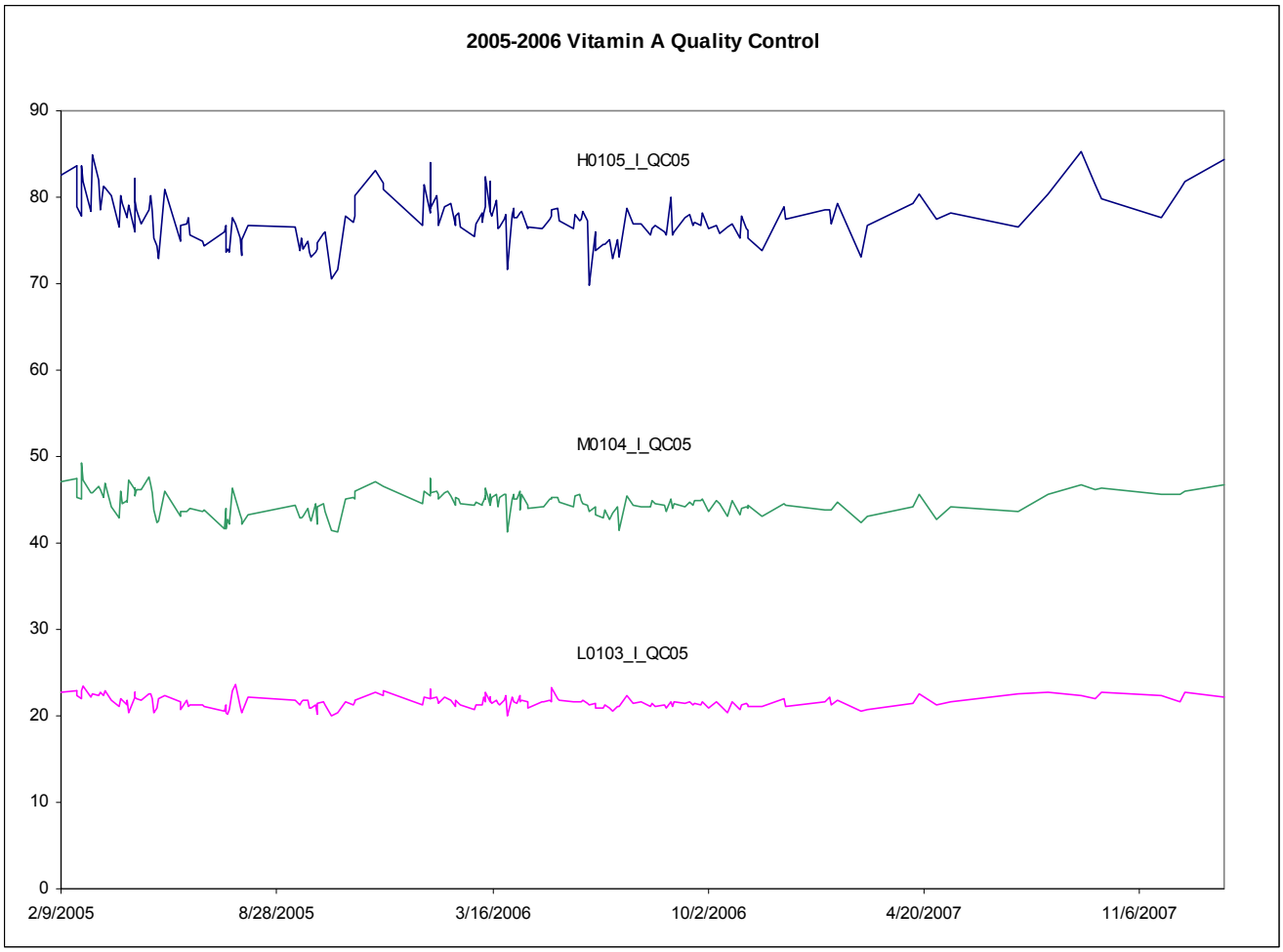
| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| L0103_I_QC05 | 195 | 2/8/2005 | 1/23/2008 | 7.3 | 0.33 | 4.5 |
| M0104_I_QC05 | 195 | 2/8/2005 | 1/23/2008 | 16.12 | 0.70 | 4.3 |
| H0105_I_QC05 | 195 | 2/8/2005 | 1/23/2008 | 21.43 | 1.00 | 4.7 |



J. Vitamin A

**Summary Statistics for Vitamin A by Lot**

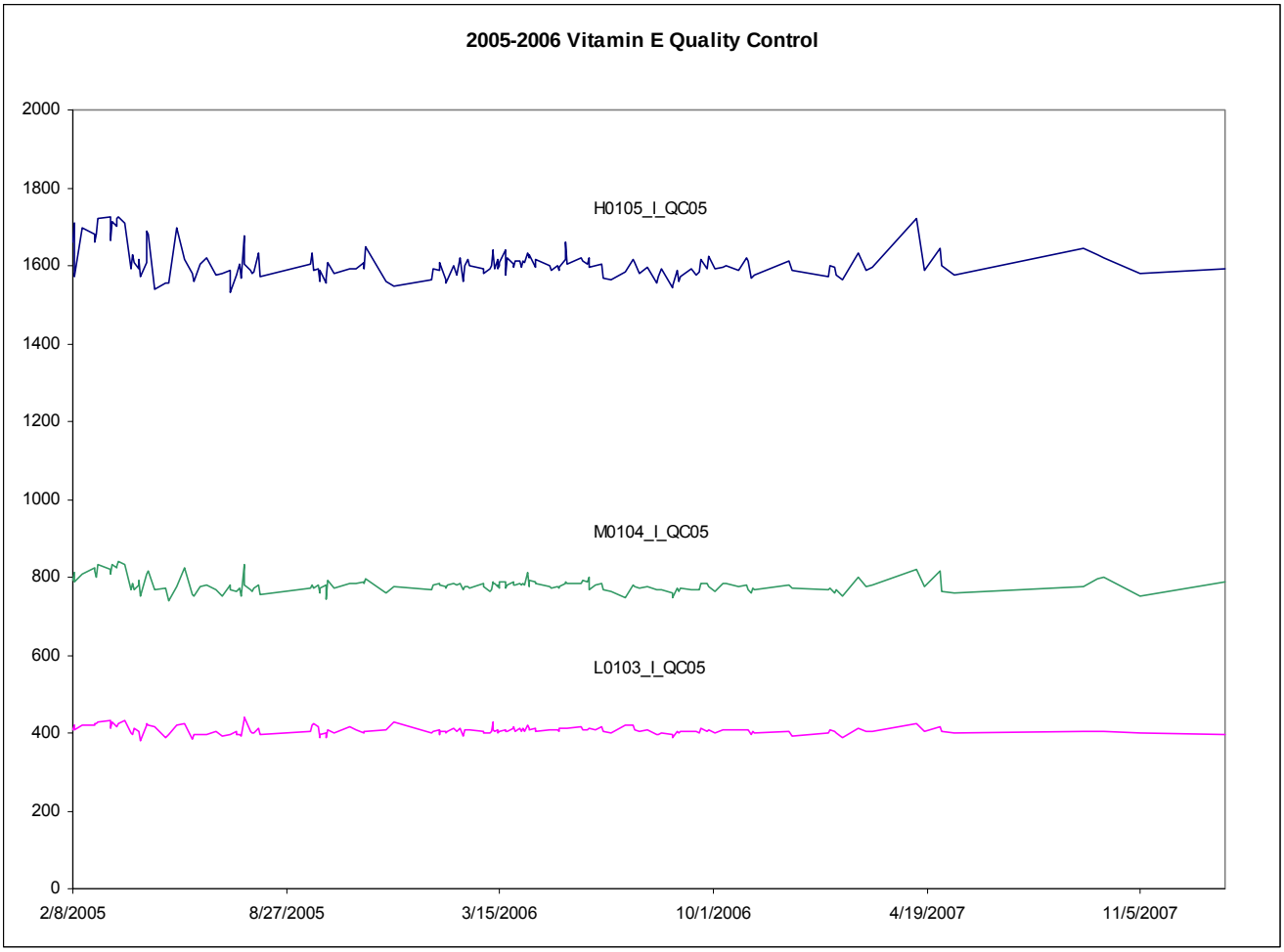
| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| L0103_I_QC05 | 183 | 2/9/2005 | 1/23/2008 | 21.64 | 0.68 | 3.1 |
| M0104_I_QC05 | 183 | 2/9/2005 | 1/23/2008 | 44.66 | 1.36 | 3.0 |
| H0105_I_QC05 | 183 | 2/9/2005 | 1/23/2008 | 77.48 | 2.67 | 3.5 |



K. Vitamin E (α-ocopherol)

**Summary Statistics for Vitamin E by Lot**

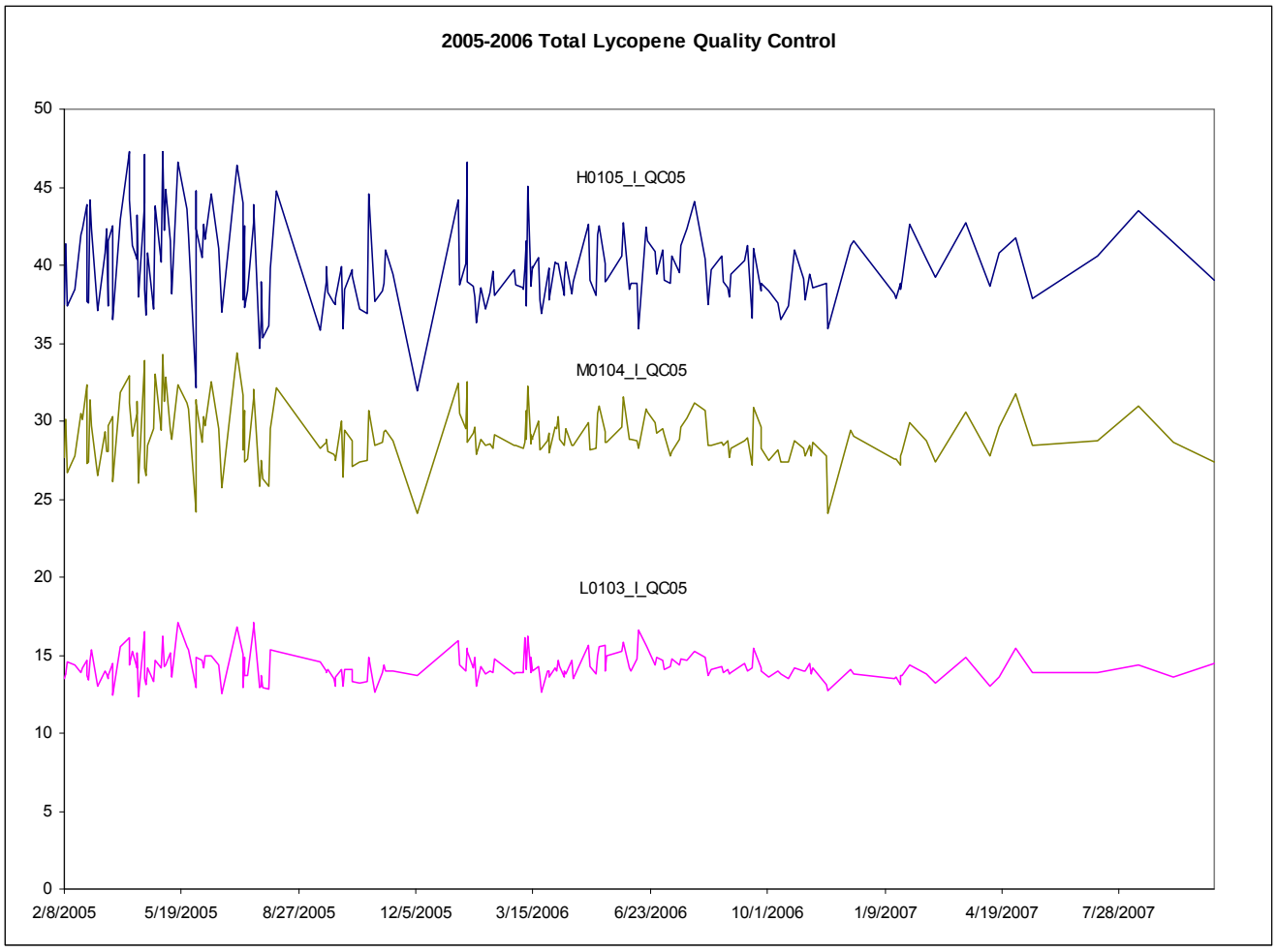
| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| L0103_I_QC05 | 171 | 2/8/2005 | 1/23/2008 | 406.98 | 10.06 | 2.5 |
| M0104_I_QC05 | 171 | 2/8/2005 | 1/23/2008 | 780.28 | 18.65 | 2.4 |
| H0105_I_QC05 | 171 | 2/8/2005 | 1/23/2008 | 1605.12 | 40.00 | 2.5 |



L. Total lycopene

**Summary Statistics for Total Lycopene by Lot**

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| L0103_I_QC05 | 197 | 2/8/2005 | 10/17/2007 | 14.252 | 0.900 | 6.3 |
| M0104_I_QC05 | 197 | 2/8/2005 | 10/17/2007 | 29.085 | 1.777 | 6.1 |
| H0105_I_QC05 | 197 | 2/8/2005 | 10/17/2007 | 39.909 | 2.651 | 6.6 |



REFERENCES

1. Sowell AL, Huff DL, Yeager PR, Caudill SP, and Gunter EW. Retinol, α-tocopherol, lutein/zeaxanthin, β-cryptoxanthin, lycopene, β-carotene, trans-β-carotene, and four retinyl