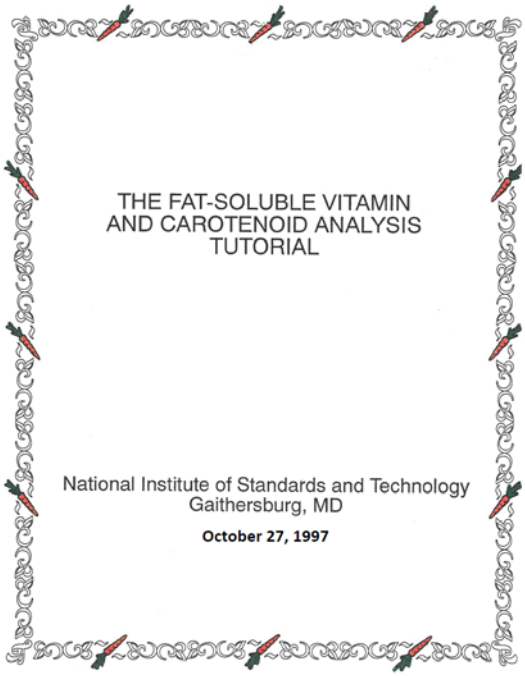
NISTIR 7880-40

NIST Micronutrients Measurement Quality Assurance Program Analysis Tutorial

Handout of the
Fat-Soluble Vitamin and Carotenoid Analysis Tutorial
October 27, 1997

Katherine E. Sharpless
Jeanice B. Thomas
David L. Duewer



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U.S. Department of Commerce
Penny Pritzker, Secretary

National Institute of Standards and Technology
Willie May, Under Secretary of Commerce for Standards and Technology and Director

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ABSTRACT

Since 1984, the National Institute of Standards and Technology (NIST) has coordinated what is now known as the Micronutrients Measurement Quality Assurance Program (MMQAP) for laboratories that measure fat-soluble vitamins and carotenoids in human serum and plasma. The MMQAP has provided participants with measurement comparability assessment through use of interlaboratory studies, Standard Reference Materials (SRMs) and control materials, methods development and validation, workshops, and tutorials. This report reproduces the printed materials provided to participants in the 1997 Fat-Soluble Vitamin and Carotenoid Analysis Tutorials, the most recent in a series of hands-on training programs for laboratorians involved in fat-soluble vitamin-related analytes. Some of the tutorial material has been updated.

KEYWORDS

Analytical methods, Retinol, Tocopherols, Carotenoids, Human serum

TABLE OF CONTENTS

INTRODUCTION

Since 1984, the National Institute of Standards and Technology (NIST) has coordinated what is now known as the Micronutrients Measurement Quality Assurance Program (MMQAP) for laboratories that measure fat-soluble vitamins and carotenoids in human serum and plasma. The MMQAP has provided participants with measurement comparability assessment through use of interlaboratory studies, Standard Reference Materials (SRMs) and control materials, methods development and validation, workshops, and tutorials. This report reproduces the printed materials provided to participants in the 1997 Fat-Soluble Vitamin and Carotenoid Analysis Tutorials, the most recent in a series of hands-on training programs for laboratorians involved in fat-soluble vitamin-related analytes. Some of the tutorial materials have been updated, and notes have been added in italics. Appendix A lists the names and chemical structures, absorptivities, and absorption wavelengths of the fat-soluble vitamin-related compounds that have been most often reported in the MMQAP interlaboratory studies, and Appendix B provides a history of SRM 968 Fat-Soluble Vitamins and Carotenoids in Human Serum.

Tutorials for fat-soluble vitamin and carotenoid analysis were held in 1994, 1995, 1996, and 1997 at NIST. Initially these tutorials were associated with workshops that were also held at NIST. When the workshops moved to venues associated with national meetings (AACC and Experimental Biology, in particular), the tutorials were eliminated. The tutorials were oriented primarily toward new laboratories or lab personnel participating in the MMQAP as well as others who were experiencing difficulties with analysis. The tutorials included discussion and demonstrations of calibration, sample preparation, and chromatographic techniques for measuring fat-soluble vitamins and carotenoids in serum.

CALIBRATION

Value of Internal Standards

- Volume correction - corrects for variable injection volume, evaporative losses of volatile solvents
- Recovery correction - corrects for spills, incomplete removal of organic phase, correction for partial solubility of organic phase in aqueous phase
- Column performance - monitor peak shape, area, and retention time in routine analysis
- Quality control - flag possible methodological errors

Internal Standard Characteristics

- Should be stable, of high purity, and readily available
- Should not react with sample components
- Should provide detector response similar to analytes'
- Should elute near analytes
- Should be completely resolved from other sample components

Internal Standards Used by Participants in the QA Program

retinyl acetate

retinyl butyrate

tocol

α-tocopheryl acetate

echinenone

ethyl-β-apo-8'-carotenoate

trans-β-apo-8'-carotenal

various oximes, including *trans*-β-apo-8'-carotenal oxime and *trans*-β-apo-10'-carotenal oxime

Synthesis of β-Apo-10'-Carotenal Oxime

- Combine β-apo-10'-carotenal dissolved in ethanol and at least 1000-fold molar excess of 1 mol/L hydroxylamine.
- Let stand overnight in the dark at room temperature.
- Extract into hexane by adding water.
- Remove hexane layer and evaporate.
- Reconstitute in ethanol.
- To purify, inject onto Vydac 201TP preparative column, mobile phase of 100% acetonitrile at 10 mL/min.
- Extract oxime from mobile phase into hexane with the addition of water.
- Remove hexane layer and evaporate.
- Reconstitute in ethanol containing 30 μg/mL.
- Store at -20 °C.

References:

G.W. T. Groenendijk, W.J. De Grip, and F.J.M. Daemen, Biochim. et Biophys. Acta, 617 (1980) 430.

G. Handelman, B. Shen, and N. Krinsky, Meth. Enzymol. 213 (1992) 336.

Analyte Ranges from Two Sets of NCI Study Samples
(approx. 1400 samples)

	Range (μg/mL)
Retinol	0.07 to 1.6
Retinyl palmitate	ND* to 1.7
Lutein	0.013 to 0.63
Zeaxanthin	0.002 to 0.20
β-Cryptoxanthin	0.001 to 0.30 (as high as 1.4 in some populations)
Lycopene	0.003 to 0.54
α-Carotene	ND* to 0.82
β-Carotene	0.003 to 2.8
δ-Tocopherol	ND to 0.90
γ-Tocopherol	ND to 11
α-Tocopherol	3.1 to 48

*ND = not detected; not present or present at levels below the limit of detection

"Normal" Physiological Concentrations

	Range (μg/mL)
Retinol	0.5 to 0.7
β-Carotene	0.18 to 0.3
α-Tocopherol	8 to 10

*Reference: Sharpless, K.S. and Duewer, D.L., "Population Distributions and Intralaboratory Variance for Fat-Soluble Vitamin-Related Compounds in Human Serum." **Anal. Chem.** 67, 4416 (1995).*

Preparation of Calibration Solutions

1. Prepare stock solutions at concentrations 5 to 10 times higher than the concentrations you'll want in your calibration solutions. The concentrations of your stock solutions do not have to be determined during this preparation step; you will be determining the concentration spectrophotometrically and correcting for impurities using chromatography.

2. Filter stock solutions and determine concentration of stock solutions using spectrophotometer.

   - blank out the solvent you're using
     (don't forget to add BHT if you used it in your stock solution)
   - run spectrum and check the maximum absorbance
   - dilute your stock solution if the maximum absorbance is approximately 1 and run the spectrum several times
   - record the absorbances at λ_{max} and take the average
     (some samples will degrade as they sit in the beam of the spectrophotometer)
   - calculate concentration using Beer's Law

   For a 1+9 dilution of β-cryptoxanthin in ethanol,

   $A = \varepsilon bc$
   $0.181 = (2356)(1)(c)$
   $c = 0.0000768$ g/dL x 1000 mg/g x 10 dL/L = 0.768 mg/L

   (Note that the absorbance here is only 0.181, which means the solution has been diluted more than it should have been.)

   Multiply by 10 to account for the 1+9 dilution. The concentration in your stock solution is 7.68 mg/L, not accounting for impurities.

3. Check filtered stock solution for impurities by running through your usual chromatographic method, but with the detector set at the wavelength where you did your Beer's Law determination (typically the wavelength of maximum absorbance for the compound you're measuring).

Analyte	λ_{max} (nm)	absorptivity (dL/g·cm)
lutein (EtOH)	445	2550
zeaxanthin (EtOH)	452	2540
β-cryptoxanthin (EtOH)	452	2356
trans-lycopene (hex)	472	3450
trans-α-carotene (hex)	444	2800
trans-β-carotene (hex)	452	2592
9-cis-β-carotene (hex)	445	2090
13-cis-β-carotene (hex)	446	2370
15-cis-β-carotene (hex)	447	1820
δ-tocopherol (EtOH)	297	91.2
γ-tocopherol (EtOH)	298	91.4
α-tocopherol (EtOH)	292	75.8
retinol (EtOH)	325	1843
retinyl palmitate (EtOH)	325	975

(EtOH = ethanol; hex = hexane)

You find that your filtered β-cryptoxanthin solution is 91.4% pure, so correct your concentration for this:

7.68 mg/L x 0.914 = 7.02 mg/L

4. Prepare calibration solutions. Say for β-cryptoxanthin, you want three calibration solutions in a range of 0.1 to 1 mg/L. To prepare 10 mL of each, you would need:

(10 mL) (0.1 mg/L) = (x mL) (7.02 mg/L): x = 0.142 mL
(10 mL) (0.5 mg/L) = (x mL) (7.02 mg/L): x = 0.712 mL
(10 mL) (1 mg/L) = (x mL) (7.02 mg/L): x = 1.42 mL

When you add your internal standard, you're going to have some dilution, so use 0.2, 0.8, and 1.5 mL to prepare your calibration solutions.

The concentrations in the calibration solutions are then:

[(0.20 mL)×(7.02 mg/L)]/10 mL = 0.140 mg/L
[(0.80 mL)×(7.02 mg/L)]/10 mL = 0.562 mg/L
[(1.50 mL)×(7.02 mg/L)]/10 mL = 1.053 mg/L

(The easiest way to do these calculations is on a spreadsheet so that everything is calculated for you automatically each time you recalibrate.)

For simplicity in this example, the calibrants have been prepared from a stock solution for which a single determination of concentration has been made. If an error is made in the determination of that concentration, that error is propagated into the entire measurement system. (This can become apparent if results for quality control materials are out of specification.) Thus, it is preferable to determine concentrations individually rather than doing serial dilutions.

5. If you don't use an internal standard, you can now inject the calibration solutions (three times each) and prepare your calibration curve.

6. If you do use an internal standard, you need to add it to an aliquot of your calibration solutions now (or you could have included it when you prepared the calibration solution). If you're adding the internal standard to an aliquot of your calibration solution, your concentrations must be corrected for the dilution caused by the addition of the internal standard. If the concentration of your internal standard is fairly high in its stock solution, you have to add less to get a good level in your calibration solutions and thus don't dilute your calibration solutions as much.

   For example, if you add 200 μL of your internal standard to 1 mL of the low calibration solution, the resulting β-cryptoxanthin concentration is:

   0.140 μg/mL x (1 mL/1.2 mL) = 0.117 μg/mL.

   For an internal standard stock concentration of 5.02 μg/mL, the resultant internal standard concentration in your calibrants will be:

   5.02 μg/mL x (0.2 mL/1.2 mL) = 0.837 μg/mL.

7. Prepare your calibration curve using the three solutions containing the internal standard.

   Calibration time is decreased if you put more than one compound in your calibration solutions. When you do this, however, you must be aware that commercial lutein preparations contain zeaxanthin as an impurity and vice versa, and α- and β-carotene also can be impurities in commercial preparations of each other. This must be taken into account when determining the final concentrations. Also, it's best to do lycopene by itself since it degrades fairly rapidly.

   If you have a lot of compounds in each calibration solution, it is often not possible to make one solution containing all the low levels, one with all the middle, and one with all the high because the volume in the high level would exceed the 10-mL total target volume. Therefore, it is sometimes necessary to mix up the levels so that the total volume of stock solution is less than 10 mL.

SAMPLE PREPARATION

Extraction Procedure

- Precipitate proteins from serum or plasma using ethanol (containing 30 μg/mL BHT anti-oxidant and internal standards)
- Vortex.
- Add hexane. Vortex to extract analytes into hexane.
- Centrifuge sample. Remove organic phase.
- Re-extract with hexane.
- Centrifuge. Remove organic phase and combine with first.
- Evaporate organic phase under nitrogen.
- Re-dissolve sample in ethanol containing 30 μg/mL BHT.

References:

Epler, K.S., Ziegler, R.G., and Craft, N.E., "Liquid Chromatographic Method for the Determination of Carotenoids, Retinoids, and Tocopherols in Human Serum and in Food." ***J. Chromatogr. (Biomed. Applications)*** *619, 37 (1993).*

Brown Thomas, J., Kline, M.C., Schiller, S.B., Ellerbe, P.M., Sniegoski, L.T., Duewer, D.L., and Sharpless, K.E., "Certification of Fat-Soluble Vitamins and Cholesterol in Human Serum: Standard Reference Material 968b." ***Fresenius J. Anal. Chem.*** *356, 1 (1996).*

Brown Thomas, J., Kline, M.C., Gill, L.M., Yen, J.H., Duewer, D.L., Sniegoski, L.T., and Sharpless, K.E., "Preparation and Value Assignment of Constituents in Standard Reference Material 968c: Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum," ***Clin. Chim. Acta*** *305, 141-155 (2001).*

EXTRACTANT	COMMENTS
Ethyl acetate/THF	May see sample-to-sample variability since solvent partitioning is not well defined; THF is not the ideal solvent for extractions since it forms peroxides, thereby leading to degradation of vitamin A and carotenoids.
Ethyl acetate/Butanol	Gives good recovery of carotenoids and effectively overcomes lipid binding.
*Hexane	Effectively competes with serum lipids for solvation of the analytes and thereby overcomes lipid binding. Hexane is not compatible with most common reversed-phase methods' mobile phases and must be evaporated under a steam of inert gas.
Alkaline hydrolysis	Method works for β-carotene analysis. Lower concentration (<1 mol/L) of KOH solution does not extract all the carotenoids when using volumes of serum greater than 0.5 mL. Additional free retinol recovered by hydrolysis of serum retinol esters cause erroneous results for all-*trans*-retinol determination. α-Tocopherol is converted to the hydrophilic phenolate anion, thereby making it impossible to recover α-tocopherol from the alkaline hydrolysate.

Storage and Handling Recommendations

- Store samples in amber vials.
- Minimize exposure to light and air.
- Store samples below room temperature (-20 °C or -80 °C). If carotenoids will be measured, -80 °C storage is preferable; carotenoids are less stable than retinoids and the tocopherols at -20 °C.
- Reconstituted serum should be used immediately or stored at -20 °C for no more than 3 days.
- Minimize repeated thawing and refreezing of serum (<5 freeze/thaw cycles).

*Reference: Brown Thomas, J., Kline, M.C., Duewer, D.L., and Sharpless, K.E. "The Stability of Fat-Soluble Vitamins and Carotenoids in Human Serum," **Clin. Chim. Acta** 276, 75 (1998).*

Effect of Light Exposure and Other Extrinsic Factors on Fat-Soluble Vitamins in Serum

SOURCE	EFFECT
Direct sunlight	Causes vitamin A and carotenoids to decompose
Artificial light	Oxidation of the analytes is minimal but evident during short-term (2 hour) exposure. As a safety precaution, direct exposure of samples to light should be avoided.
Air, heat, metal ions	Vitamin A and carotenoids are readily destroyed by oxidation in the presence of air. Heat and the presence of metal ions such as iron and copper also add to this phenomenon.

QUALITY CONTROL

Standard Reference Material 968c
Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum

Certified:	Non-certified:
retinol	trans-lutein
trans-β-carotene	total lutein
total β-carotene	total zeaxanthin
δ-tocopherol	total β-cryptoxanthin
γ-tocopherol (includes β-tocopherol)	trans-lycopene
α-tocopherol	total lycopene
cholesterol	trans-α-carotene
total α-carotene	25-hydroxyvitamin D

SRM 968c is no longer available. SRM 968e is the currently available (2016) version. Table B1 in Appendix B describes the production history of the SRM 968 versions. Table B2 identifies the analyte information delivered by each version.

There are also several other SRMs of potential relevance to those interested in measurement of fat-soluble vitamin-related analytes. Table B3 in Appendix B lists these materials.

The information on how to order NIST SRMs provided in the original tutorial is no longer valid and is not reproduced. To obtain information on, and to order, NIST's currently available SRMs, visit http://www.nist.gov/srm/index.cfm and enter "vitamin" in the "Keyword" search box.

CHROMATOGRAPHY

In the early 1990s we tested 65 reversed-phase columns to evaluate the selectivity of various C_{18} stationary phases with methanol- and acetonitrile-based mobile phases and to see whether the carotenoids eluted from the columns or were retained. Much of the content of this section is drawn from the results of that study, published in:

> *Epler, K.S., Sander, L.C., Wise, S.A., Craft, N.E., and Ziegler, R.G., "Evaluation of Reversed-Phase Liquid Chromatography Columns for Recovery and Selectivity of Selected Carotenoids."* ***J. Chromatogr.*** *595, 89 (1992).*

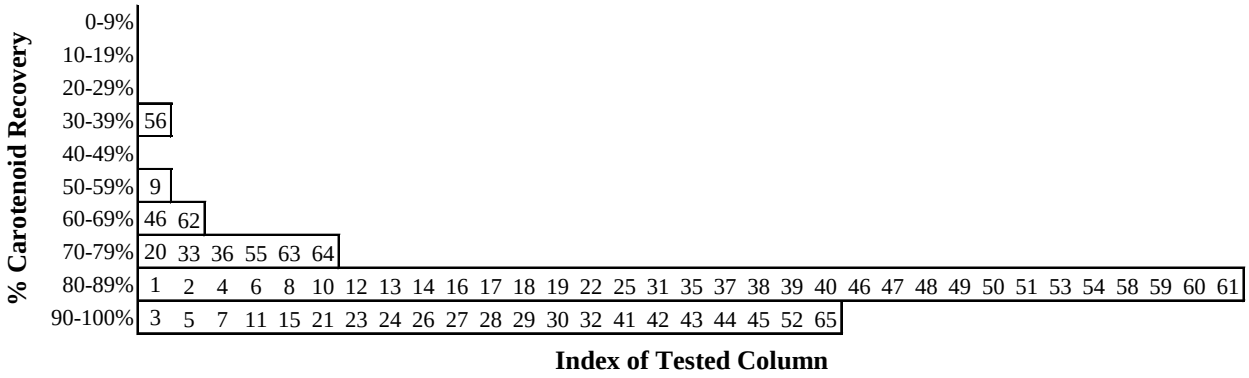
This publication is included in the freely available compendeum:

> *Methods for Analysis of Cancer Chemopreventive Agents in Human Serum, Brown Thomas, J. and Sharpless, K.E., editors, NIST Special Publication 874, U.S. Government Printing Office Washington: 1995*

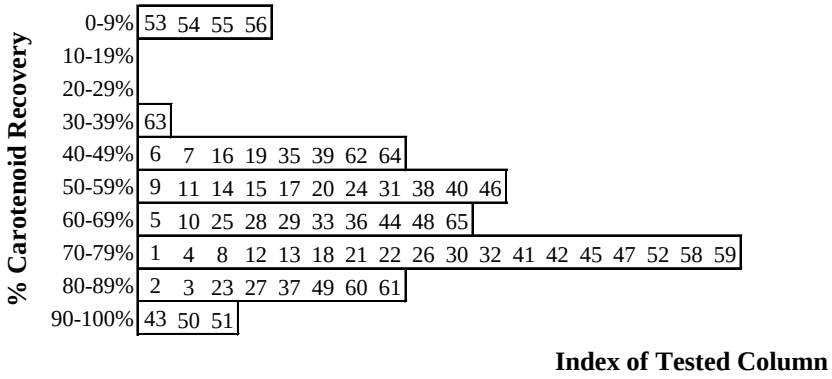
Percent Recovery of Carotenoid Mixture on Tested Columns

Mobile Phase

Using Methanol or THF/Methanol:



Using Acetonitrile or THF/Acetonitrile:



THF = tetrahydrofuran

Table 1: Reversed-Phase Columns Tested

Columns are C_{18} except where noted.

Supplier	Column name	RR[a]	Class[b]
Beckman	Ultrasphere ODS	1.92	Monomeric
Beckman	Ultrasphere ODS	2.01	Monomeric
Beckman	Ultrasphere ODS DABS	2.00	Monomeric
Analytichem	Sepralyte C18	1.82	Monomeric
Separations Group	Vydac 218TP	0.83	Polymeric
Separations Group	Vydac 201TP	0.78	Polymeric
Separations Group	Vydac 201TP	0.80	Polymeric
Biotage	Unisphere-PBD[c]	3.06	Monomeric
ES Industries	Gamma Bond C18	1.86	Monomeric
ES Industries	Chromegabond C22 (C_{22})	1.83	Monomeric
ES Industries	Chromegabond MC18	1.73	Monomeric
Supelco	LC-18	2.03	Monomeric
Supelco	LC-18	2.02	Monomeric
Supelco	LC318	2.05	Monomeric
Supelco	LC318	2.04	Monomeric
Supelco	LC-PAH	0.70	Polymeric
Analytichem	Sepralyte C18	1.92	Monomeric
Alltech	Adsorbosphere C18	2.01	Monomeric
EM Science	LiChrospher 100 RP-18	1.45	Intermediate
Serva	Octadecyl	1.84	Monomeric
Serva	Triacontyl (C_{30})	[d]	
J&W	Accusphere ODS	2.07	Monomeric
J&W	Accusphere ODS	1.96	Monomeric
MacMod	Zorbax RX C8 (C_8)	2.33	Monomeric
Perkin Elmer	Pecosphere C18	2.00	Monomeric
YMC	A303	1.97	Monomeric
YMC	AMP303	2.01	Monomeric
YMC	AP303	2.06	Monomeric
J.T. Baker	exper WP C18[e]	0.57	Polymeric
J.T. Baker	exper WP C18[e]	0.93	Polymeric
J.T. Baker	exper WP C18[e]	0.73	Polymeric
J.T. Baker	exper WP C18[e]	1.42	Intermediate
J.T. Baker	exper WP C18[e]	0.22	Polymeric

Supplier	Column name	RR[a]	Class[b]
ES Industries	Chromegabond PFP[f]	0.88	Polymeric
ES Industries	Chromegabond BF-C18	1.04	Intermediate
J.T. Baker[g]	Bakerbond C18	1.25	Intermediate
J.T. Baker[g]	Bakerbond WP C18	0.51	Polymeric
J.T. Baker[g]	Bakerbond WP C18	0.54	Polymeric
J.T. Baker[g]	Bakerbond WP C18	1.09	Intermediate
Keystone Sci.	ODS Hypersil	1.95	Monomeric
Shiseido	Capsell Pak C18	1.99	Monomeric
Nacalai Tesque	Cosmosil 5C18-P Waters	2.04	Monomeric
Nacalai Tesque	Cosmosil 5C18 Waters	1.85	Monomeric
Waters	Nova-Pak C18	1.97	Monomeric
YMC	ASP303-S	2.05	Monomeric
Hewlett-Packard	ODS Hypersil	1.98	Monomeric
Hewlett-Packard	LiChrospher 100 RP-18	1.50	Intermediate
Brownlee	Spheri-S ODS	1.26	Intermediate
Bio-Rad	Hi-Pore RP318	0.59	Polymeric
Macherey-Nagel	Nucleosil 5 PAH	0.36	Polymeric
Rainin[g]	Microsorb C18	1.78	Monomeric
Phase Separations	Spherisorb SS PAH	0.82	Polymeric
Phase Separations	Spherisorb SS ODS	1.68	Intermediate
Phase Separations	Spherisorb SS ODS	1.50	Intermediate
Carlo Erba	Erbasil 5 C18/L	1.76	Monomeric
Carlo Erba	Erbasil 5 C18/M	1.28	Intermediate
Carlo Erba[h]	Erbasil 5 C18/H	0.91	Polymeric
Brownlee	Spheri-5 RP-18	1.92	Monomeric
MacMod	Zorbax RX C18	1.50	Intermediate
Phenomenex	Ultracarb 5 ODS20	1.95	Monomeric
Phenomenex	Ultracarb 5 ODS30	2.01	Monomeric
J.T. Baker	Bakerbond WP C18	0.89	Polymeric
J.T. Baker	Bakerbond WP C18	0.67	Polymeric
Brownlee	Spheri-5 ODS	1.42	Intermediate
MacMod[g]	Zorbax ODS	1.80	Monomeric

a Relative retention of 1,2,3,4,5,6,7,8-tetrabenzonaphthalene (TBN) to benzo[*a*]pyrene (BaP)
b As determined by evaluation of SRM 869.
c PBD = polybutadiene.
d Returned to supplier before value could be determined.
e Experimental columns-with varying surface coverage.
f PFP = pentafluorophenol.
g Used in laboratory prior to evaluation in this study.
h Not tested due to excessive backpressure (> 200 bar).

Reference: Epler, K.S., Sander, L.C., Wise, S.A., Craft, N.E., and Ziegler, R.G., "Evaluation of Reversed-Phase Liquid Chromatography Columns for Recovery and Selectivity of Selected Carotenoids." ***J. Chromatogr.*** *595, 89 (1992).*

Average Percent Recovery of Carotenoid Mixture

Stationary Phase

Eluent	Polymeric	Intermediate	Monomeric
MeOH and THF/MeOH	83%	79%	88%
ACN and THF/ACN	62%	46%	67%

ACN = acetonitrile; MeOH = methanol; THF = tetrahydrofuran

Reference: Epler, K.S., Sander, L.C., Wise, S.A., Craft, N.E., and Ziegler, R.G., "Evaluation of Reversed-Phase Liquid Chromatography Columns for Recovery and Selectivity of Selected Carotenoids." ***J. Chromatogr.*** *595, 89 (1992).*

Percent Recovery of Carotenoid Mixture

Buffer Treatment

Column	Recovery before buffer treatment	Recovery after buffer treatment	Recovery after buffer, 0.05% TEA in eluent
1	48%	74%	102%
2	1%	30%	>99%
3	7%	87%	106%
4	15%	69%	104%
5	3%	12%	>87%

TEA = trimethylamine

Reference: Epler, K.S., Sander, L.C., Wise, S.A., Craft, N.E., and Ziegler, R.G., "Evaluation of Reversed-Phase Liquid Chromatography Columns for Recovery and Selectivity of Selected Carotenoids." ***J. Chromatogr.*** *595, 89 (1992).*

Recovery of a Mixture of Carotenoids from the Bakerbond C_{18} Column

In the column comparison study:

100% methanol	75%
5% THF/95% acetonitrile	66%

Using a gradient of acetonitrile, methanol, and ethyl acetate:

No ammonium acetate, no TEA	68%
0.05 mol/L NH_4OAc in the methanol	92%
0.1% TEA in all solvents, no NH_4OAc in the methanol	89%
0.1% TEA in all solvents, 0.05 mol/L NH_4OAc in the methanol	101%
0.05% TEA in all solvents, 0.05 mol/L NH_4OAc in the methanol	94%

NH_4OAc = ammonium acetate; TEA = trimethylamine; THF = tetrahydrofuran

Reference: Epler, K.S., Sander, L.C., Wise, S.A., Craft, N.E., and Ziegler, R.G., "Evaluation of Reversed-Phase Liquid Chromatography Columns for Recovery and Selectivity of Selected Carotenoids." ***J. Chromatogr.*** *595, 89 (1992).*

Critical Factors in Chromatographic Analysis of Carotenoids

- Use polymeric (or some intermediate) C_{18} columns if you want to be able to separate lutein and zeaxanthin.
- Use a monomeric column, which on average provides better recovery than polymeric columns, if you do not need to measure lutein and zeaxanthin.
- Use methanol-based solvent systems rather than acetonitrile-based systems.
- If you have to use an acetonitrile-based system, add ammonium acetate and triethylamine to improve carotenoid recovery.
- Stainless steel frits may cause on-column carotenoid losses.
- Residual acids and metal activity on column packing materials cause carotenoid losses.
- Be aware that there are a lot of carotenoid peaks in serum.

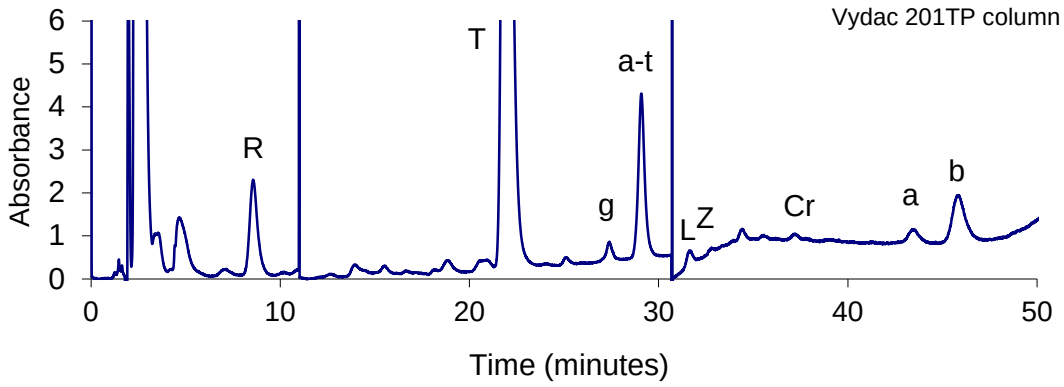
Reference: *Methods for Analysis of Cancer Chemopreventive Agents in Human Serum, Brown Thomas, J. and Sharpless, K.E., editors, NIST Special Publication 874, U.S. Government Printing Office Washington: 1995*

Column: Vydac 201TP

Gradient: 67:10:23 / 86.5:10:3.5 Methanol:Butanol:Water with 10 mmol/L HOAc (pH 5.5) over 25 min

Flow rate: 1.5 mL/min

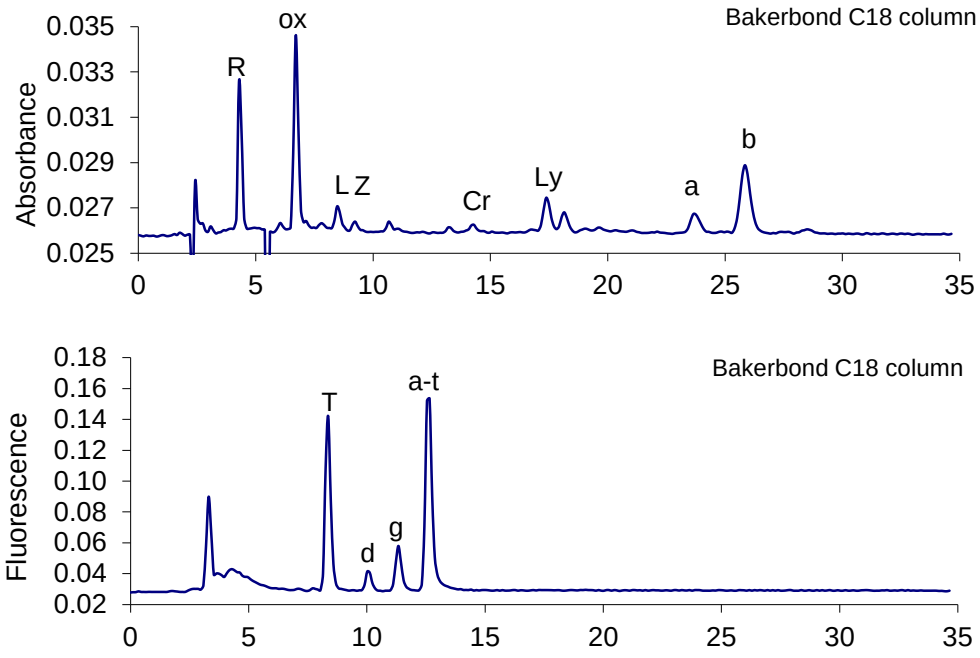
Detection: Retinol - 325 nm
Tocopherols - 292 nm
Carotenoids - 452 nm



R = retinol
T = tocol
g = γ-tocopherol
a-t = α-tocopherol
L = lutein
Z = zeaxanthin
Cr = β-cryptoxanthin
a = α-carotene
b = β-carotene

Reference: Brown Thomas, J., Kline, M.C., Gill, L.M., Yen, J.H., Duewer, D.L., Sniegoski, L.T., and Sharpless, K.E., "Preparation and Value Assignment of Constituents in Standard Reference Material 968c: Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum," ***Clin. Chim. Acta*** *305, 141-155 (2001).*

Column:	Bakerbond C_{18}
Guard column:	Vydac 201TP
Temperature:	29 °C
Flow rate:	1.5 mL/min
Solvent A:	acetonitrile containing 0.05% triethylamine (TEA)
Solvent B:	methanol containing 0.05 mol/L ammonium acetate and 0.05% TEA
Solvent C:	ethyl acetate containing 0.05% TEA
Gradient:	98% A/2% B to 75% A/18% B/7% C at 10 min 75% A/18% B/7% C to 68% A/25% B/7% C at 15 min Hold at 68% A/25% B/7% C for 15 min Return to initial conditions (98% A/2% B) over 5 min Re-equilibrate for 5 min
Detection:	Retinol and retinyl palmitate - absorbance at 325 nm Carotenoids - absorbance at 450 nm Tocopherols - fluorescence - excitation at 295 nm, emission at 335 nm

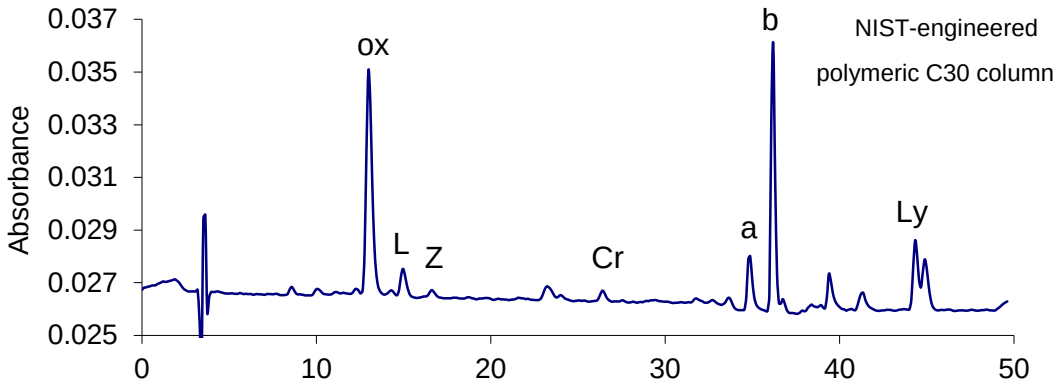


R = retinol
ox = *trans*-β-apo-8'-carotenal oxime (internal standard)
L = lutein
Z = zeaxanthin
Cr = β-cryptoxanthin
Ly = lycopene

a = α-carotene
b = β-carotene
T = tocol
d = δ-tocopherol
g = γ-tocopherol
a-t = α-tocopherol

Reference: Brown Thomas, J. et al., ***Clin. Chim. Acta*** *305, 141-155 (2001).*

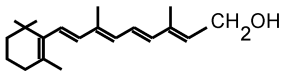
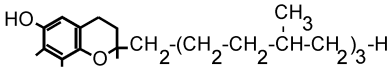
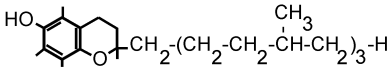
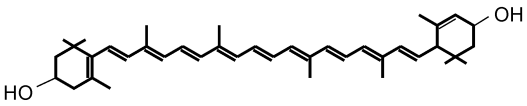
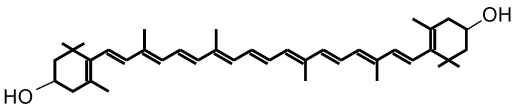
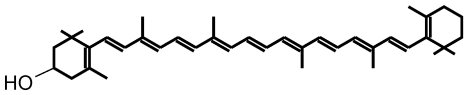
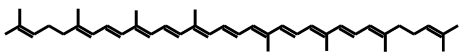
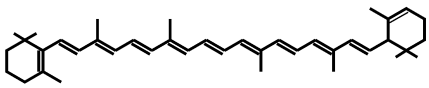
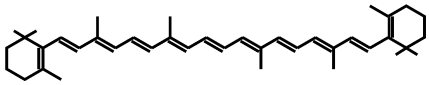
Column:	polymeric C_{30} column (3-µm particles, 15-cm length) prepared in-house
Guard column:	in-line titanium frit
Temperature:	room temperature
Flow rate:	1.5 mL/min
Solvent A:	8% water/ 92% methanol containing 0.05 M ammonium acetate and 0.05% triethylamine
Solvent B:	methyl t-butyl ether
Gradient:	83% A/17% B to 59% A/41% B at 29 min 59% A/41% B to 30% A/70% B at 34 min Hold at 30% A/70% B for 11 min Return to initial conditions (83% A/17% B) over 5 min Re-equilibrate for 10 min
Detection:	Carotenoids - absorbance at 450 nm



ox = *trans*-β-apo-8'-carotenal oxime (internal standard)
L = lutein
Z = zeaxanthin
Cr = β-cryptoxanthin
a = α-carotene
b = β-carotene
Ly = lycopene

*Reference: Brown Thomas, J., Kline, M.C., Gill, L.M., Yen, J.H., Duewer, D.L., Sniegoski, L.T., and Sharpless, K.E., "Preparation and Value Assignment of Constituents in Standard Reference Material 968c: Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum," **Clin. Chim. Acta** 305, 141-155 (2001).*

APPENDIX A: Chemical Structures and Absorptivities[1]

COMPOUND	STRUCTURE	λ_{max}	ABSORPTIVITY
trans-retinol		325 nm	1843 dL/g·cm in ethanol
γ-tocopherol		298 nm	91.4 dL/g·cm in ethanol
α-tocopherol		292 nm	75.8 dL/g·cm in ethanol
trans-lutein		445 nm	2550 dL/g·cm in ethanol
trans-zeaxanthin		452 nm	2540 dL/g·cm in ethanol
trans-β-cryptoxanthin		452 nm	2356 dL/g·cm in ethanol
trans-lycopene		472 nm	3450 dL/g·cm in hexane
trans-α-carotene		444 nm	2800 dL/g·cm in hexane
trans-β-carotene		452 nm	2592 dL/g·cm in hexane

[1]Absorptivities were taken from the following references:

Schierle, J.; Härdi, W.; Faccin, N.; Bühler, I.; Schüep, W.; *Geometrical Isomers of β,β-Carotene*; In: *Carotenoids: Isolation and Analysis*; Britton, G., Liaaen-Jensen, S., and Pfander, H., eds., Basel: Birkhäuser Verlag, Vol. 1A, pp. 265-272 (1995).

Robeson, C.D.; Cawley, J.D.; Weisler, L.; Stern, M.H.; Eddinger, C.C.; Chechak, A.J.; *The Synthesis of Geometric Isomers of Vitamin A via Methyl β-Methylglutaconate*; J. Am. Chem. Soc., Vol. 77**,** p. 4111 (1955).

Windholz, M.; ed., *The Merck Index of Chemicals and Drugs*; 9th ed., Rahway, NJ, Merck, p. 1221 (1976).

Schudel, P.; Mayer, H.; Isler, O.; *Tocopherols*; In: *The Vitamins: Chemistry, Physiology, Pathology, Methods*; Sebrell, W.H., Jr.; Harris, R.S.; eds., New York, Academic Press, pp. 168-218 (1967).

DeRitter, E.; Purcell, A.E.; *Carotenoid Analytical Methods*; In: *Carotenoids as Colorants and Vitamin A Precursors*, Bauernfeind, J.C., ed., Orlando, FL, Academic Press, pp. 883-923 (1981).

Absorptivity for β-cryptoxanthin in ethanol is calculated from the values for β-cryptoxanthin in petroleum ether and β-carotene in ethanol and petroleum ether provided in DeRitter and Purcell.

APPENDIX B: Fat-Soluble Vitamins SRMs

Table B1: Production History of the SRM 968 series

Version	Issued	Number Levels	Number Analytes	Form	Spiked Analytes
968	1989	3	5	Lyophilized	Retinol, Tocopherols
968a	1991	3	12	Lyophilized	Retinol, Tocopherols
968b	1995	3	15	Lyophilized	Retinol, Tocopherols
968c	1999	2	21	Lyophilized	Retinol, Tocopherols
968d	2008	1	12	Liquid frozen	None
968e	2010	3	17	Liquid frozen	None
968f	2017?	2	??	Liquid frozen	None

Table B2: Analyte Information Delivered by the SRM 968 series

Analyte	968	968a	968b	968c	968d	968e
Retinol	C	C	C	C	C	C
Retinyl Palmitate			C	NC		
α-Tocopherol	C	C	C	C	C	C
γ/β-Tocopherol	NC	NC	NC	C	C	C
δ-Tocopherol			NC	C		NC
Total β-Carotene	C	C	C	C	C	C
Trans-β-Carotene	NC	NC	C	C		NC
Total *cis*-β-Carotene				NC		NC
Total α-Carotene			C	NC	NC	NC
Trans-α-Carotene		NC	NC	NC		
Total Lycopene		NC	NC	NC	NC	NC
Trans-Lycopene		NC	NC	NC	NC	NC
Total α-Cryptoxanthin		NC		NC		NC
Total β-Cryptoxanthin			NC	NC	NC	C
Total Lutein		NC	C	NC	NC	C
Trans-Lutein				NC		
Total Zeaxanthin		NC	NC	NC	NC	C
Phylloquinone (Vitamin K)						NC
Coenzyme Q_{10}					NC	NC
25-Hydroxyvitamin D				NC		
25-Hydroxyvitamin D_2				NC		
25-Hydroxyvitamin D_3				NC		
Cholesterol		NC	C	C	C	C

C: Certified analyte value (traceable to the SI)

NC: Non-certified value (traceable to the material if analyzed with method(s) similar to those used to provide the value

Table B3: Other SRMs of Potential Interest

Number	Name
972a	Vitamin D Metabolites in Frozen Human Serum
1950	Metabolites in Frozen Human Plasma
2972a	25-Hydroxyvitamin D Calibration Solutions
2973	Vitamin D Metabolites in Frozen Human Serum (High Level)
3950	Vitamin B6 in Frozen Human Serum

These materials are available as of July 2016. For further information on these materials, visit
http://www.nist.gov/srm/index.cfm
and enter the SRM number in the "SRM/RM Number:" search box, click the hyperlink listed under the "Description" header, then click the hyperlink next to the "Certificate" bullet. Pricing and ordering information is also provided on the webpage containing the "Certificate" bullet.

To obtain information on all currently available vitamin-related SRMs, visit
http://www.nist.gov/srm/index.cfm
and enter "vitamin" in the "Keyword" search box and follow the relevant hyperlinks.