Laboratory Procedure Manual

Analyte: **Fat Soluble Micronutrients (Vitamins A, E and Carotenoids)**

Matrix: **Serum**

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

Method No.:

Revised:

as performed by: Craft Technologies, Inc.
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Important Information for Users

Craft Technologies 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
l45vit_c	LBXATC	α-Tocopherol(µg/dL)
	LBDATCSI	α -Tocopherol(µmol/L)
	LBXALC	α-Carotene(µg/dL)
	LBDALCSI	α-Carotene(µmol/L)
	LBXACY	α -Cryptoxanthin(µg/dL)
	LBDACYSI	α -Cryptoxanthin(mol/L)
	LBXBEC	trans-β-carotene((µg/dL)
	LBDBECSI	trans-β-carotene(µmol/L)
	LBXBCC	Combined β-Carotene(µg/dL)
	LBDBCCSI	Combined β-Carotene(µmol/L)
	LBXCBC	cis-β-carotene(µg/dL)
	LBDCBCSI	cis-β-carotene(µmol/L)
	LBXCLC	cis-Lycopene(µg/dL)
	LBDCLCSI	cis-Lycopene(µmol/L)
	LBXCLZ	cis- Lutein/Zeaxanthin(ug/dL)
	LBDCLZSI	cis- Lutein/Zeaxanthin(umol/L)
	LBXCRY	β-cryptoxanthin(µg/dL)
	LBDCRYSI	β-cryptoxanthin(µg/dL)
	LBXDTC	δ -Tocopherol(µg/dL)

	LBDDTCSI	δ -Tocopherol(µmol/L
	LBXGTC	Gamma tocopherol (µg/dL)
	LBDGTCSI	Gamma tocopherol (µmol/L)
	LBXLCC	Combined Lycopene(µg/dL)
	LBDLCCSI	Combined Lycopene(µmol/L)
	LBXLUT	Lutein(µg/dL)
	LBDLUTSI	Lutein(µmol/L
	LBXLUZ	Combined Lutein/zeaxanthin(µg/dL)
	LBDLUZSI	Combined Lutein/zeaxanthin(µmol/L)
	LBXLYC	trans-lycopene(µg/dL)
	LBDLYCSI	trans-lycopene(µmol/L)
	LBXPHF	Phytofluene(µg/dL)
	LBDPHFSI	Phytofluene(µmol/L)
	LBXPHE	Phytoene(µg/dL)
	LBDPHESI	Phytoene(µmol/L)
	LBXRPL	Retinyl palmitate(ug/dL)
	LBDRPLSI	Retinyl palmitate (µmol/L)
	LBXRST	Retinyl stearate (µg/dL)
	LBDRSTSI	Retinyl stearate (µmol/L)
	LBXVIA	Retinol (µg/dL)
	LBDVIASI	Retinol (µmol/L)
	LBXZEA	Zeaxanthin(µg/dL)
	LBDZEASI	Zeaxanthin(µmol/L)

1. Summary of Test Principle and Clinical Relevance

Serum concentrations of vitamin A (retinol, Retinyl palmitate, Retinyl stearate), vitamin E (α-, δ-, and γ-tocopherol), and eleven carotenoids (lutein, zeaxanthin, α-cryptoxanthin, β-cryptoxanthin, *Trans* lycopene, *cis* lycopene, α-carotene, *Trans* β-carotene, *cis* β-carotene; also phytoene and phytofluene) are measured using high performance liquid chromatography with multi wavelength photodiode-array absorbance detection. A small volume (150 μL) of serum/plasma is mixed with an equal volume of buffer, and then mixed with 2 volumes of ethanol containing the internal standard (tocol). The analytes are extracted from the aqueous phase into hexane. The combined hexane extracts are dried under vacuum. The extract is re-dissolved in ethyl acetate and diluted in mobile phase. An aliquot is injected onto a C18 reversed phase column and eluted isocratically. The analytes all possess absorbance and/or fluorescence which are proportional to their concentration in solution; therefore these properties are used for quantitative analysis. The mode of detection is chosen to provide the highest sensitivity and selectivity. Carotenoids are measured by absorbance at 450 nm. Retinol, retinyl esters, phytoene and phytofluene are measured by UV absorbance near their absorption maxima of 325 nm, 280 nm and 340 nm. Tocopherols have absorption maxima between 292 and 300 nm. Chromatograms are recorded using a computer data system. Analytes are quantified by external standard quantitation using neat standards to calculate response factors based on the peak area of the analyte. The quantities of analytes are corrected for recovery post-run based upon tocol as an internal standard.

2. Hazards for the Procedures and Safety Precautions for Handling and Disposal of Hazardous Materials. Protective Clothing and Safety Requirements.

The CDC guidelines for handling of serum should be obeyed (Guidelines for Prevention of Transmission of Human Immunodeficiency Virus and Hepatitis B. *MMWR* 1988; 37 (suppl. 4):1-22).

All serum samples received for analysis should be considered potentially positive for infectious agents including HIV and hepatitis B viruses. Observe universal precautions including the use of gloves, lab coat, and protective eyewear while handling all human blood products. The hepatitis B vaccine series is recommended and is offered to all analysts working in the laboratory. Sample handling should be performed in a hood. Disposable plastic, glass, latex and paper items (pipette tips, gloves, etc.) that contact biological samples should be placed in biohazard disposal. All work surfaces should be wiped down with 0.1% benzalkonium chloride solution (Zephiran) when work is finished and any disposable absorbent materials used to cover work surfaces should be discarded. Organic solvents should be handled in a well-ventilated area or, as required, in a chemical fume hood.

All reagents and solvents used in this study are listed in Section 6e. Material Safety Data Sheets (MSDS) for these chemicals are readily accessible as hard copies in the CTI laboratory library. If needed, MSDS for other chemicals can be obtained on the Internet.

3. Computerization; Data System Management

A. When the sample data have been integrated a text file (designated by the PC1000 software as a 'Trend Report') is transferred to another PC for processing. The file is in a comma-delimited format (.CSV) that includes significant amounts of text that must be stripped out prior to importing into a spreadsheet.

B. The CSV file is parsed using a REXX program.

1. The program makes the following calculations:

a. Correction based upon Tocol concentration

b. Molecular weight of retinyl palmitate and retinyl stearate

2. The program looks for results that are equal to or less than the minimum detection limit for the analyte. It automatically "corrects" the output result to that LOD and then inserts a '37' in the comment field.

C. The output file is imported into a Lotus 1-2-3 spreadsheet for examination. If the data is approved the output file is imported into a Lotus Approach database application which merges the data with a previously downloaded file from the Westat lab. The newly created database record is exported in the approved format to Westat.

.D. Data are backed up to tape each evening. Once per month all data are copied to a CD.

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

Craft Technologies, Inc. is not involved in specimen collection. Samples are received from each NHANES stand on a weekly basis.

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 ultraviolet radiation.

B. Specimens for fat soluble vitamin analysis may be fresh or frozen. Serum should be harvested from blood collected in red-top or royal blue-top Vacutainer brand or equivalent tubes by standard venipuncture procedures. Hemolysis may interfere or cause low values for these analytes.

C. A 0.5-mL sample of serum is preferable, but a minimum sample volume of 150 μL is required for analysis.

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

E. Specimens collected in the field are frozen, and then are shipped on dry ice by overnight mail. Frozen serum is stored at -70 °C. Retinol and a-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 that have been through more than five freeze-thaw cycles, that have been refrigerated for more than 24 hours, or that 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 not informative.

H. Specimen handling conditions are outlined in the NHANES protocol for whole blood collection and handling (National Health and Nutrition Examination Survey III, Cycle 2: Manual for Medical Technicians, 1992). Collection, transport, and special equipment are discussed there. In general, serum should be transported and stored at no more than -20 °C. Samples thawed and refrozen fewer than five times are not considered compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of serum will have been transferred into a sterile Nalge cryovial labeled with the participant's ID at the time of collection.

I. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking:

CTI assigns Track #'s to each shipment of sample that arrives. The number and type of sample, analysis requested and freezer location are recorded on the sheet. All information on the track sheets is recorded in computer files and archived on the network. Records, including related QA/QC data, will be maintained for up to 10 years after completion of the NHANES study. Only numerical identifiers will be used (e.g., case ID numbers). Residual serum from the NHANES samples will be maintained at −70ºC for 1 year and will be available to CDC after NHANES analyses are completed.

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: Acetonitrile (ACN, HPLC grade), methanol (MeOH, HPLC grade), isopropanol (IPA, HPLC grade), p-dioxane (stabilized with 200 ppm BHT upon opening) are obtained from a scientific distributor. For each liter of mobile phase, 60 mL MeOH and 60 mL IPA are mixed. Ammonium acetate (0.926 g) is added to provide 100 mM. To a 1 L beaker add 792 mL ACN and 148 mL dioxane. Slowly, with stirring, add 60 mL of the alcohol mixture. Add 1 mL triethylamine (TEA, 98% min) to yield 0.1 %. Filter through 0.45 μm membrane (PVDF, Pall Gelman).

2. 2% Ascorbic Acid, 4% NaCl, and EDTA: L-Ascorbic Acid (ACS Certified), 2.0 g, 4.0 g NaCl (Analytical Grade), and 1 mg EDTA are dissolved in a small amount of deionized water in a 100-mL graduated cylinder. The volume is brought up to 100 mL by addition of water. The solution can normally can be used for 2 weeks or until a noticeable yellow color appears. Store the solution at room temperature blanketed under nitrogen, capped, and in the dark.

B. Standards Preparation

1. Stock Solutions. Stock solutions of all standards, except retinyl stearate, α-cryptoxanthin, phytoene and phytofluene, are prepared by dissolving a small amount of neat standard in solvent then filtering through 0.45 μm syringe filters. The stock is diluted to appropriate levels, measured spectrophotometrically, then corrected for purity as determined by HPLC. Solutions should be >90% pure by HPLC.

   For retinol, lutein, zeaxanthin, β-cryptoxanthin, tocol, δ-tocopherol, γ-tocopherol, and α-tocopherol, a small amount (<1 mg) is dissolved individually in ~50 mL reagent alcohol. The absorbance of the solution is measured using a UV/visible spectrophotometer. The concentrations are calculated based on the extinction coefficients (EC) listed in Table 1. The stock solutions are diluted with mobile phase and the purity determined by injecting on the HPLC while monitoring the wavelength maximum of the analyte (Table 1). Solutions should be >90% pure by HPLC. Note: γ-tocopherol must be chromatographically purified to remove δ-tocopherol or corrected mathematically for its presence.

   For lycopene, α-carotene, β-carotene, and retinyl palmitate, a small amount (<1 mg) is dissolved individually in ~50 mL of 10% tetrahydrofuran in reagent alcohol. The absorbance of the solution is measured using a UV/visible spectrophotometer. The concentrations are calculated based on the extinction coefficients (EC) listed in Table The stock solutions are diluted with mobile phase and the purity determined by injecting on the HPLC while monitoring the wavelength maximum of the analyte (Table 1). Solutions should be >90% pure by HPLC.

   For analytes which are not commercially available as neat standards, CTI either synthesizes them or isolates them from a rich, natural source (e.g., phytoene and

phytofluene from tomato paste). An extract is separated by HPLC and the central third of the peak ("middle cut") is collected. Pooled middle cuts from several injections are diluted to 10 mL with ethanol. Alternatively, compounds can be isolated by thin-layer chromatography. The absorbance of the solution is measured with the spectrophotometer and the solution is diluted with reagent alcohol to obtain the correct stock concentration.

Retinyl esters (e.g., retinyl stearate) are synthesized by reacting retinol with fatty acid anhydrides using the following procedure: To 0.87 mmol of retinol in a 50-mL round bottom flask add 3 mL of TEA, 20 mL of n-hexane and 1.00 mmol of the appropriate acid anhydride. The reaction mixture is stirred for 3.5 hours at 60 °C, cooled, and then the hexane and TEA are removed under vacuum. The residue is purified by HPLC on a diol column, and the retinyl esters are isolated as colorless material with greenish-yellow fluorescence.

Table 1. Extinction Coefficients Used to Calculate Concentrations of Standard Stock Solutions

Analyte	MW	Extinction Coefficient* (dL / (g •cm)	CTI Analysis Wavelength (nm)	Target Concentration of Stock Solutions
Retinol	286.5	1850 (NHANES/CTI)	325	15 μg/mL
Retinyl Palmitate	524.9	1850 (NHANES)	325	
		975 (CTI)	325	6 μg/mL
Retinyl Stearate	552.9	1850 (NHANES)	325	
		975 (CTI)	325	6 μg/mL
δ-Tocopherol	402.7	91.2 (CTI)	290	25 μg/mL
γ-Tocopherol	416.7	91.4 (NHANES/CTI)	290	100 μg/mL
α-Tocopherol	430.7	75.8 (NHANES/CTI)	290	250 μg/mL
Lutein	568.9	2550 (NHANES/CTI)	450	8 μg/mL
Zeaxanthin	568.9	2540 (NHANES/CTI)	450	6 μg/mL
α-Cryptoxanthin	552.9	2520 (CTI)	450	6 μg/mL
β-Cryptoxanthin	552.9	2350 (CTI)	450	6 μg/mL
		2370 (NHANES)		
Lycopene	536.9	3450 (NHANES/CTI)	450	8 μg/mL
α-Carotene	536.9	2800 (CTI)	450	6 μg/mL
		2725 (NHANES)		

β-Carotene	536.9	2560 (NHANES/CTI)	450	12 μg/mL
Phytoene	544.9	915 (CTI)	290	6 μg/mL
Phytofluene	542.9	1577 (CTI)	325	6 μg/mL
Tocol (internal standard)	388.6	90 (CTI)	290	

A $^{1\%}_{1cm}$ is defined as the theoretical absorbance of a 1 % solution (1g/100 mL) in a cell of 1 cm path length. If extinction coefficients in Table 1 are different from those used in previous NHANES studies, data will be mathematically adjusted to match using formulas within Excel. For instance, CTI uses 2350 for β-cryptoxanthin and NHANES used 2370. We will multiply our value by 2350 and divide by 2370 to obtain the adjusted value. For the NHANES samples, concentrations of retinyl esters will be adjusted and reported as retinol equivalents to be consistent with previous NHANES studies.

Mixed Standards (Calibration Solutions): Mixed standards are prepared in four sets in order to avoid combining very similar analytes that may be contaminated by one another (e.g., lutein with zeaxanthin).

The Vitamin A mixed standard level 3 is prepared by mixing 560 μL stock tocol solution with 550 μL of retinol, 520 μL of retinyl palmitate and 440 μL of retinyl stearate plus 250 μL of zeaxanthin stock solutions then diluting to 5000μL with a 50:50 mix of ethanol:acetonitrile.

The vitamin E mixed standard level 3 is prepared by mixing 560 μL of stock tocol solution with 500 μL of α-tocopherol, γ-tocopherol, and δ-tocopherol stock solutions then diluting to 5000 μL with 50:50 ethanol: acetonitrile.

The Carotenoid mixed standard A level 3 is prepared by mixing 560 μL of stock tocol solution with 500 μL of lutein, β-cryptoxanthin, lycopene, 250 μL of α-carotene, and 1.00 mL of phytoene stock solutions then diluting to 5000 μL with 50:50 ethanol:acetonitrile. The Carotenoid mixed standard B level 3 is prepared by mixing 560 μL of stock tocol solution with 500 μL of α-cryptoxanthin, 700 μL of β-carotene, and 960 μL of phytofluene stock solutions then diluting to 5000 μL with 50:50 ethanol:acetonitrile.

Mixed standard solutions are stored in glass vials under nitrogen at -20 °C and are stable for at least eight weeks.

To obtain mixed standard level 2, 1000 μL of level 3 is diluted to 5000 μL with 50:50 ethanol: acetonitrile containing a 1:20 dilution of stock tocol solution.

To obtain mixed standard level 1, 1000 μL of level 2 is diluted to 4000 μL with 50:50 ethanol: acetonitrile containing a 1:20 dilution of stock tocol solution.

C. Preparation of Quality Control (QC) Materials

All pools are filtered through cheesecloth, followed by glass wool and then Lifeguard borosilicate glass micro fiber prefiiters, followed by Polysep borosilicate glass micro fiber filters (nominal 0.2-um pore size). The filtered serum is aliquoted into 2.0-mL cryovials, blanketed with nitrogen, and capped. The QC pools vials are stored in zip-lock plastic freezer bags at -70 °C.

The low QC pool is prepared by selecting and pooling sera that contain low levels of all analytes (all analytes below the mean concentrations in the US population).

The medium QC pool is prepared by pooling sera that contain most of the analytes at levels close to the mean levels observed in the normal US population.

The high QC pool is prepared by pooling sera that contain higher than normal levels of most analytes. In some instances, dog serum, which typically has a high retinyl ester concentration, is added to the high pool. In addition, analytes in ethanol solution are spiked into a bovine l ipoprotein solution, allowed to mix overnight, and then added to a serum pool.

Other types of subjects useful for blending into the high pool are Type II diabetics who, in the absence of good glycemic control, may have high concentrations of lutein, zeaxanthin, β-cryptoxanthin, and γ-tocopherol.

D. Instrumentation

1. ThermoSeparation HPLC System (ThermoSeparation Products, CA)

   a. Model SCM 1000 solvent conditioning module (degasser)

   b. Model P 1000 isocratic pump

   c. Model AS 3000 autosampler with vial and column temperature control

   d. Model UV6000 LP photodiode-array UV/visible detector

   e. Model SN 4000 data interface

   f. Pentium computer with TSP PC 1000 data system

   g. VWR multi-tube vortex mixer

   h. Cary 1 spectrophotometer (Varian Instruments, Palo Alto, CA)

   i. Speedvac SC200H System (Savant Instrument Co., Farmingdale, NY)

   j. Precision Scientific Model VP-190 Direct Drive Vacuum Pump (Precision Scientific Inc., Chicago, IL)

k. Refrigerated vapor trap, model RT-490 (Savant Instrument Co., Farmingdale, NY)

l. Magnetic stirrer (American Scientific Products)

m. Barnstead Thermolyne Repipettor, to deliver 1.0 mL

n. Gilson Microman pipet, 1000 μL positive displacement (Woburn, MA)

o. Gilson Microman pipet, 250 μL positive displacement (Woburn, MA)

p. Branson 2210 Ultrasonic Cleaner, (Terre Haute, IN)
q. Gas tight syringe, 100 μL (Hamilton, Reno, NV)

r. Genie vortex mixer(Redmond, WASH)

E. Materials

1. 25-cm x 4.0-mm Spherisorb ODS2, 3-μm HPLC column with Ti frits (ES Industries, West Berlin, NJ)

2. Phenomenex C18 ODS guard column, (Torrance, CA)

3. Hexane UV (EM Science, Chicago, Ill)

4. Acetonitrile, non-UV grade (EM Science, Chicago, Ill)

5. Reagent alcohol, (EM Science, Chicago, Ill)

6. p-Dioxane, grade (EM Science, Chicago, Ill)
7. Ethyl acetate, (EM Science, Chicago, Ill)

8. Triethylamine, 98% minimum (Fisher Scientific, Allentown, PA or VWR Scientific, WestChester, PA)

9. Retinol (Sigma Chemical Co., St. Louis, MO)

10. Retinyl Palmitate (Sigma-Aldrich, St. Louis, Missouri)

11. α-Tocopherol (Eastman Chemical Co., Kingsport, TN)

12. γ-Tocopherol (Cognis, Tokyo, Japan)

13. δ-Tocopherol (Cognis Tokyo, Japan)

14. Lutein (Kemin Industries, Des Moines, IA)

15. Zeaxanthin (Hoffmann la Roche, Basel Switzerland)

16. β-Cryptoxanthin (Hoffmann La Roche, Nutley, NJ)
17. Lycopene.(Sigma-Aldrich, St. Louis, Missouri)
18. α-Carotene (Sigma-Aldrich, St. Louis, Missouri)
19. β-Carotene (Sigma-Aldrich, St. Louis, Missouri)
20. L-Ascorbic acid, ACS grade (Sigma-Aldrich, St. Louis, Missouri)
21. EDTA (Sigma-Aldrich, St. Louis, Missouri)
22. Stearic anhydride (Sigma-Aldrich, St. Louis, Missouri)
23. Alumina, Grade III (obtained from various sources)
24. Nitrogen (Air Products, Inc., Wilson , NC)
25. 10- x 75-mm disposable glass culture tubes (Corning Glassworks, Corning, NY)
26. 12- x 75-mm disposable glass culture tubes (Corning Glassworks, Corning, NY)
27. (Xxvii). Pipette tips for Gilson pipets (Gilson, Lewis Center, Ohio)
28. Tetrahydrofuran, stabilized with BHT (EM Science, Chicago, Ill)
29. Solvent filters, 0.45-um pore size (Pall Gelman)
30. Autosampler vials 12- x 31-mm (National Scientific, Duluth GA), 250-uL glass inserts, and screw caps with teflon/silicone septa (National Scientific, Rockwood, TN)
31. Actinic glassware (various sources)
32. Nunc cryovials (Nalge Nunc, Inc., Rochester, NY)
33. Tocol (Matreya, Pleasant Gap, PA)
34. Butylated hydroxytoluene (Sigma-Aldrich, St. Louis, Missouri)
35. Isopropyl alcohol (EM Science, Chicago, Ill)
36. Methanol (EM Science, Chicago, Ill)

7. Calibration and Calibration Verification Procedures

At the beginning and end of each run, one each of the level 1, 2 and 3 calibration solution is injected. The values for sentinel analytes (retinol, alpha-tocopherol, and beta-carotene) must agree within 15 % of the calibrator values.

Table 2. Concentrations of Calibration Solutions

Analyte	Level 1 μg / mL	Level 2 μg / mL	Level 3 μg / mL
Retinol	0.075	0.3	1.5
Retinyl Palmitate	0.03	0.12	0.6
Retinyl Stearate	0.03	0.12	0.6
α-Tocopherol	1.25	5.0	25.0
γ-Tocopherol	0.5	2.0	10.0
δ-Tocopherol	0.125	0.5	2.5
Lutein	0.04	0.16	0.8
Zeaxanthin	0.03	0.12	0.6
β-Cryptoxanthin	0.03	0.12	0.6
Lycopene	0.04	0.16	0.8
α-Carotene	0.03	0.12	0.6
β-Carotene	0.06	0.24	1.2
Phytoene	0.03	0.12	0.6
Phytoene	0.03	0.12	0.6

CTI participates in a proficiency testing program for retinol, retinyl palmitate, α- and γ-tocopherol, lutein, zeaxanthin, β-cryptoxanthin, lycopene, and α- and β-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. In addition, standard reference materials (SRM), NIST SRM 968c: Fat-Soluble Vitamins and Cholesterol in Human Serum are analyzed for retinol, α-tocopherol and β-carotene to confirm the CTI method. NIST SRM is analyzed during methods validation and at intervals to check the extraction and analysis. If the results by the CTI method do not fall within the 95% confidence interval for the mean of these analytes as determined by NIST, NHANES specimens are not analyzed until the system has been adjusted to produce results within the 95% confidence interval (excluding the presence of a known bias such as separation of isomers).

8. Procedure Operating Instructions; Calculations; Interpretation of Results

A. **Preliminaries**

1. Allow frozen serum, quality control serum, working standards and the internal standard to reach ambient temperature.

2. Prepare the mobile phase as described in 6.a.1 above.

B. Sample Preparation

1. Prepare two sets of labeled 12- x 75-mm glass tubes for the pools and unknowns.

2. Using a 250 μL Microman pipettor, dispense 150 μL of serum into a tube followed by 150 μL of 2 % ascorbic acid/4 % NaCl solution. Briefly vortex mixes each tube.

3. Using a 1.0 mL Microman pipettor, add 300 of internal standard solution in alcohol to each tube and vortex mix to precipitate proteins.

4. With the Repipettor, add 1000 uL of hexane containing BHT to each tube.

5. Vortex the mixture for5 minutes on the multi-tube vortexer, and centrifuge 3 minutes at 2200 rpm.

6. Decant the upper organic layers into the second set of labeled 12- x 75-mm tubes.

7. Repeat steps 4-6.

8. Evaporate the combined hexane extracts in the Speedvac (without heat).

9. Add 35uL of ethyl acetate to the tubes containing the dried extracts.

10. Vortex the tubes for 15 seconds then add 100 μL of mobile phase.

11. Ultrasonically agitate for 15 sec then vortex the tubes for 30 seconds.

12. Transfer the extract into a 250-μL glass insert using a fine-tip disposable pipette.

13. Centrifuge the inserts to pellet any particulate in the bottom.

14. Place the inserts into autosampler vials, cap, and place the vials in the autosampler AS3000 trays at 15ºC.

C. Instrument Preparation

1. <u>Acquisition Method</u> (create by clicking on the Acquisition Icon)

   a. Enter 100% for the reservoir containing the mobile phase.

b. Enter the flow rate as 1.0 mL/min.

c. Go to the next page and enter autosampler conditions
Column temperature: 35ºC
Tray temperature: 15 ºC
Syringe rinse volume: 400 μL
Needle height: 2mm

d. Proceed to the Detector 1 set-up page and enter diode-array detector program
(1) Channel A: 450 nm

(2) Channel B: 325 nm

(3) Channel C: 290 nm

(4) Set sampling rate at 5.0 pts/sec

e. Proceed to the Column page and enter column mfg, ID, lot #, dimensions, etc.

2. Calculation Method (create by clicking on the Calculation Icon)

a. Enter minimum integration parameters until a sample is injected to determine retention times, thresholds, etc. On Signal 1, set initial bunching factor to 1, peak threshold to 10. On Signal 2, set bunching factor to 2, threshold to 100. Inhibit integration on both signals until 2.8 min.

b. The integration parameters will vary with lamp age, column age, and other factors. All components are calibrated on the basis of area using a linear curve forced through the origin. The retention times will vary slightly with age of the column and from column to column. Actual retention times for a given column/instrument combination should be determined individually, monitored on a regular basis, and the component table updated as necessary. After response factors have been generated for the components in the calibration solutions, manually enter response factors for *cis*-lycopene and *cis* β-carotene that are equal to the calculated response factor for their *trans* isomers under the same chromatographic conditions.

3. Report Method (create by clicking on the Report Icon)

a. Enter 'Report Comment' (e.g., NHANES Vitamin A/E and Carotenoids)

b. 'Method Report" = none

c. 'Spectral Analysis' = none

d. 'Vial Summary Report' = none

e. 'Trend Report' = Samples only

f. 'Analysis Report' = single w/Cgram

g. 'System Suitability' = none
h. 'Calibration Report' = Text and Plots

i. Place a 'check mark' in ASCII file

j. Under 'Options', select 'Postrun processing', place a check mark in 'Trend Report' and in the command line type "TSP_AUTO Z:\NHANES %RES"

4. Sample Queue (create by clicking the Sample Queue Icon)

   a. Enter the filename for the sample queue consisting of the CDC container number and sequential sample set, the HPLC system letter. Enter sample information into the sample queue. Edit the Sample Queue so that all of the samples are correctly identified and the appropriate vial numbers entered. Enter the Acquisition, Calculation, and Report Methods.

   b. Save the Sample Queue as a Template.

   c. Flush autosampler syringe

   d. Start the run.

5. Post-Run Integration

   It is necessary for the operator to check the baseline for each peak and to correct it if necessary. Consistency in drawing baselines is essential for reproducibility.

   In some analyses, components may overlap. For example, β-cryptoxanthin (maximum absorbance and quantitation in the 450-nm channel) may overlap with γ-tocopherol (maximum absorbance and quantitation in the 290-nm channel). This contributes extraneous area to the γ-tocopherol peak and also confuses peak-naming by the PC-1000 data system. It has been determined that the contribution from β-cryptoxanthin to the γ-tocopherol peak at 290 nm is approximately 10% of the γ-tocopherol peak area. To correct this, a) place a Dropline on the trailing side (right side) of the γ-tocopherol peak so as to cut off 10% of its area; this will correct the peak area of the γ-tocopherol and will be named β-cryptoxanthin by the data system. b) Place a small Peak Rider baseline on the leading side (left side) of the β-cryptoxanthin; this will be named γ-tocopherol by the data system but will not affect the peak area.

D. Maintenance

1. Speedvac. The trap temperature is maintained at -35 °C and is checked daily before turning on the vacuum pump. The vapor trap of the Speedvac is emptied when full, usually weekly. The vacuum pump oil is changed when the quality of the vacuum deteriorates or the oil becomes turbid.

2. HPLC System. Clean the autosampler needle with methanol every two weeks. Guard columns are changed monthly or when backpressure increases 300-400 psi. HPLC columns are replaced when chromatography deteriorates or back-pressure increases and neither is corrected by back-flushing the column or replacing the column frits. Preventive maintenance on the HPLC system is performed semi-annually.

E. Calculations

PC1000 software performs all external standard calculations. Calibration curves are linear, forced through zero, and based on multiple injections of three standard concentrations. All analytes are present in one set of the standard solutions used to calculate response factors.

The concentration of the components in Level 3 of the mixed standards is equal to 1/10 of the concentration of the stocks. The concentration of the components in Level 2 of the mixed standards is equal to 1/5 of Level 3 and concentration of the components in Level 1 of the mixed standards is equal to 1/20 of Level 3.

F. Method Modifications

This method is a modification of a method that has been used by CTI for over 8 years and is detailed in several publications (Nomura et al., 1997a; Nomura et al., 1997b; DeRoos et al., 2001; Toyoda et al., 2002; Roe et al., 2002; Jordan et al., 2002; Sedjo et al., in press).

9. Reportable Range of Results

This method is linear for the carotenoids in the range 0.01-1.5 μg/mL, for retinol and the retinyl esters in the range 0.01-1.5 μg/mL, and for alpha-tocopherol in the range 0.05-40 μg/mL. The CVs for vitamins A and E and β-carotene are less than 5%. The CVs for the minor carotenoids are less than 11%.

10. Quality Control Procedures

A. Blind controls
Blind controls will be inserted in sample batches prior to their receipt at CTI.

B. Bench controls
Up to three serum pools are used as bench controls. These controls represent high, medium, and low levels of the analytes in serum. All three QC sera are analyzed at the

beginning and end of the day. The run is considered 'within control' or 'out of control' based on the quality control scheme provided below. The initial upper and lower limits are established from the results of analyzing the QC pools 20 consecutive times at a point when the NIST SRM 968 sample have been measured and the certified values have been achieved. The mean values and upper and lower limits are updated annually

C. Quality Control Scheme for HPLC Fat-Soluble Vitamin Analysis

The CTI fat-soluble vitamins assay measures vitamins A and E and carotenoids. Each of these classes of nutrients is measured using different detection wavelengths or detectors. We are measuring three of the five analytes (retinol, a-tocopherol, lutein, lycopene, and β-carotene) which are generally present in significant amounts in most sera. Most methodological problems affect analytes within a class in a similar manner, however, not necessarily all classes are affected. Due to the number of total analytes and the wide range of concentrations, specific analytes within a class are selected as 'Sentinel' analytes for the class.

The three classes of analytes are listed below and the sentinel analyte for each group is emboldened.
Vitamin A Group - **Retinol**, Retinyl Palmitate, Retinyl Stearate
Vitamin E Group - α**-Tocopherol**, y-Tocopherol, δ-tocopherol
Carotenoid Group - Lutein, Zeaxanthin, α-cryptoxanthin, β-Cryptoxanthin, Lycopene, α-Carotene, **β-Carotene** (also phytoene and phytofluene)

1. Three serum pools are used which represent low, medium and high concentrations of the analytes indicated in bold-type above. These analytes were chosen because a) they are typically the one of most prominent components in their class; b) they are representative of each class; and c) SRMs are available for each of these.

2. The means and ranges of all analytes will be tracked using Levy-Jennings plots. Whether a run is in control or not will be based on these three analytes. The sentinel analytes will be judged in control using single analyte rules as follows:

   If the mean for one or more of the three pools is out of the 99 % limits, the analyte is out of control.

   If the mean for two or more pools is out of the 95 % limits, the analyte is out of control.

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

A. Check to make sure that the hardware is functioning properly.
Make sure the pump is operating at the correct flow rate with appropriate, steady pressure.

Check the autosampler to ensure the vials are in the correct location, the injections are being made as programmed, the syringe is not worn, and the injection valve is not worn.

Run the detector diagnostic programs.

Check for extraction and pipetting errors. These can be determined by abnormalities in the carotenoid profile and internal standard area.

B. Run NIST SRM 968.

If the steps outlined above do not correct the "out of control" values for QC materials, the supervisor should be consulted for other appropriate corrective actions. Analytical results should not be reported for runs out of statistical control.

12. Limitations of Method; Interfering Substances and Conditions

A. Linear range and limits of detection (LOD): Our methods are linear for the range 1 - 150 μg/dL for carotenoids, 1 - 150 μg/dL for retinoids and 50 - 5000 μg/dL for alpha-tocopherol. CVs for vitamins A and E and for beta-carotene are less than 5%; for minor carotenoids, CVs are less than 11%.

Lower limits of detection were determined by injecting diminishing levels of retinol, lutein, beta-carotene, alpha-tocopherol, and retinyl palmitate. LOD is defined as three times the signal: noise ratio. Limits of detection are:

retinol	0.003 ug/mL
retinyl esters	0.005 ug/mL
tocopherols	0.1 ug/mL
xanthophylls	0.002 ug/mL
carotenes	0.003 ug/mL

B. Interfering substances: Under conditions where the retention times for *cis* β-carotene and retinyl palmitate are similar, the carotenoid absorption at 325 nm may result in an overestimation of retinyl palmitate. The retinyl palmitate value in these samples is unreliable. β-Cryptoxanthin may coelute and contribute to the peak area of γ-tocopherol at 290 nm.

C. The following substitutions may be made for the specified instrumentation: Instead of drying the hexane extracts with a Speedvac system, the samples may be dried under a stream of nitrogen.

13. Reference Ranges (Normal Values)

Reference ranges have not been established for retinyl esters in serum. The values in Table 3 are used for the reference ranges for retinol, a-tocopherol, and the carotenoids. These values are approximate, based on the 1-99 percentile ranges for 2480 specimens analyzed for NHANES III.

Table 3. Concentration means and ranges of analytes (μg/mL) from 2480 free-living male and female subjects, aged 4 to 93 years (NHANES III)

Analyte	Mean	5-95 percentile range
Lutein + Zeaxanthin	0.20	0.08 - 0.42
beta-Cryptoxanthin	0.12	0.03 - 0.29
Lycopene	0.21	0.06 - 0.43
alpha-Carotene	0.04	0.01 - 0.12
beta-Carotene	0.18	0.04 - 0.47
Retinyl ester (total)	0.09	0.016 - 0.20
Retinol	0.54	0.30 - 0.84
alpha-Tocopherol	11.1	5.97 - 20.2

Calculated from data of Sowell, A. L., Huff, D. L., Yeager, P. R., Caudill, S. P. & Gunter, E. W. (1994) Retinol, alpha-tocopherol, lutein/zeaxanthin, beta-cryptoxanthin, lycopene, alpha-carotene, *trans*-beta-carotene, and four retinyl esters in serum determined simultaneously by reversed-phase HPLC with multi wavelength detection. *Clin.Chem.* 40: 411-416.

14. Critical Call Results ("Panic Values")

The collaborating agency with access to patient identifiers or the responsible medical officer is notified by FAX or email by the supervisor on any vitamin A result that is < 10 μg/dL or any vitamin A/retinyl ester profile that suggests hypervitaminosis A with hepatotoxicity (i.e., fasting serum with retinol elevated for the donor's age or gender and total retinyl esters ≥50% of serum retinoids). Copies of Faxes and emails sent concerning abnormal results are retained by CTI for the duration of the study.

15. Specimen Storage and Handling during Testing

Specimens are allowed to reach room temperature during preparation. After an aliquot has been removed from the samples, the unused portion of the patient specimen is returned to the freezer at −70ºC. When the samples are ready to run, the extracts are placed in the AS3000 autosampler at 15 °C.

16. Alternative Methods for Performing Test or Storing Specimens If Test System Fails

Samples can be measured using another HPLC system if the dedicated system fails. Electrochemical detection is an alternative mode of detection for all the analytes.

CouloChem and CoulArray detectors are available at CTI. If the analytical system fails and can be repaired within 48 hr, then the extracted specimens will be stored at $\leq$-20ºC until HPLC analysis.

17. Test Result Reporting System; Protocol for Reporting Critical Calls (if applicable)

The collaborating agency with access to patient identifiers or the responsible medical officer is notified by FAX or email 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 (Westat) at a frequency and by a method determined by the study coordinator. Data from this analysis are sent as an ASCII text file electronically to the data coordinator for incorporation into the NHANES database.

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

The instrument control computer is used to store short-term chromatographic data, with automatic transfer and longer-term data storage on a separate networked computer. Data are archived on magnetic tape at least weekly; once per month all data are archived to compact disk (CD). Records, including related QC data, are maintained for 10 years after completion of the study. Only numerical identifiers are used.

19. Additional Notes and Precautions:

A. Inadequate sample volume: If less than 150 µL of sample is available, take up the entire sample volume into the Microman pipettor. Adjust the volume dial of the pipettor downwards until the sample is just contained in the pipet tip. Read the sample volume. Dispense the sample into the tube for extraction. Add exactly the same volume of ascorbic acid/NaCl solution into the tube. Add a proportionate amount of internal standard (tocol) solution to the extraction tube; e.g. if only 100 μL of serum is available instead of 150 μL, add 200 μL of internal standard solution (300 μL x 100/150). Proceed with remaining steps of the sample preparation procedure as usual.

A sample with volume < 80 μL is too small to analyze. Provide a place holder" (sample vial containing mobile phase only). Report sample with appropriate code on the Report Sheet.

B. Missing sample: Provide a place holder" (sample vial containing mobile phase only). Report sample with appropriate code on the Report Sheet.

C. Repeat samples: Identification of samples to be repeated is prepared by a random-number generator. If sample volume is inadequate for repeated sampling, take the preceding sample number.

D. File naming: When re-queuing or re-processing samples after a stoppage, be watchful that the PC-1000 data system does not change windows; this can result in different names for the .RES and .REM files.
Re-injection from the same sample vial: Use the suffix "i" on the sample name.
Re-extraction from the original sample: Use the suffix "e" on the sample name.

E. Sample queue sizes: The least polar (latest eluting) sample components precipitate from the injection solvent on prolonged standing. Run groups of no more than 30 samples, to minimize need for fresh sample preparations in case of instrument problems.

20. Summary Statistics and QC Graphs

A. α-Tocopherol(ug/dL)

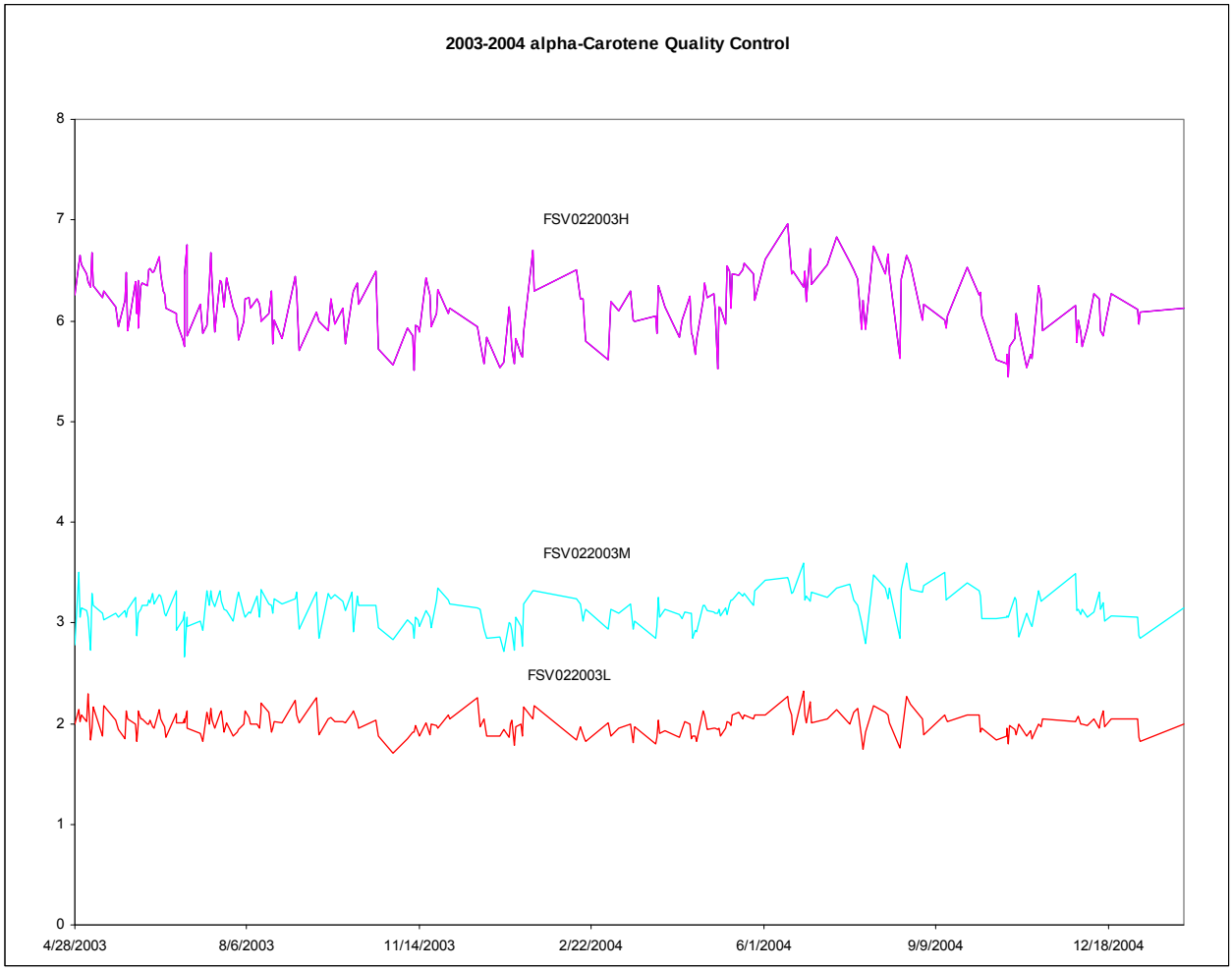
Summary Statistics for alpha-Tocopherol (Vitamin E) by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003L	140	4/28/2003	1/31/2005	559.5	28.9	5.2
FSV022003M	221	4/28/2003	1/31/2005	703.7	18.4	2.6
FSV022003H	221	4/28/2003	1/31/2005	1428.2	35.7	2.5

B. α –Carotene

Summary Statistics for alpha-Carotene by Lot

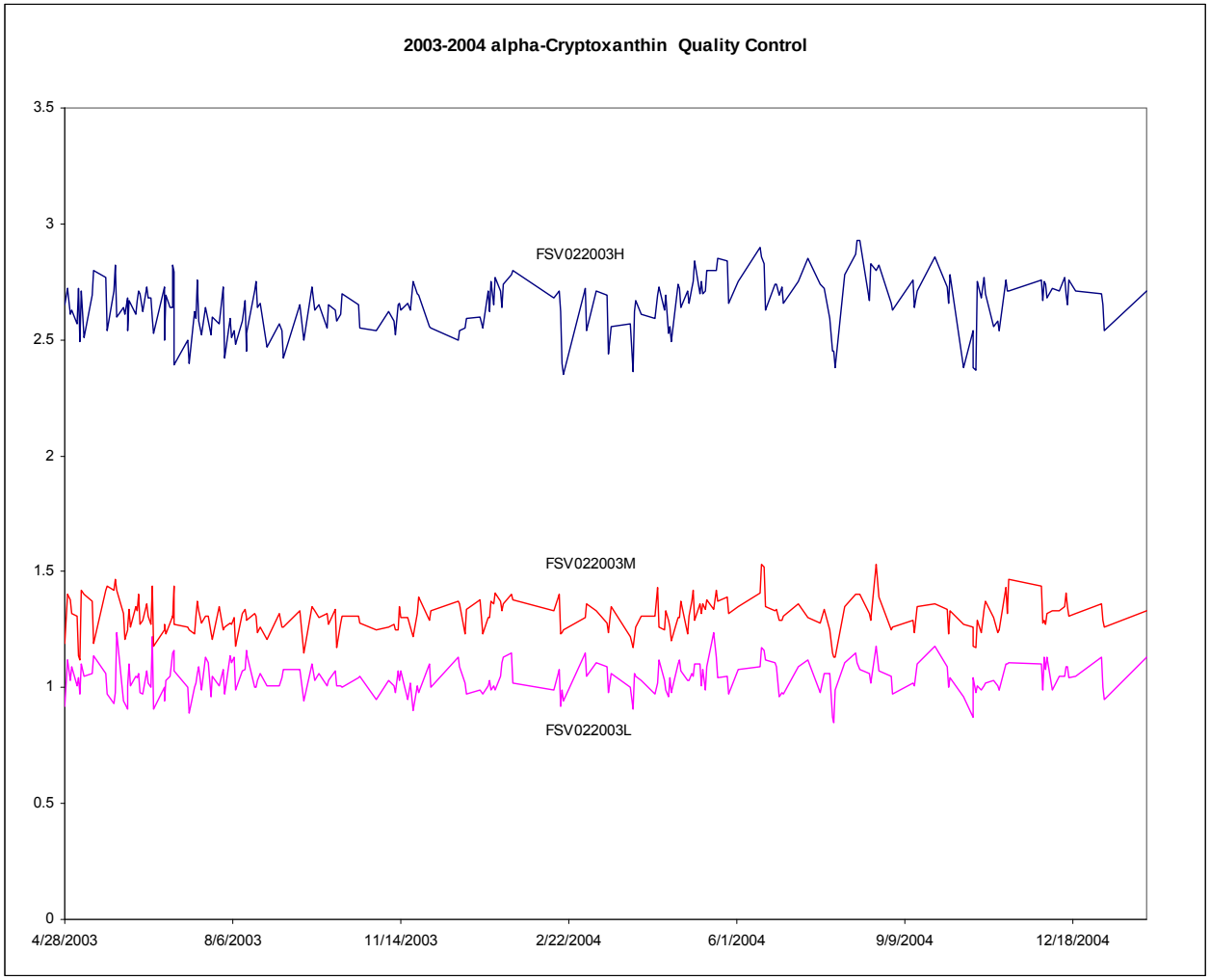
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003L	221	4/28/2003	1/31/2005	1.997	0.109	5.5
FSV022003M	221	4/28/2003	1/31/2005	3.134	0.165	5.3
FSV022003H	221	4/28/2003	1/31/2005	6.140	0.308	5.0



D. α –Cryptoxanthin

Summary Statistics for alpha-Cryptoxanthin by Lot

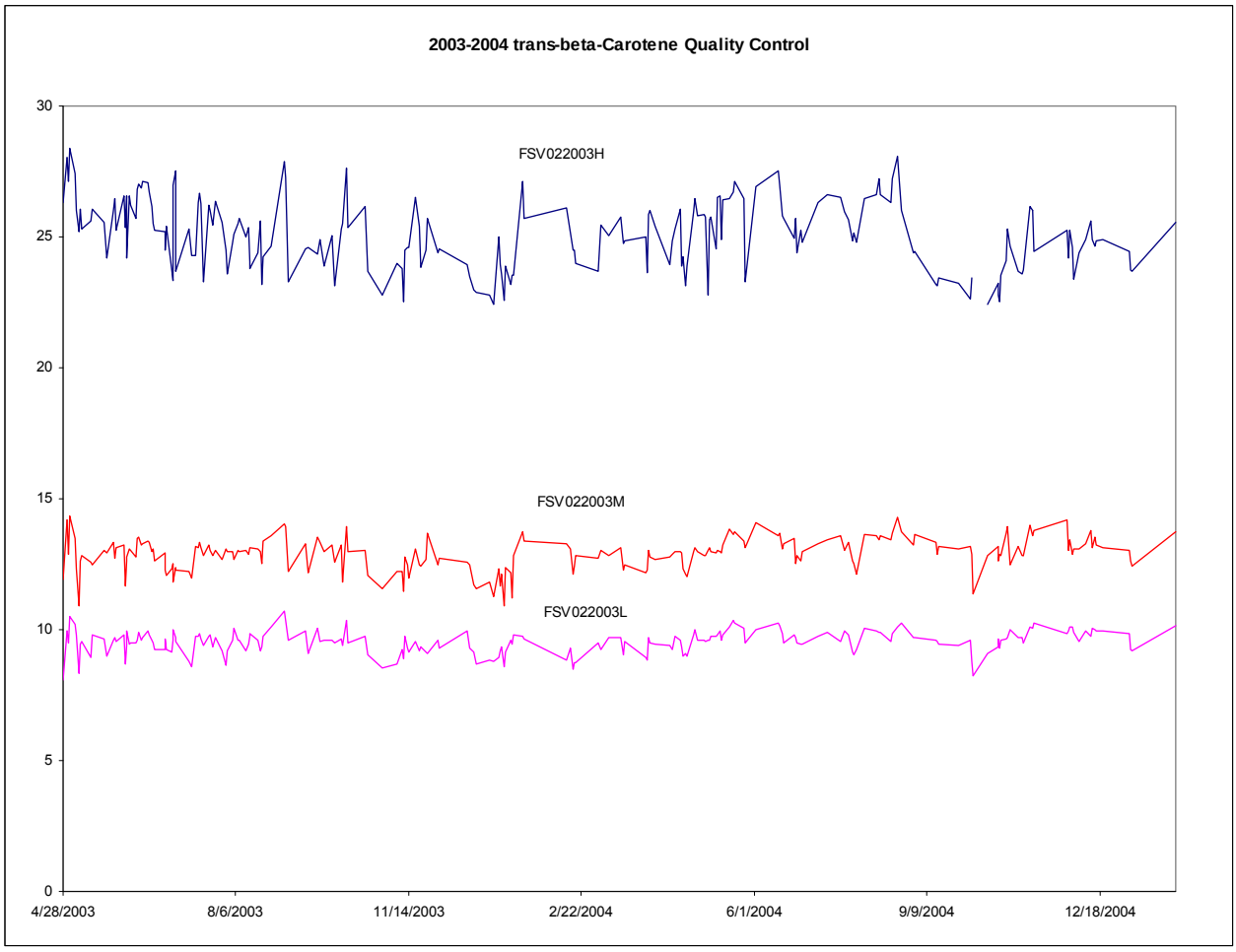
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003L	221	4/28/2003	1/31/2005	1.041	0.068	6.6
FSV022003M	221	4/28/2003	1/31/2005	1.310	0.073	5.6
FSV022003H	221	4/28/2003	1/31/2005	2.652	0.114	4.3



E. trans-β-carotene

Summary Statistics for trans-beta-Carotene by Lot

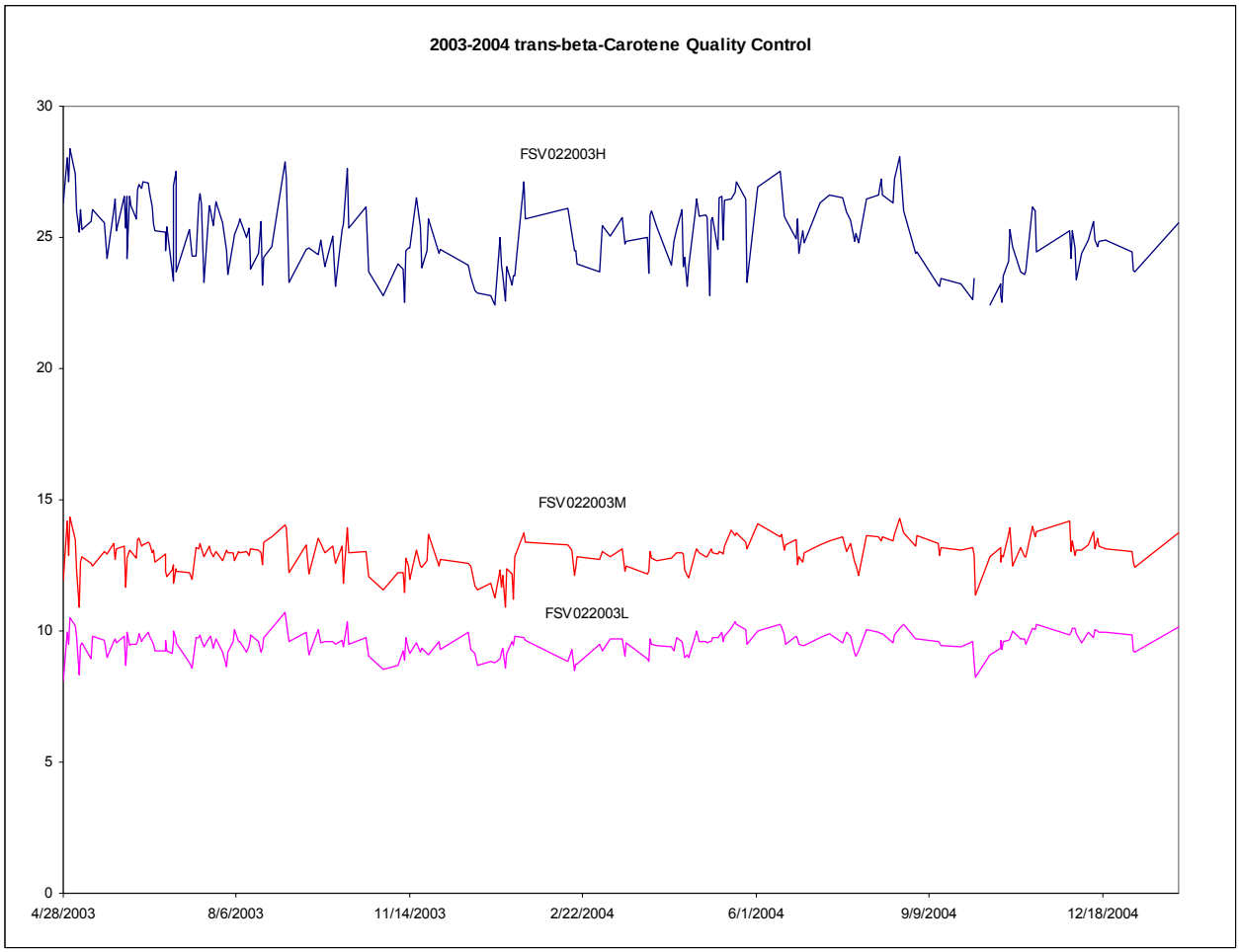
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003L	221	4/28/2003	1/31/2005	9.542	0.433	4.5
FSV022003M	221	4/28/2003	1/31/2005	12.905	0.616	4.8
FSV022003H	221	4/28/2003	1/31/2005	25.091	1.317	5.2



F. Combined β-Carotene

Summary Statistics for Combined beta-Carotene by Lot

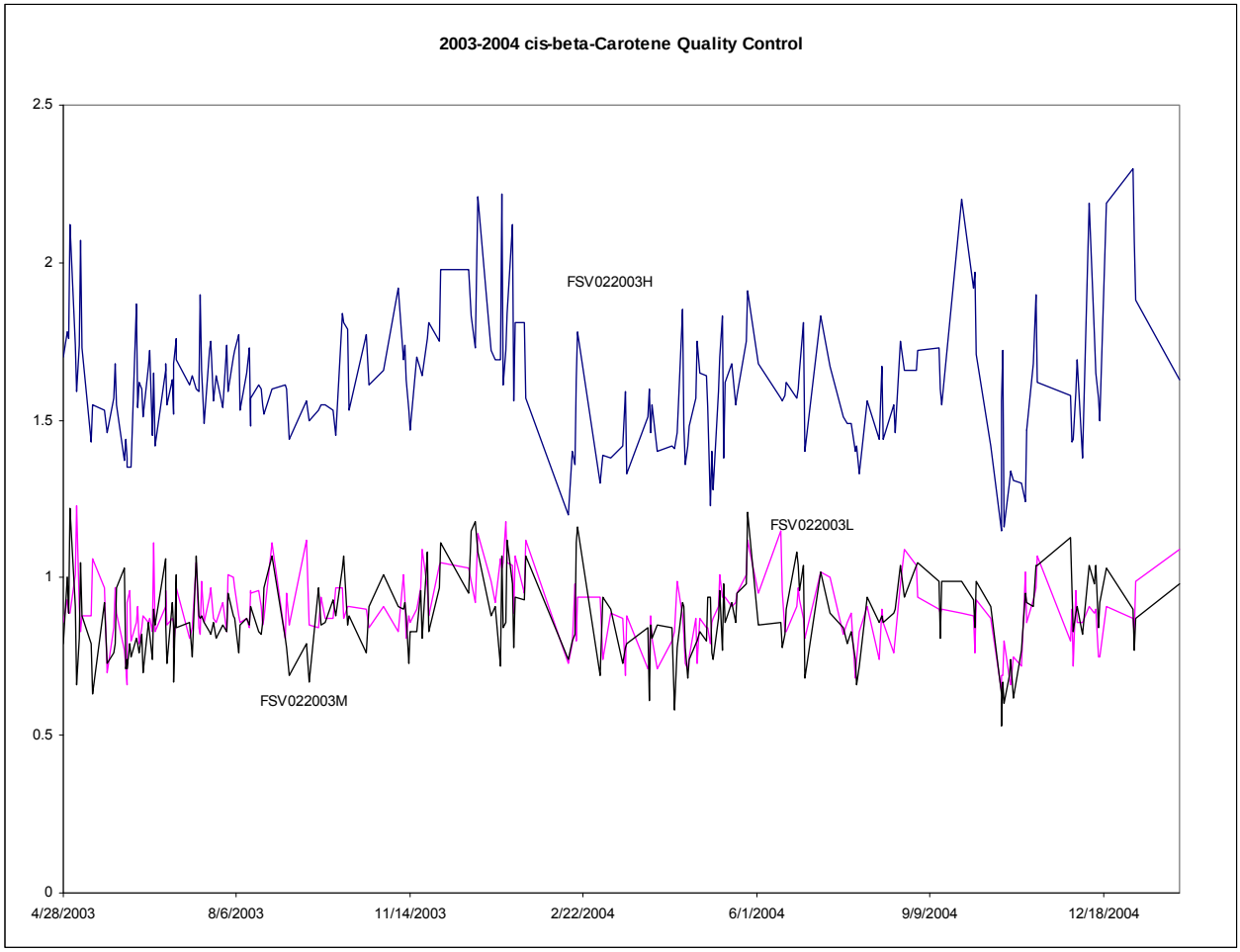
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003L	221	4/28/2003	1/31/2005	10.433	0.463	4.4
FSV022003M	221	4/28/2003	1/31/2005	13.776	0.646	4.7
FSV022003H	220	4/28/2003	1/31/2005	26.705	1.335	5.0



G. cis-β-carotene

Summary Statistics for cis-beta-Carotene by Lot

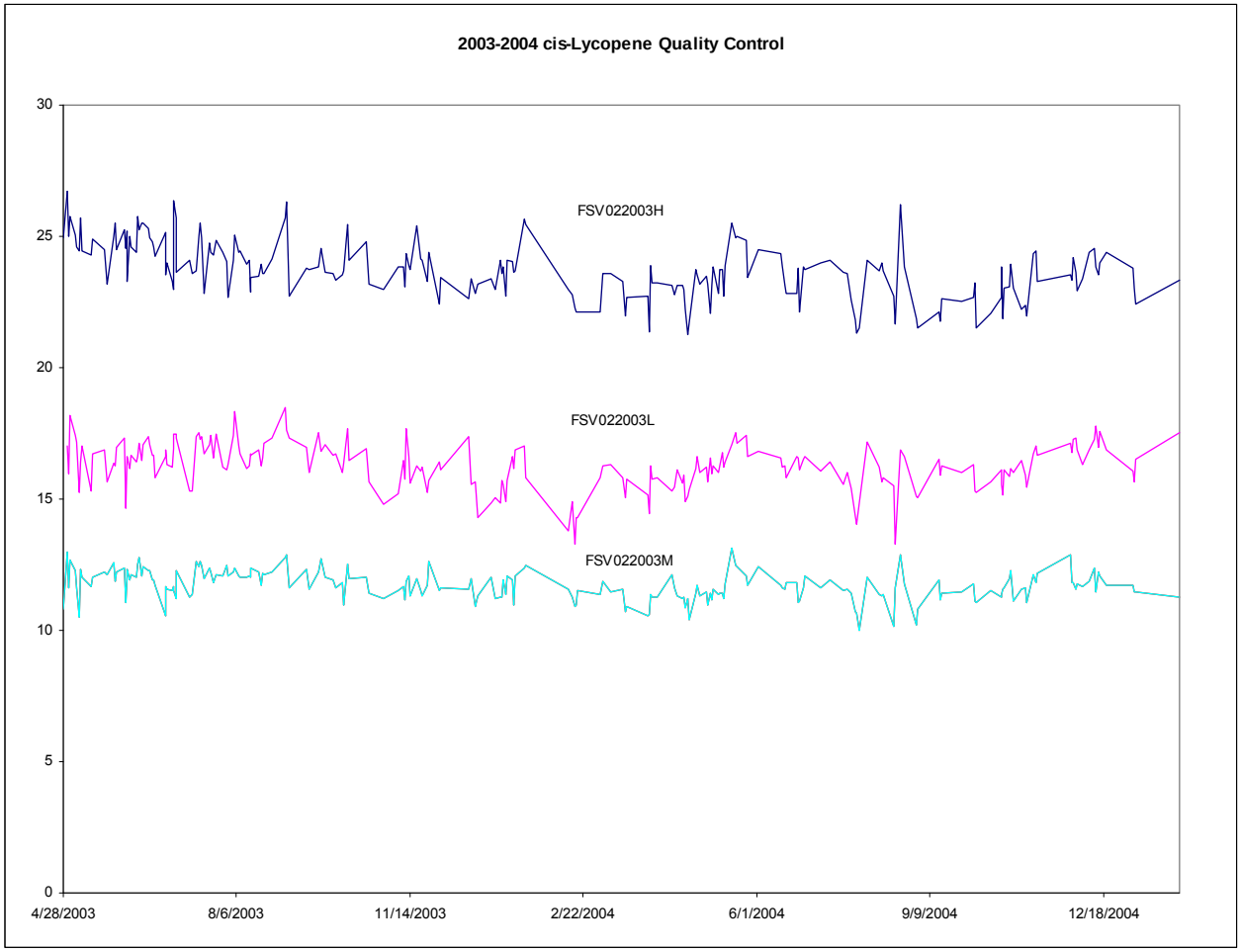
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003M	221	4/28/2003	1/31/2005	0.872	0.124	14.2
FSV022003L	221	4/28/2003	1/31/2005	0.893	0.107	11.9
FSV022003H	221	4/28/2003	1/31/2005	1.617	0.200	12.4



H. cis-Lycopene

Summary Statistics for cis-Lycopene by Lot

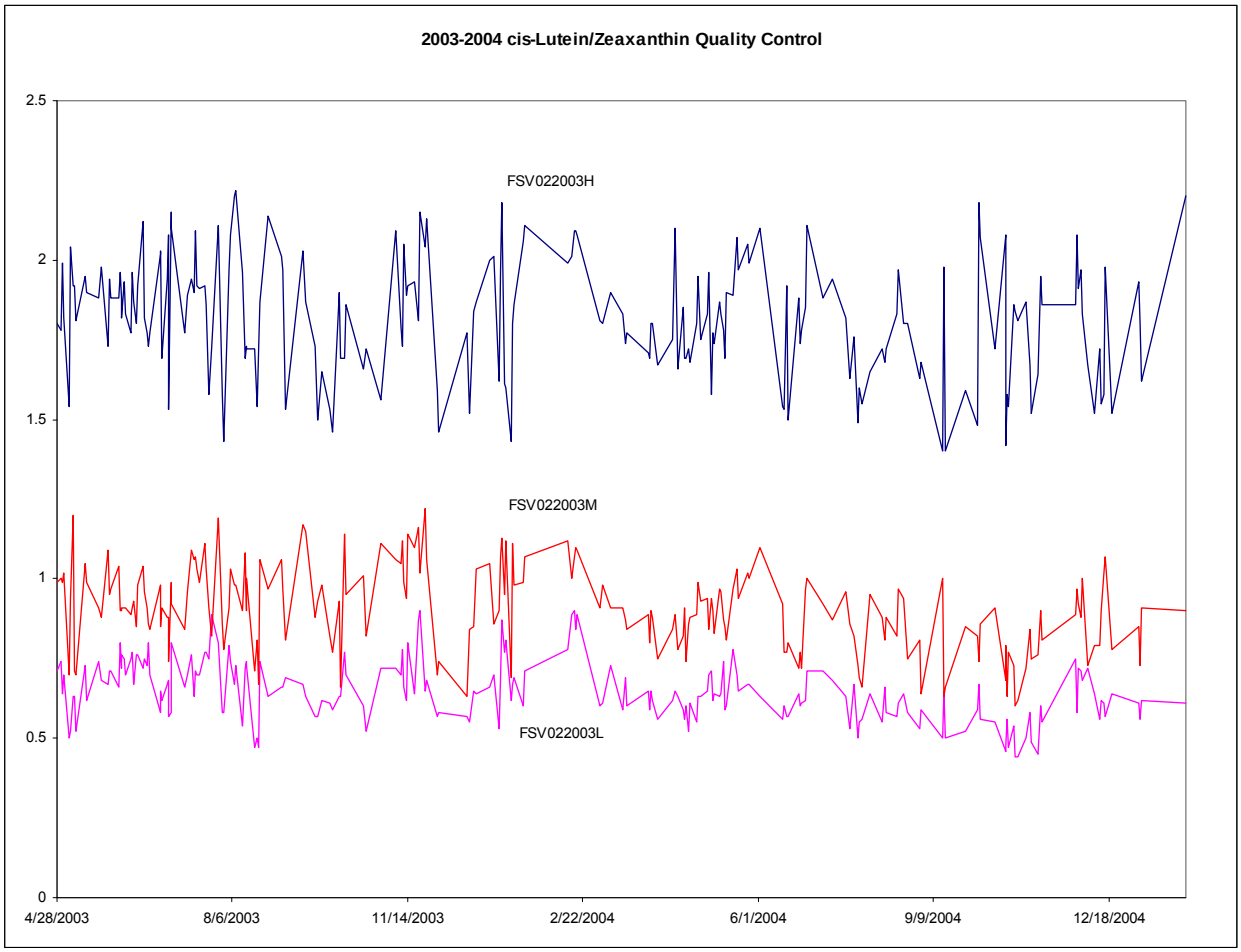
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003M	221	4/28/2003	1/31/2005	11.714	0.558	4.8
FSV022003L	221	4/28/2003	1/31/2005	16.271	0.880	5.4
FSV022003H	220	4/28/2003	1/31/2005	23.716	1.074	4.5



I. cis-Lutein/Zeaxanthin

Summary Statistics for cis-Lutein/Zeaxanthin by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003L	221	4/28/2003	1/31/2005	0.648	0.093	14.4
FSV022003M	221	4/28/2003	1/31/2005	0.907	0.130	14.3
FSV022003H	221	4/28/2003	1/31/2005	1.819	0.187	10.3



J. β-cryptoxanthin

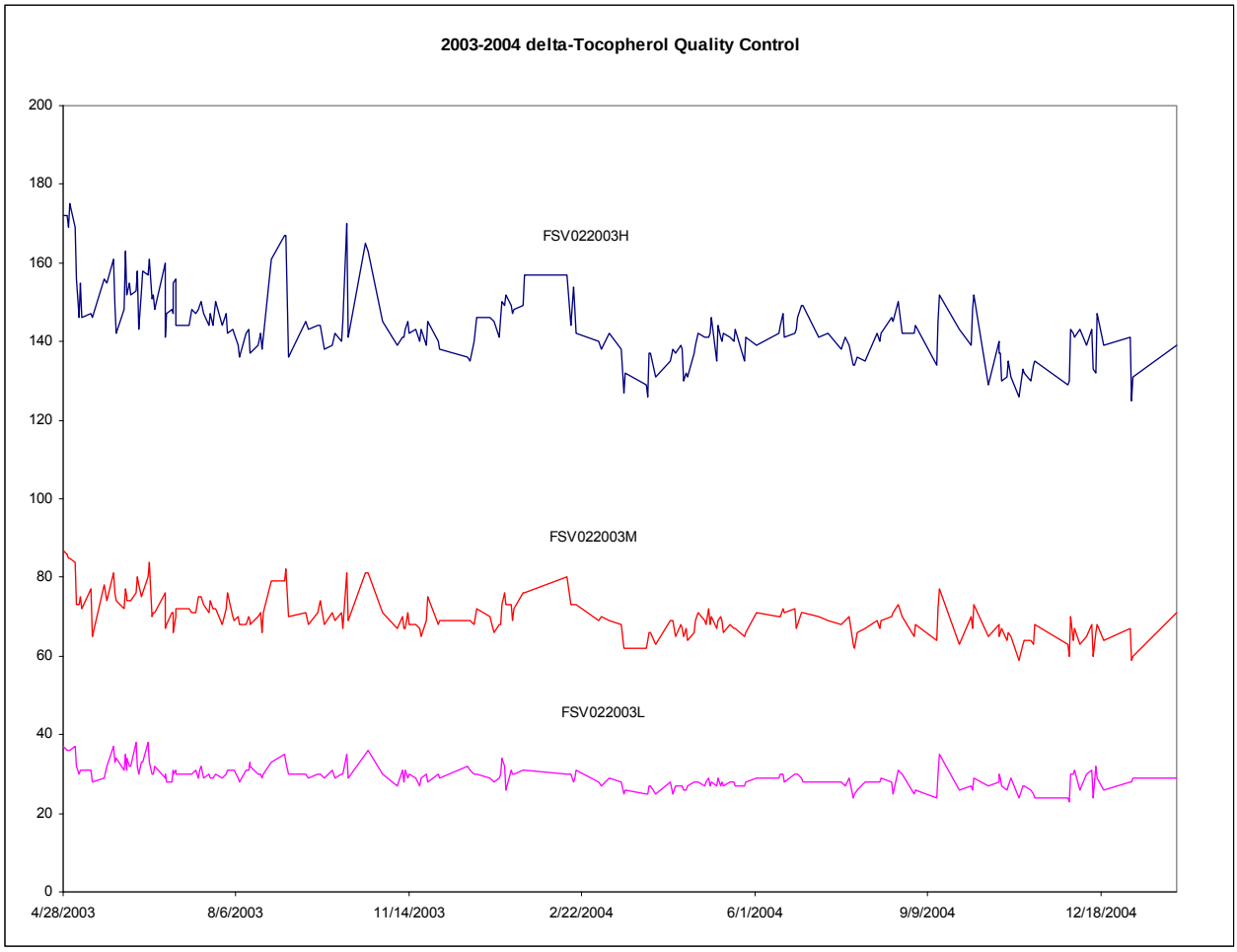
Summary Statistics for beta-Cryptoxanthin by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003L	221	4/28/2003	1/31/2005	5.403	0.244	4.5
FSV022003M	221	4/28/2003	1/31/2005	11.644	0.464	4.0
FSV022003H	221	4/28/2003	1/31/2005	23.454	0.847	3.6

K. δ –Tocopherol

Summary Statistics for delta-Tocopherol by Lot

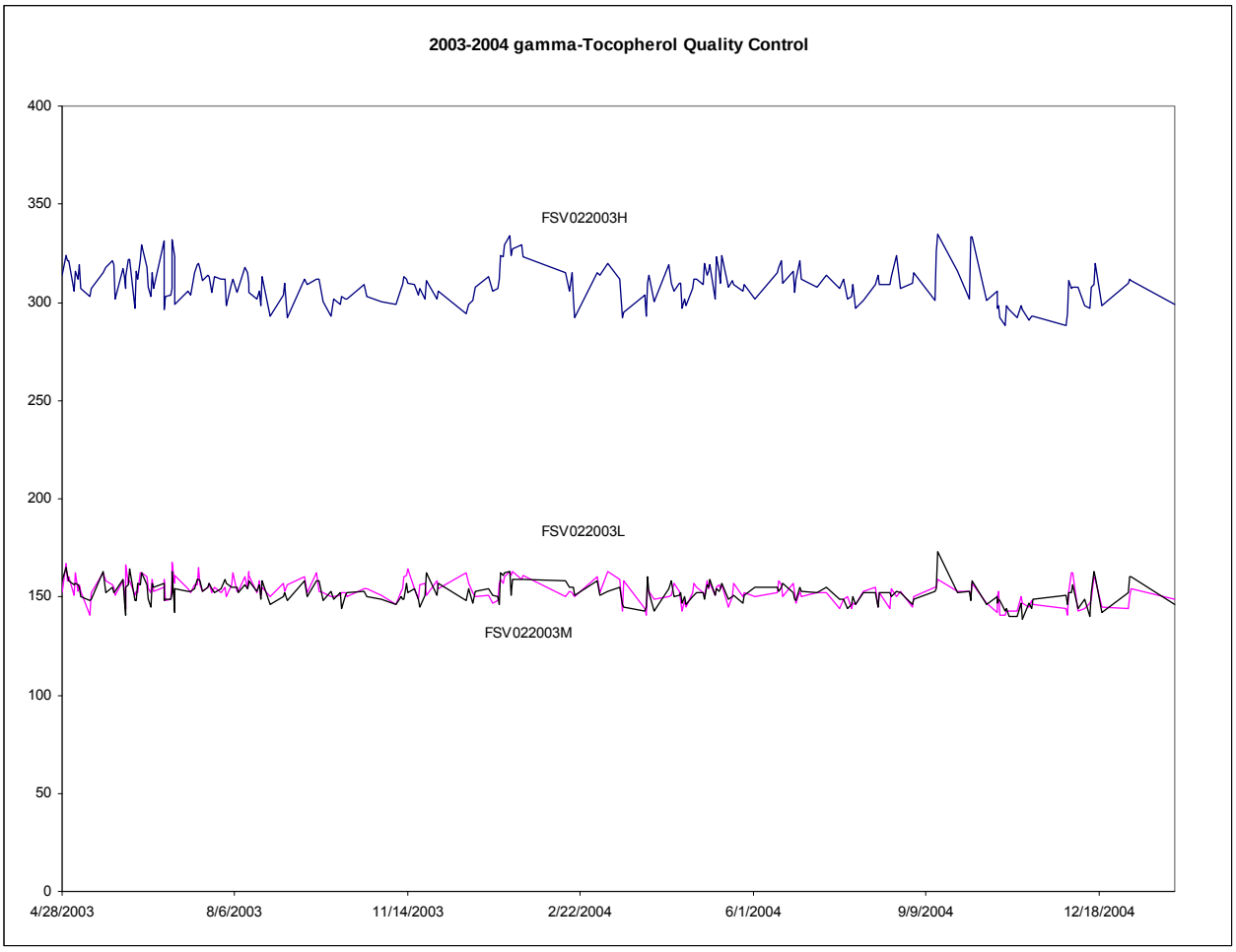
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003L	221	4/28/2003	1/31/2005	29.4	2.8	9.5
FSV022003M	221	4/28/2003	1/31/2005	70.1	5.1	7.2
FSV022003H	221	4/28/2003	1/31/2005	143.8	9.0	6.3



L. γ-tocopherol

Summary Statistics for gamma-Tocopherol by Lot

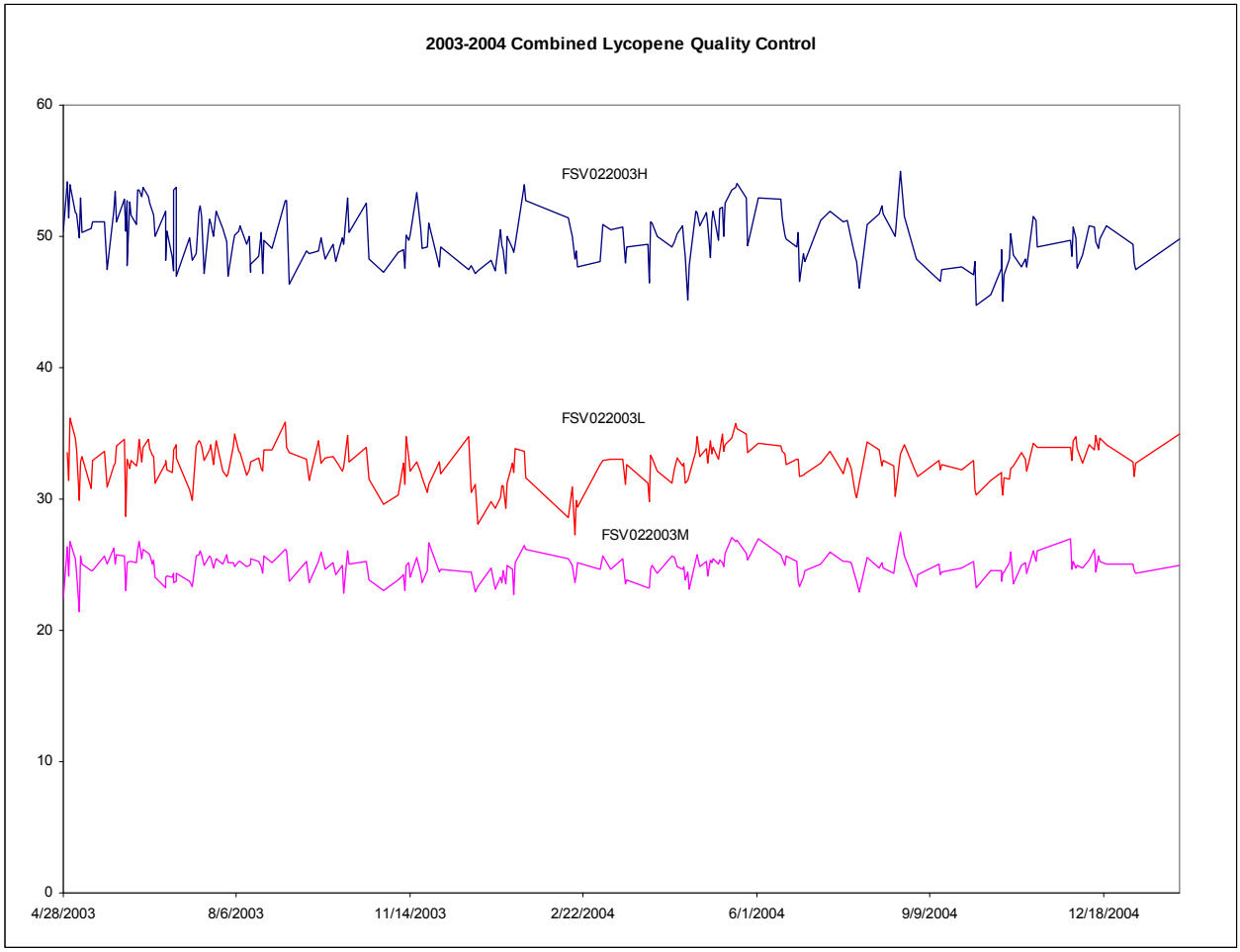
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003M	221	4/28/2003	1/31/2005	152.3	5.3	3.5
FSV022003L	221	4/28/2003	1/31/2005	153.1	5.6	3.7
FSV022003H	221	4/28/2003	1/31/2005	309.3	9.5	3.1



M. Combined Lycopene

Summary Statistics for Combined Lycopene by Lot

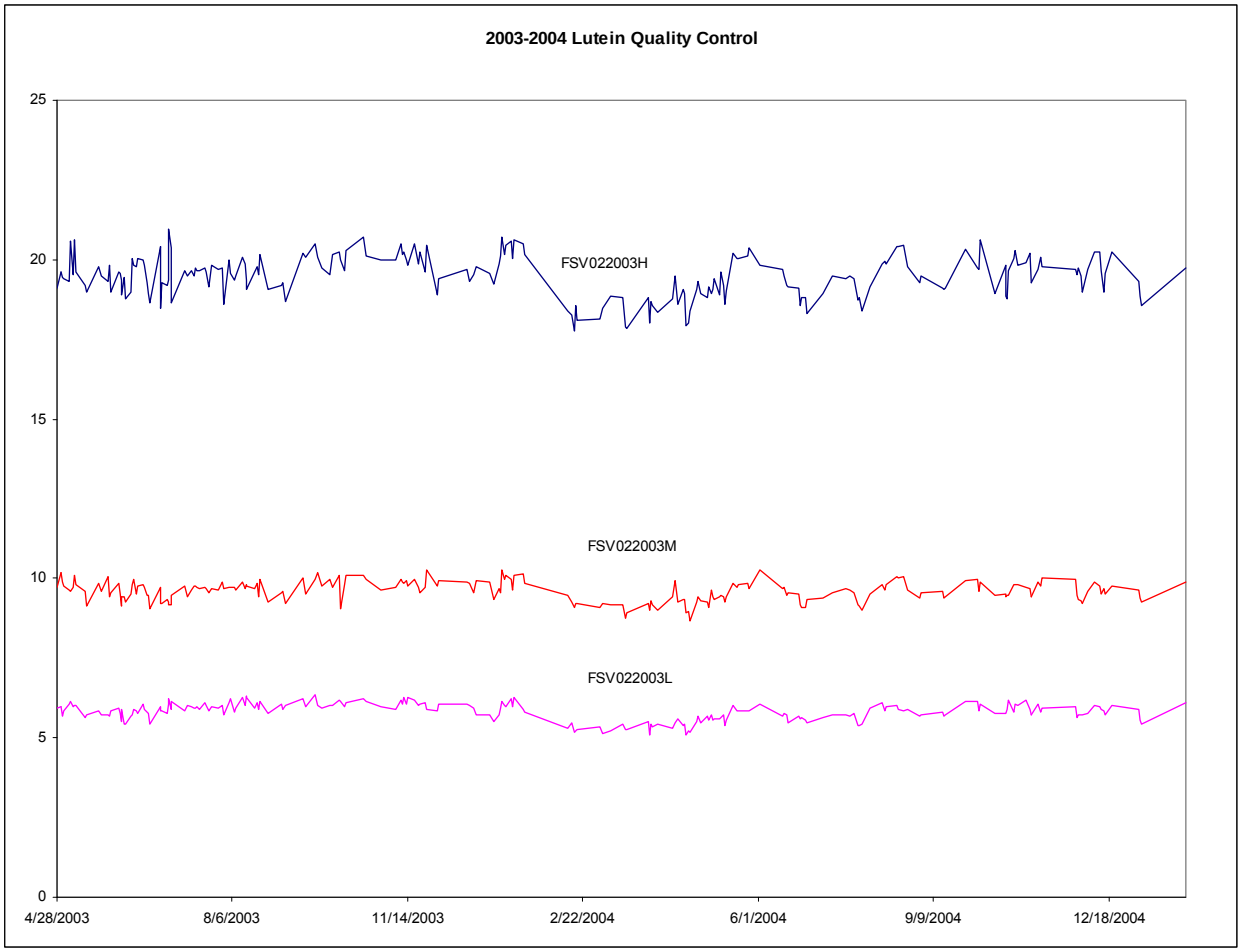
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003M	221	4/28/2003	1/31/2005	24.862	0.952	3.8
FSV022003L	220	4/28/2003	1/31/2005	32.680	1.496	4.6
FSV022003H	221	4/28/2003	1/31/2005	49.942	2.034	4.1



N. Lutein

Summary Statistics for Lutein by Lot

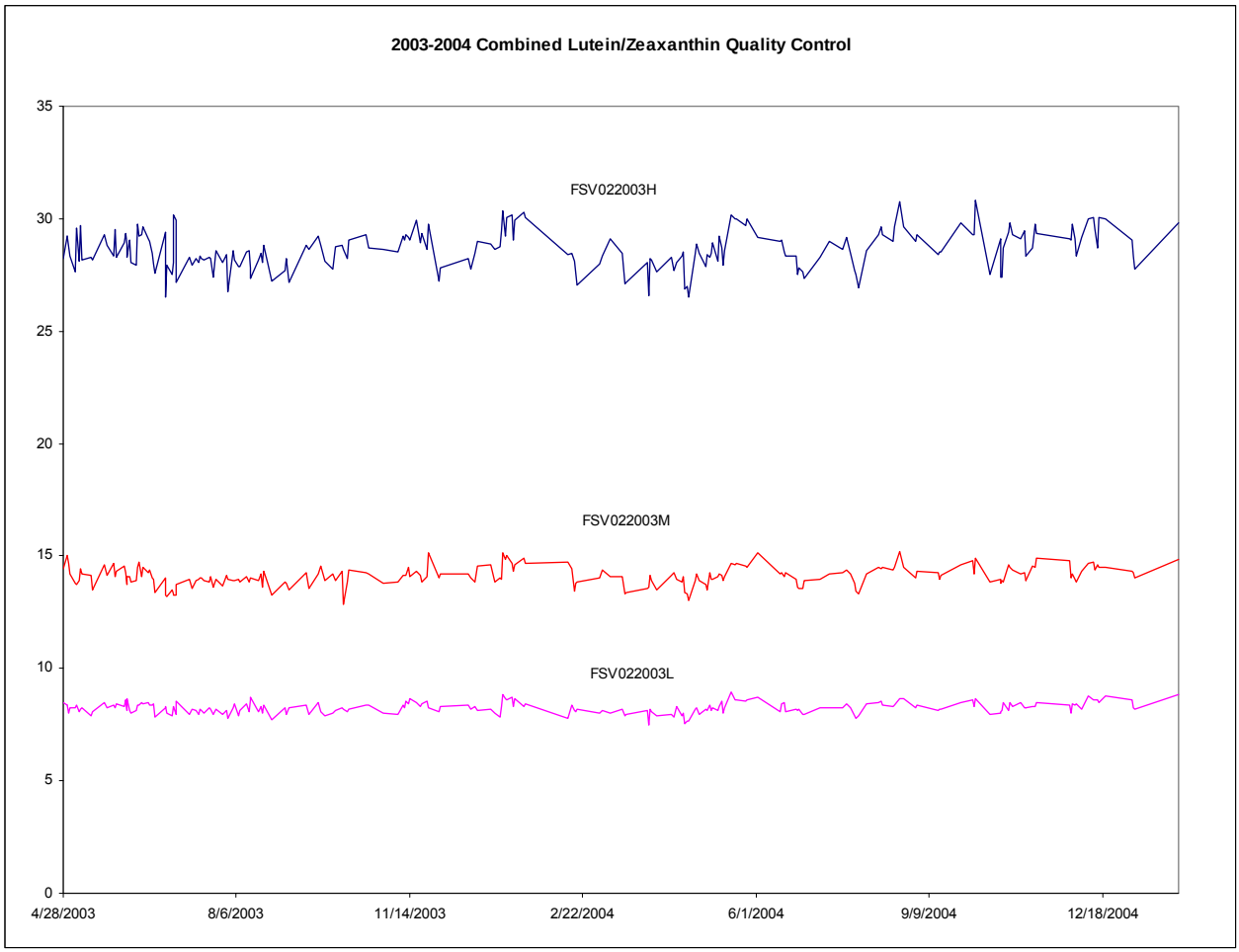
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003L	221	4/28/2003	1/31/2005	5.817	0.272	4.7
FSV022003M	221	4/28/2003	1/31/2005	9.597	0.309	3.2
FSV022003H	221	4/28/2003	1/31/2005	19.483	0.646	3.3



O. Combined lutein/zeaxanthin

Summary Statistics for Combined Lutein/ Zeaxanthin by Lot

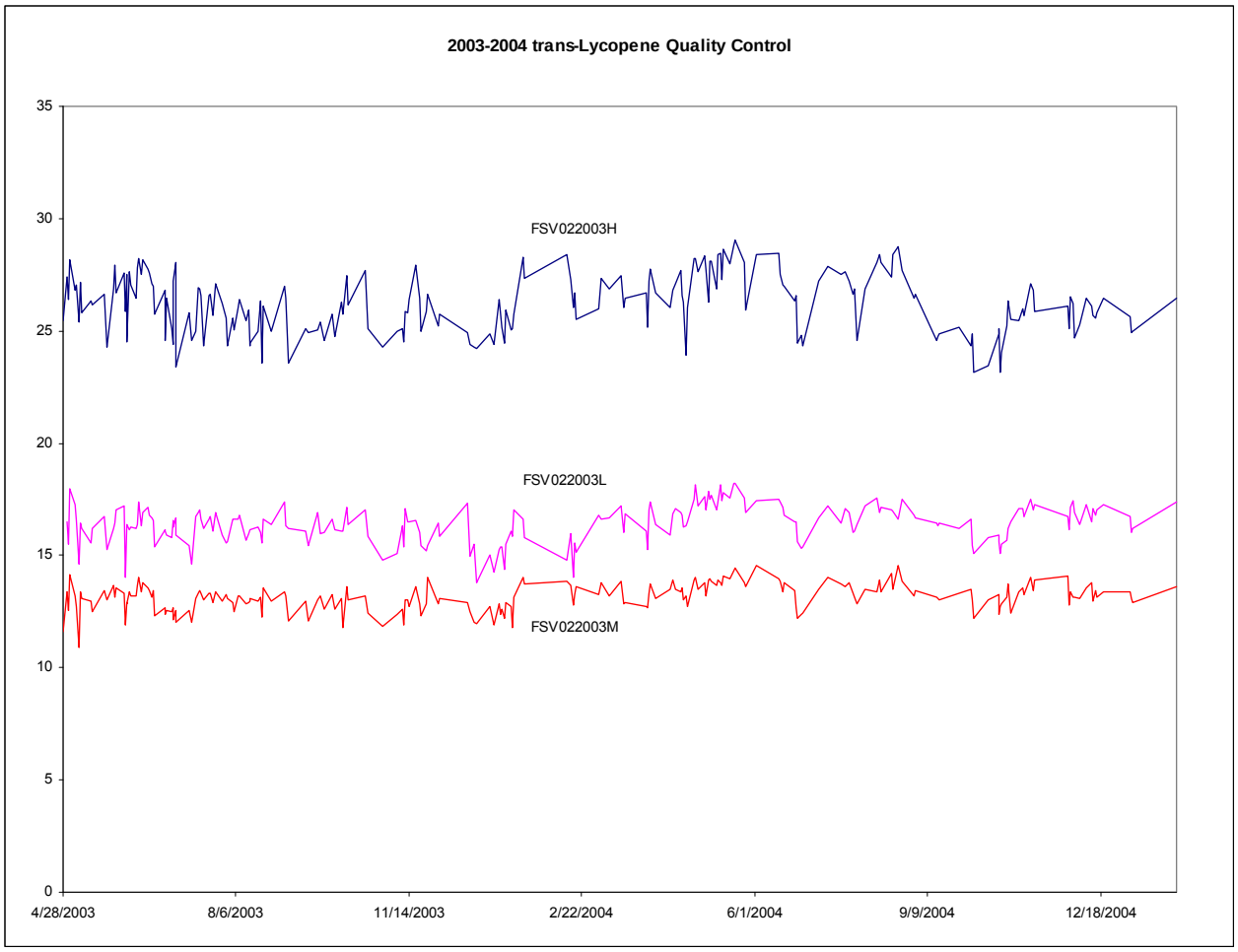
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003L	221	4/28/2003	1/31/2005	8.251	0.251	3.0
FSV022003M	221	4/28/2003	1/31/2005	14.107	0.421	3.0
FSV022003H	221	4/28/2003	1/31/2005	28.629	0.844	2.9



P. trans-lycopene

Summary Statistics for trans-Lycopene by Lot

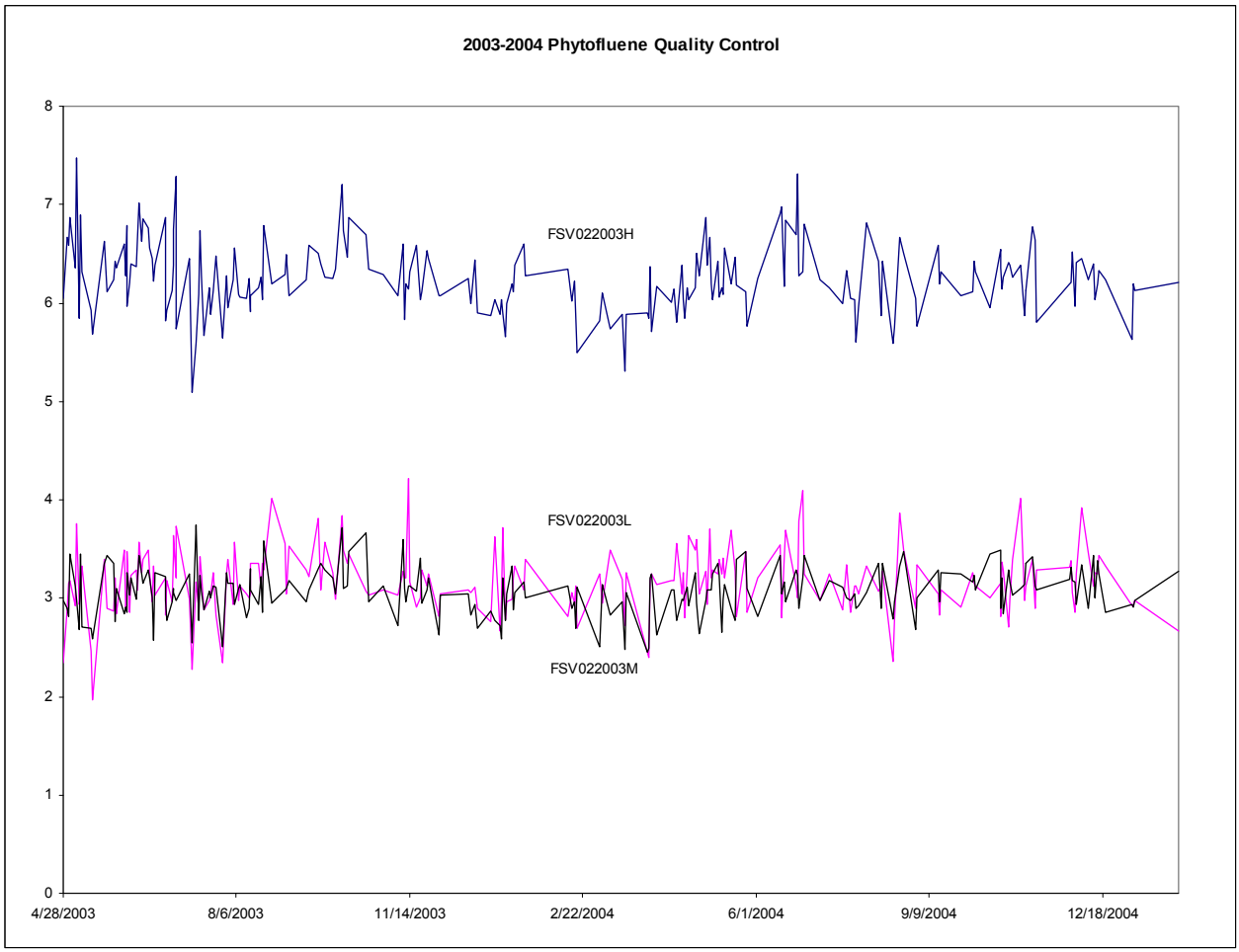
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003M	221	4/28/2003	1/31/2005	13.149	0.597	4.5
FSV022003L	220	4/28/2003	1/31/2005	16.411	0.816	5.0
FSV022003H	221	4/28/2003	1/31/2005	26.227	1.307	5.0



Q. Phytofluene

Summary Statistics for Phytofluene by Lot

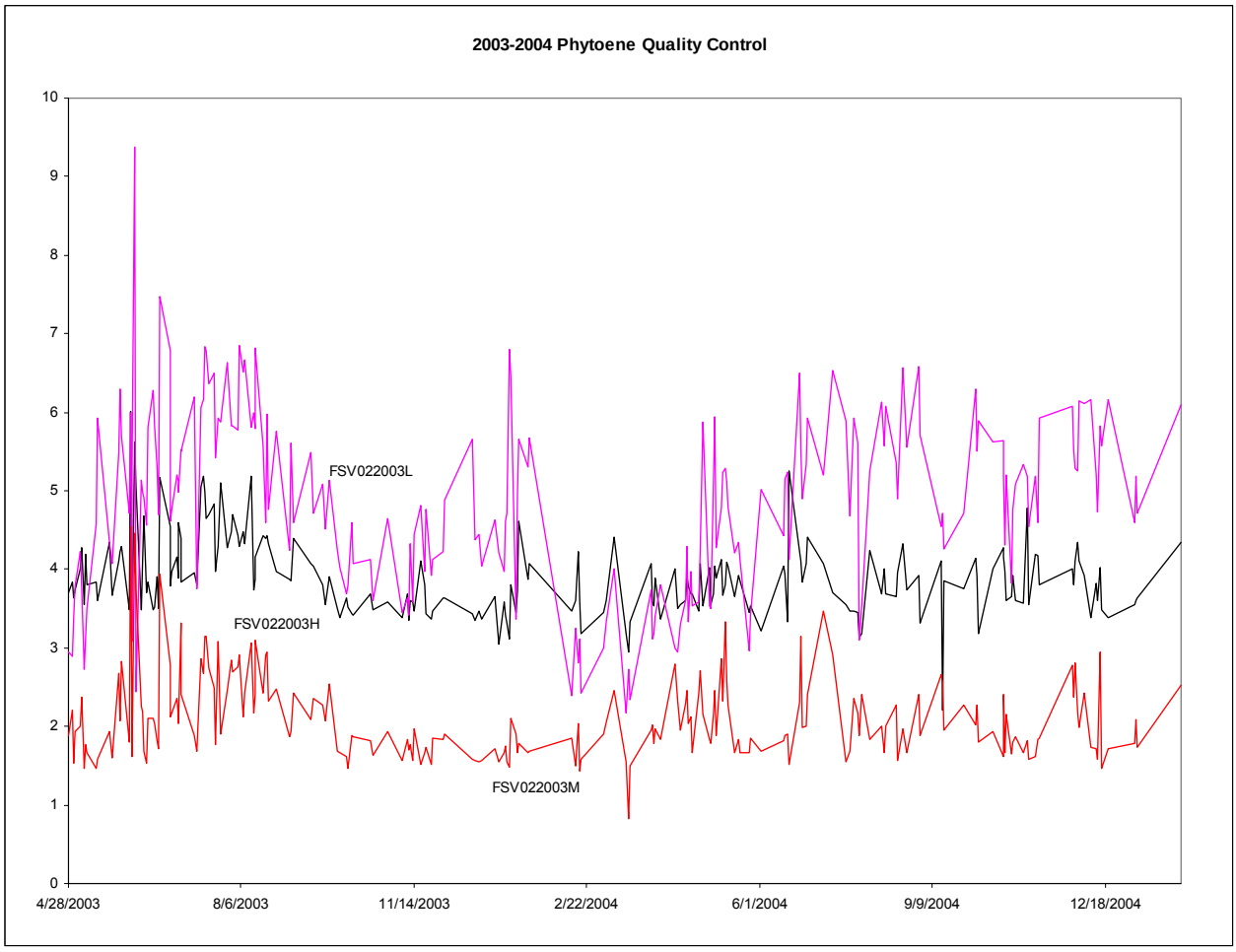
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003M	221	4/28/2003	1/31/2005	3.067	0.241	7.8
FSV022003L	221	4/28/2003	1/31/2005	3.168	0.324	10.2
FSV022003H	221	4/28/2003	1/31/2005	6.252	0.356	5.7



R. Phytoene

Summary Statistics for Phytoene by Lot

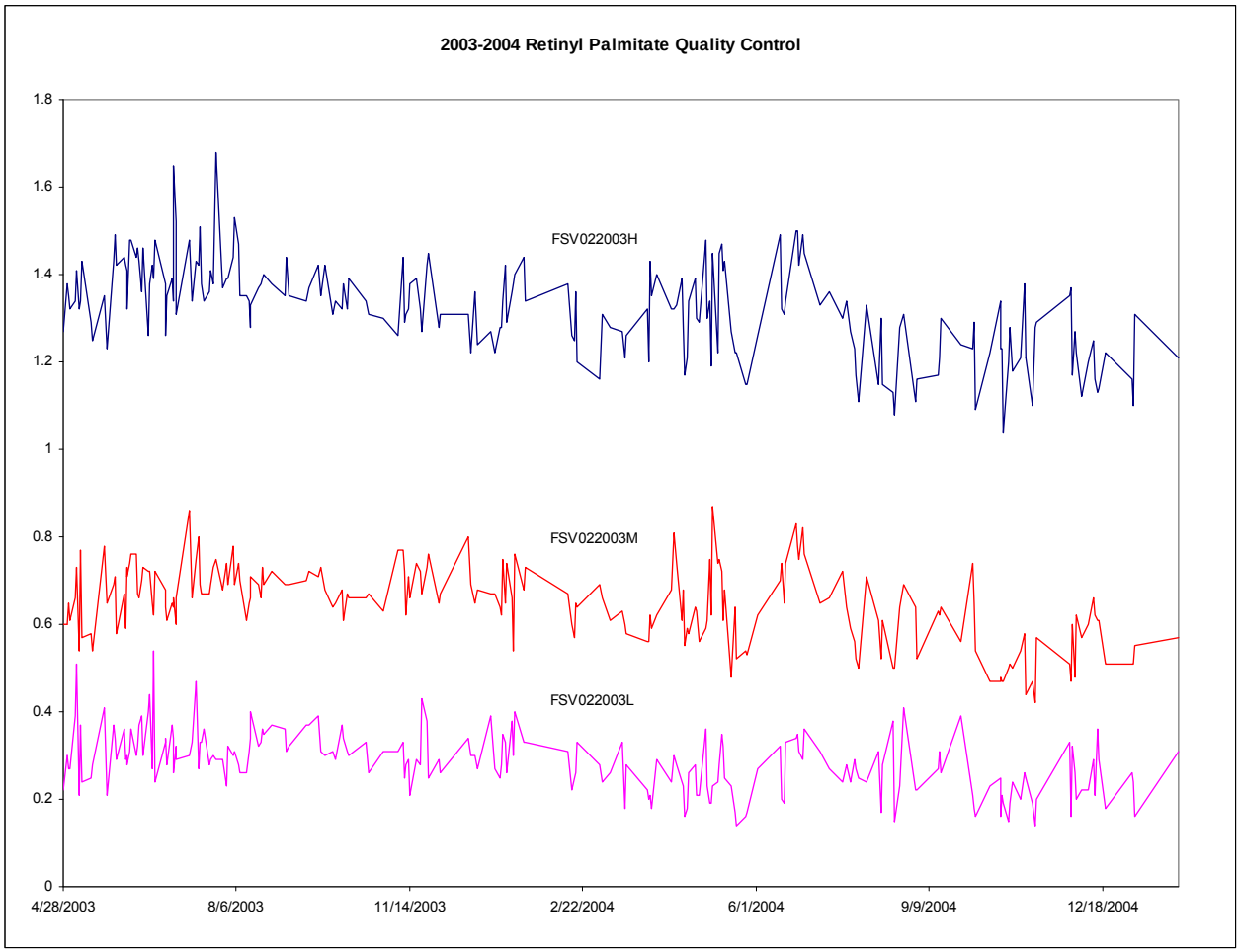
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003M	221	4/28/2003	1/31/2005	2.094	0.535	25.5
FSV022003H	221	4/28/2003	1/31/2005	3.881	0.490	12.6
FSV022003L	221	4/28/2003	1/31/2005	4.856	1.147	23.6



S. Retinyl palmitate

Summary Statistics for Retinyl Palmitate by Lot

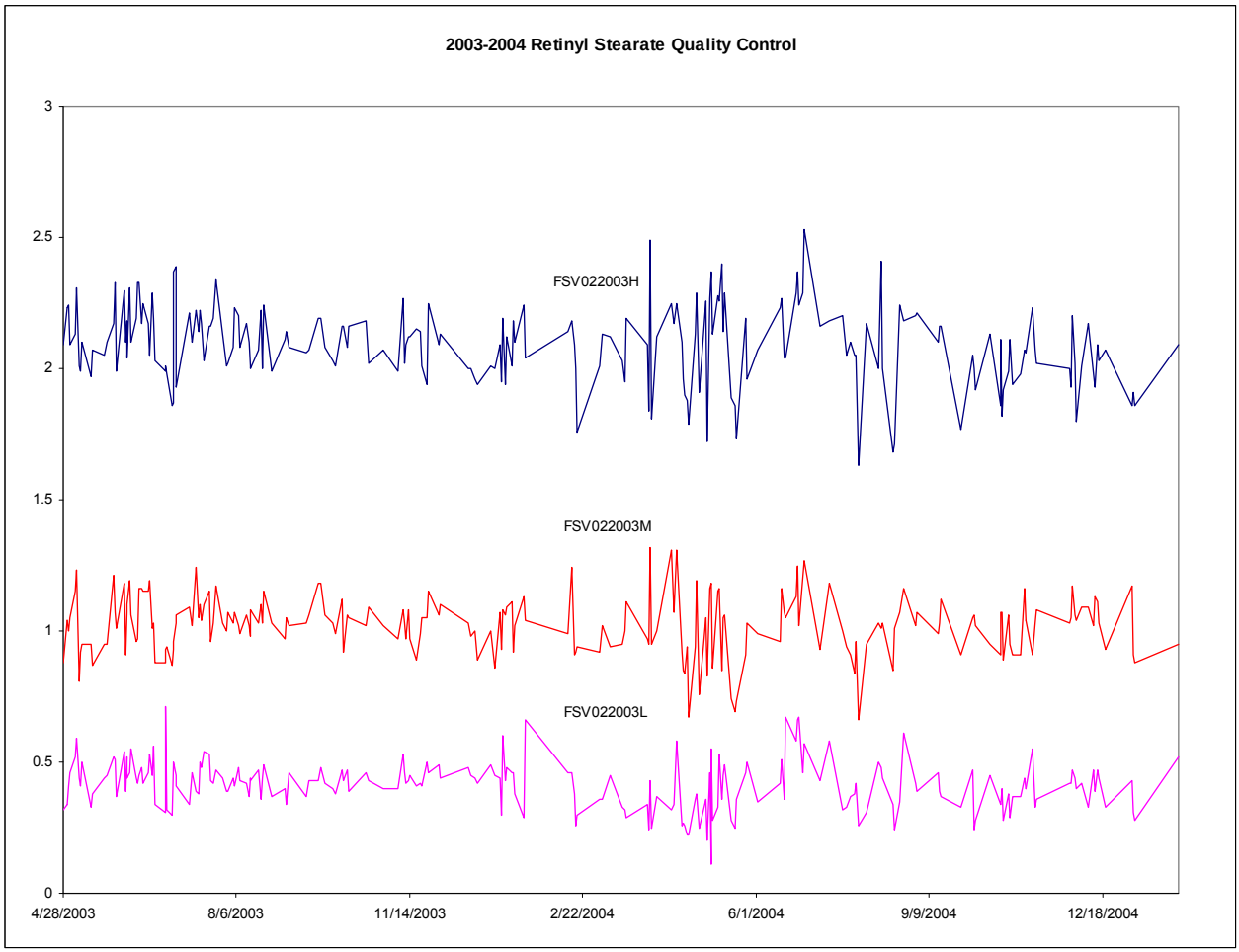
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003L	221	4/28/2003	1/31/2005	0.284	0.069	24.4
FSV022003M	221	4/28/2003	1/31/2005	0.646	0.085	13.2
FSV022003H	221	4/28/2003	1/31/2005	1.322	0.105	8.0



T. Retinyl stearate

Summary Statistics for Retinyl Stearate by Lot

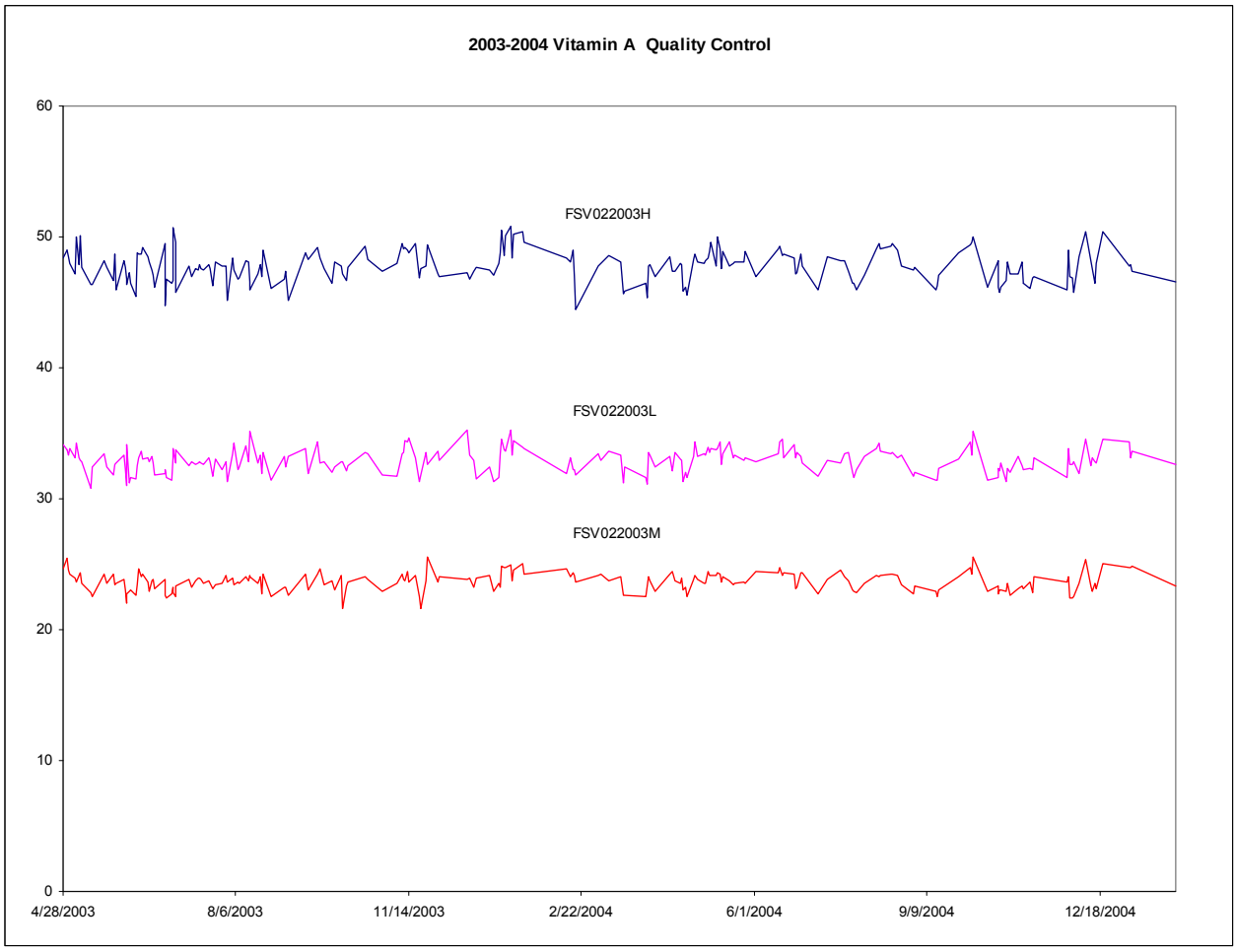
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003L	221	4/28/2003	1/31/2005	0.415	0.093	22.3
FSV022003M	221	4/28/2003	1/31/2005	1.022	0.112	11.0
FSV022003H	221	4/28/2003	1/31/2005	2.092	0.150	7.1



U. Retinol (Vitamin A)

Summary Statistics for Vitamin A (Retinol) by Lot

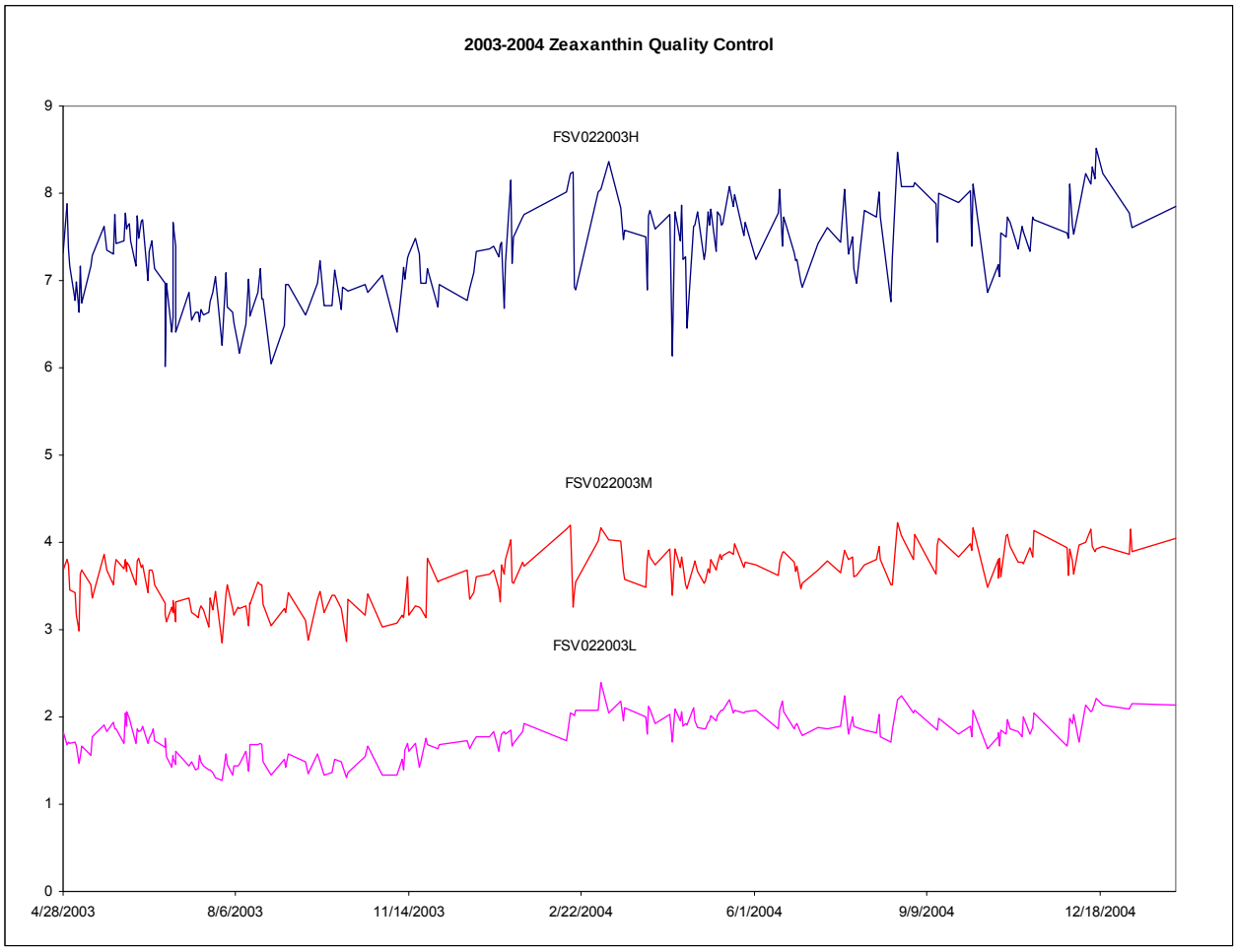
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003M	221	4/28/2003	1/31/2005	23.673	0.698	2.9
FSV022003L	221	4/28/2003	1/31/2005	32.933	0.921	2.8
FSV022003H	221	4/28/2003	1/31/2005	47.773	1.228	2.6



V. Zeaxanthin

Summary Statistics for Zeaxanthin by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
FSV022003L	221	4/28/2003	1/31/2005	1.789	0.241	13.4
FSV022003M	221	4/28/2003	1/31/2005	3.606	0.300	8.3
FSV022003H	221	4/28/2003	1/31/2005	7.325	0.513	7.0



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