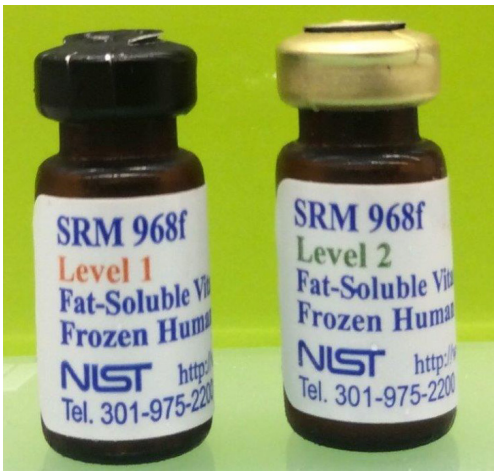
NIST Special Publication 260-188

Certification of Standard Reference Material® 968f

Fat-Soluble Vitamins in Frozen Human Serum



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Abstract

Standard Reference Material (SRM) 968f is intended for use in validating methods for determining fat-soluble vitamins in human serum and plasma and value assigning in-house produced control materials analyzed using those methods. A unit of SRM 968f consists of one vial each of two concentration levels of frozen human serum. This publication documents the production, analytical methods, and statistical evaluations involved in realizing this product.

Keywords

Human serum;
Standard Reference Material (SRM);
Retinol (Vitamin A); α-Tocopherol (Vitamin E); γ+β-Tocopherol

Technical Information Contact for this SRM

Please address technical questions about this SRM to srms@nist.gov where they will be assigned to the appropriate Technical Project Leader responsible for support of this material. For sales and customer service inquiries, please contact srminfo@nist.gov.

Table of Contents

Purpose and Description

This Standard Reference Material (SRM) is intended for use in validating methods for determining fat-soluble vitamins in human serum and plasma and qualifying control materials produced in-house and analyzed using those methods. A unit of SRM 968f consists of one vial each of two concentration levels of frozen human serum, each vial containing at least 1.05 mL serum.

NIST is guided by and adheres to the ethical principles set forth in the Belmont Report [1]. SRM 968f was developed after an appropriate human subjects' research determination.

Warning: SRM 968f is a Human Source Material

SRM 968f is a human source material. *Handle as a biohazardous material capable of transmitting infectious disease.*

SRM 968f was prepared from source plasma obtained from Interstate Blood Bank, Inc., Memphis, TN, USA. Each donor unit of plasma used in the preparation of this product was tested by FDA-licensed tests and found to be negative for human immunodeficiency virus (HIV), HIV 1 antigen, hepatitis B surface antigen, and hepatitis C. However, no known test method can offer complete assurance that hepatitis B virus, hepatitis C virus, HIV, or other infectious agents are absent from this material. Accordingly, this human blood-based product should be handled at the biosafety level 2 or higher as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control and Prevention/National Institutes of Health (NIH) Manual [2].

Preparation

All plasma units used to prepare SRM 968f were stored at −80 °C or shipped on dry ice until use. Levels of total retinol, α-tocopherol, and γ+β-tocopherol were measured at NIST in tubes of plasma obtained from the individual units at the time of plasmapheresis, and blending protocols were specified to result in two materials with different concentration levels of these analytes. The plasma was shipped by NIST to Solomon Park Research Laboratories (Kirkland, WA, USA), where it was thawed and filtered through filter paper twice to convert it to serum. The serum was pooled, blended, bottled in 1.05-mL aliquots, and stored at −80 °C prior to shipment back to NIST. Analyte concentrations were *not* adjusted by spiking.

Storage and Use

Until required for use, SRM 968f should be stored in the dark at or between −20 °C and −80 °C. If carotenoids are to be measured, the unit should be stored at or below −70 °C in the dark. Carotenoids appear to be less stable than the retinoids and the tocopherols at −20 °C [3].

The frozen vials of serum should be allowed to thaw at room temperature for at least 30 min under subdued light. The contents of a vial should then be gently mixed prior to removal of a test portion for analysis. Precautions should be taken to avoid exposure to strong ultraviolet (UV) light and direct sunlight. The certification only applies to the initial use. The same results are not guaranteed if the remaining material is used later. Results obtained in

analyses should include their own estimates of uncertainty and can be compared to the certified values using procedures described in [4, 5].

History and Background

Standard Reference Material (SRM) 968f Fat-Soluble Vitamins in Frozen Human Serum is the seventh member of the SRM 968 series of certified reference materials [6-14]. These materials were developed to help address the clinical, epidemiological, and nutritional communities' need for well-characterized reference materials. Table 1 summarizes preparative and sales characteristics of each of the SRM 968 series.

Table 1: Preparative and Sales Characteristics of the SRM 968 Series

Parameter	**Value**						
SRM Series	968	968a	968b	968c	968d	968e	968f
Issued	1989	1991	1995	1999	2008	2010	2017
Number Levels / Unit	3	3	3	2	1	3	2
Number Vials / Level	2	2	1	1	2	1	1
Matrix [a]	Ly	Ly	Ly	Ly	LqFz	LqFz	LqFz
Augmented Analytes [b]	R,α	R,α	R,α,γ,rp	R,α,γ,δ	Native	Native	Native
Serum / Vial, mL	1.00	1.00	1.00	1.00	1.00	1.00	1.1
Vial Volume, mL	3.5	3.5	3.5	2	10	5	2
Total Units Sold	>218[c]	642	1248	2407	257	1959	TBD
Average Units / Year Sold	150	225	302	292	138	322	TBD

a) Ly = Lyophilized; LqFz = Liquid Frozen

b) R = *trans*-Retinol, α = α-Tocopherol, γ = γ-Tocopherol, δ = δ-Tocopherol, rp = Retinyl Palmitate, Native = endogenous levels of all analytes. Not all levels of a given SRM were augmented, nor were all augmented levels necessarily augmented with the same analytes.

c) Incomplete total: SRM 968 was issued 4/21/89, the first available sales record is 5/24/1990.

The number of measurands reported in the Certificates of Analysis (COA) for the various SRM 968 reference materials has evolved over time, as has the status of their values. Table 2 lists the measurands and, if reported, whether their values were certified or non-certified.

Table 2: Measurands Listed in Certificates of Analysis for the SRM 968 Series

Measurand	Status of Listed Values[a]						
	968	968a	968b	968c	968d	968e	968f
Trans-Retinol				C			
Total Retinol[b]	C	C	C		C	C	C
Retinyl Palmitate			C	I			O
α-Tocopherol	C	C	C	C	C	C	C
γ+β-Tocopherol[b]	I	I	I	C	C	C	C
δ-Tocopherol			I	C		I	
Trans-β-Carotene	I	I	C	C		R	O
Total β-Carotene	C	C	C	C	C	C	O
Total *cis*-β-Carotene						I	O
9-*cis*-β-Carotene			I				
13+15-*cis*-β-Carotene			I				
15-*cis*-β-Carotene			I				
Trans-α-Carotene			I	R			
Total α-Carotene[b]		I	C	R	R	R	O
Total α-Cryptoxanthin				I		I	
Total β-Cryptoxanthin[b]		I	I	R	R	C	O
Total *cis*-β-Cryptoxanthin				I			
Trans-Lutein				R			
Total Lutein[b]		I	C	R	R	C	O
Trans-2',3'-Anhydrolutein				I			
Total Zeaxanthin[b]		I	I	R	R	C	O
Total Lutein+Zeaxanthin[b]					R		
Trans-Lycopene[b]		I	I	R	I	R	O
Total Lycopene		I	I	R	R	R	O
Phytofluene				I			
25-Hydroxyvitamin D				R			O
25-Hydroxyvitamin D_2				I			O
25-Hydroxyvitamin D_3				I		C	O
3-*epi*-25-Hydroxyvitamin D_3							O
Phylloquinone (Vitamin K_1)						I	O
Coenzyme Q_{10}					I	I	O
Cholesterol		I	C	C	C	C	
Serum Density						T	O

a) C = Certified, I = Information (non-certified, no uncertainty evaluation), O = Other (non-certified; limits of data, uncertainty, and traceability described), R = Reference (non-certified; uncertainty estimated), T = Text (non-certified, value provided in COA text). See [15] for formal criteria for C, R, and I designations.

b) Variably designated combination of isomers; name reflects current understanding of measurand

Differences Between SRM 968f and Prior Materials

The plasma pools used to produce SRM 968f were obtained from the same providers and at the same time as those used to produce SRM 968d and 968e. However, there are differences in the analytes certified and how values are presented in the COA.

β-Carotene

Total and/or *trans*-β-carotene values were certified in all prior materials of the SRM 968 series. Except for SRM 968d, each unit of these SRMs provided at least one material with a mid-to-high-normal β-carotene content. SRM 968d was designed to deliver low-, mid-, and high-normal levels of *trans*-β-carotene, but the mid- and high-level materials were unacceptably heterogenous; the low-normal material was issued as a bridge until SRM 968e could be produced and certified. Production of SRM 968e consumed all of the β-carotene-enriched plasma that had been purchased.

Due to prior commitments, the commercial provider from whom NIST had previously obtained β-carotene-enriched human plasma was unable to supply similar materials to use with SRM 968f. Other providers that were contacted were unable to select even marginally carotenoid-enriched candidate materials. To enable timely introduction of SRM 968f before (or soon after) the supply of SRM 968e was exhausted, SRM 968f was redesigned to focus on the most clinically relevant analytes: retinol and α-tocopherol.

The available plasma pools were adequate to produce two materials, one with low-normal levels of retinol, α-tocopherol, and γ+β-tocopherol and the other with mid-to-high-normal levels of these analytes. However, both materials have similar low-normal β-carotene content. Because these materials are of limited utility to users interested in the carotenoids and given limited analytical resources, the β-carotene content of the SRM 968f materials has not been evaluated at NIST.

However, both SRM 968f materials were evaluated in three NIST Micronutrient Measurement Quality Assurance Program (MMQAP) interlaboratory comparability improvement studies [16,17]. The SRM 968f COA lists consensus results from these studies for total and *trans*-β-carotene and 11 other analytes. While these consensus results do not meet NIST's criteria for certification, the consensus results for samples of well-characterized composition (including all three levels of SRM 968e) distributed in these studies as blind controls agreed very well with their expected values.

Cholesterol

Cholesterol concentration values were certified in SRM 968b through SRM 968e to facilitate use of the materials with lipid-adjusted vitamin indices [18]. However, no participant in any of the 82 MMQAP interlaboratory comparability studies reported cholesterol values for any sample. Given limited analytical resources and no compelling community demand, the cholesterol content of the SRM 968f materials has not been evaluated.

NIST currently supports SRM 1951c Lipids in Frozen Human Serum, a set of two materials with certified values for both cholesterol and total glycerides which are used in estimating lipid-adjusted values. NIST also supports two other serum-based SRMs that provide

certified cholesterol values: SRM 909c Frozen Human Serum and SRM 1952a Cholesterol in Freeze-Dried Human Serum.

Vitamin D Metabolites

Vitamin D metabolite values were established in SRM 968c from results reported by six participants, using a total of three analytical methods. The values in SRM 968f were determined at NIST using a reference measurement procedure approved by the Joint Committee for Traceability in Laboratory Medicine [19].

Density

SRM 968e was the first member of this series to specify serum densities, but they were provided in the textual description without metrological context. SRM 968f is the first member of the series to explicitly report serum density as a measurand. The values delivered by SRM 968f were determined at NIST using a well-established method [20].

Certified Values

The certified values in all members of the SRM 968 series have met the formal, international accepted definitions for values delivered by Certified Reference Materials [21] and the definition in [15]:

> "A NIST Certified Value represents data for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been fully investigated or accounted for by NIST."

However, SRM 968f is the first member of the series to explicitly state that the certified values are metrologically traceable to the International System of Units (SI) through the molar absorptivities of the calibration standards [14]. These values are stated in the mass concentration units of μg/mL and the amount-of-substance concentration units of μmol/L [22].

Non-Certified Values

Prior to SRM 968f, non-certified values were described as "Reference" or "Information," where these terms are defined in [15]:

- "A NIST Reference Value is a best estimate of the true value provided on a NIST Certificate of Analysis where all known or suspected sources of bias have not been fully investigated by NIST. … The uncertainty associated with a NIST Reference Value may not include all sources of uncertainty and may represent only a measure of the precision of the measurement method(s)."
- "NIST Information Value: A NIST Information Value is considered to be a value that will be of interest and use to the SRM user, but insufficient information is available to assess the uncertainty associated with the value. Typically, the information value has no reported uncertainty listed on the certificate …"

For the SRM 968 series, in practice the "Reference" designation indicated that the value was the result of a single NIST assay or the consensus result from numerous MMQAP study participants. The "Information" designation indicated values reported by one (to a few) participants having established measurement expertise for the given measurand.

While NIST certified values provide metrological traceability [21] to the International System of Units [23], non-certified results are by definition not traceable to the SI but may be traceable to other useful reference systems such as particular analytical measurement methods. The COAs for prior materials of the SRM 968 series do not always provide sufficient information to determine the limitations of the listed result nor its traceability status.

With SRM 968f we designate all values that do not meet NIST's criteria for certification as "Non-Certified Values" and describe them as "values that do not meet NIST's criteria for certification but are the best currently available estimates for measurands of potential interest." All results with the same metrological traceability characteristics are listed in their own tables. Results that are not deemed traceable to a useful reference system, such as values from a non-validated measurement system or from a single interlaboratory study participant, are not listed in the COA.

Uncertainty Propagation

Certified Values

A certified value for an analyte *X* delivered by SRM 968f is described in the COA as a value, x_{srm}, with an approximate 95 % confidence expanded uncertainty $U_{95\%}(x_{srm})$. To propagate this uncertainty, treat the certified value as a normally distributed random variable with mean x_{srm} and standard deviation $s_{srm} = U_{95\%}(x_{srm})/2$ [24, 25].

For example, if you measure *X* in an SRM using a method known to provide a relative standard deviation of 2 %, then your measurement standard deviation is $s_{you} = (2/100)\, x_{you}$, the standard uncertainty in your measurement is

$$u(x_{you}) = \sqrt{s_{srm}^2 + s_{you}^2}\,,$$

and the approximate 95 % expanded uncertainty on your measurement is $U_{95}(x_{you}) = 2 \cdot u(x_{you})$ where "2" is the usual metrological approximation to the more exact 1.96 coverage factor for normal (Gaussian) distributions.

If your measurement standard deviation is estimated from a number, *n*, of repeated independent determinations of x_{you}, then the appropriate way to compute a 95 % expanded uncertainty is a bit more complicated. Rather than multiplying $u(x_{you})$ by 2, the appropriate coverage factor should come from the Student's *t* distribution with *v* degrees of freedom: $t_{0.975,v}$, the 97.5th percentile of the Student's *t* distribution with *v* degrees of freedom, where *v* is the effective number of degrees of freedom associated with $u(x_{you})$. If s_{you} is much larger than s_{srm}, then $v = n\text{-}1$; if not, then *v* can be estimated from the Welch-Satterthwaite formula [24-27]

$$v = u^4(x_{you}) \Big/ \left(\frac{s_{srm}^4}{60} + \frac{s_{you}^4}{n-1}\right).$$

Table 3 lists approximate $t_{0.975,v}$ values for $v = 1$ to 60. The "60" in the above equation comes from the observation that $t_{0.975,60} = 2.0$.

Table 3: Student's t 95 % Coverage Factors for $v = 1$ to $v = 60$

υ	$t_{0.975,\upsilon}$	υ	$t_{0.975,\upsilon}$	υ	$t_{0.975,\upsilon}$	υ	$t_{0.975,\upsilon}$	υ	$t_{0.975,\upsilon}$	υ	$t_{0.975,\upsilon}$
1	12.706	11	2.201	21	2.080	31	2.040	41	2.020	51	2.008
2	4.303	12	2.179	22	2.074	32	2.037	42	2.018	52	2.007
3	3.182	13	2.160	23	2.069	33	2.035	43	2.017	53	2.006
4	2.776	14	2.145	24	2.064	34	2.032	44	2.015	54	2.005
5	2.571	15	2.131	25	2.060	35	2.030	45	2.014	55	2.004
6	2.447	16	2.120	26	2.056	36	2.028	46	2.013	56	2.003
7	2.365	17	2.110	27	2.052	37	2.026	47	2.012	57	2.002
8	2.306	18	2.101	28	2.048	38	2.024	48	2.011	58	2.002
9	2.262	19	2.093	29	2.045	39	2.023	49	2.010	59	2.001
10	2.228	20	2.086	30	2.042	40	2.021	50	2.009	60	2.000

Non-Certified Values Derived from MMQAP Results

The non-certified results derived just from the MMQAP results have been estimated as the weighted medians of individual laboratory means, where the weights are based on a Laplace random effects model [28]. The uncertainty of each weighted median, $U_{95\%}(x_{srm})$, was estimated using a bootstrap procedure based on a Laplace random effects model for the between-lab and within-lab effects [24,28-31]. To propagate this uncertainty, treat x_{srm} as a normally distributed random variable with mean x_{srm} and standard deviation $s_{srm} = U_{95\%}(x_{mmqap})/2$ and combine as described above.

Non-Certified Values Derived from a Limited Number of NIST Results

The non-certified values for three vitamin D metabolites are stated with the number of measurements, n, underlying the reported value, x_{srm}, and its 95 % expanded uncertainty, $U_{95\%}(x_{srm})$. These $U_{95\%}(x_{srm})$ have been estimated using the Student's t expansion factor $t_{0.975,n-1}$. To propagate this uncertainty, combine $s_{srm} = U_{95\%}(x_{srm})/t_{0.975,n-1}$ as in the above section, but substituting "n-1" for "60" in the effective degrees of freedom calculation.

Non-Certified Values Derived from a Single Method

The non-certified values for the serum densities are stated with a 95 % expanded uncertainty, $U_{95\%}(x_{srm})$, based upon a conservative estimate of the variability of the method. To propagate this uncertainty, treat x_{srm} as a normally distributed random variable with mean x_{srm} and standard deviation $s_{srm} = U_{95\%}(x_{mmqap})/2$ and combine as described above.

Computation

The standard and expanded uncertainties for your measurements can also be computed using Monte Carlo methods [29]. The *NIST Uncertainty Machine* [32] is a web-based application freely available at uncertainty.nist.gov that performs uncertainty propagations according to the GUM [24] and the GUM Supplement 1 [29].

Certificate of Analysis

A NIST COA is defined as:

"In accordance with ISO Guide 31: 2000, a NIST SRM certificate is a document containing the name, description, and intended purpose of the material, the logo of the U.S. Department of Commerce, the name of NIST as a certifying body, instructions for proper use and storage of the material, certified property value(s) with associated uncertainty(ies), method(s) used to obtain property values, the period of validity, if appropriate, and any other technical information deemed necessary for its proper use. A Certificate is issued for an SRM certified for one or more specific physical or engineering performance properties and may contain NIST reference, information, or both values in addition to certified values. A Certificate of Analysis is issued for an SRM certified for one or more specific chemical properties. Note: ISO Guide 31 is updated periodically; check with ISO for the latest version." [https://www.nist.gov/srm/srm-definitions]

For the most current version of the COA for NIST SRM 968f Fat-Soluble Vitamins in Frozen Human Serum COA, please visit: https://www-s.nist.gov/srmors/view_detail.cfm?srm=968f.

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Appendix A

Data Submitted for Statistical Analysis

Tables A1 through A.6 present the data used in the statistical evaluation of the certified values listed in Table 1 of the Certificate of Analysis (COA) for SRM 968f, the MMQAP consensus values listed in Table 2 of the COA, and NIST's isotope-dilution mass-spectroscopic values listed in Table 3 of the COA. Table A.7 lists the organizations that participated in the 2016 and 2017 NIST Micronutrients Measurement Quality Assurance Program (MMQAP).

Table A.1: NIST Certification Data for SRM 968f Levels 1 and 2
Source: ROA 646.02-17-045

SRM 968f Level 1

Box	Vial Location	Analysis Order	Total retinol µg/mL	α-Tocopherol µg/mL	γ+β-tocopherol µg/mL
11	First	1	0.333	5.24	1.14
12	Center	2	0.326	5.21	1.11
13	Last	3	0.326	5.37	1.11
14	Last	4	0.329	5.30	1.05
16	Last	5	0.335	5.35	1.08
1	Center	6	0.322	5.18	1.10
2	Center	7	0.326	5.22	1.08
3	Center	8	0.326	5.37	1.04
4	First	9	0.324	5.19	1.06
20	Center	10	0.318	5.16	1.14
19	Last	11	0.320	5.24	1.10
18	First	12	0.325	5.23	1.14
17	Last	13	0.336	5.31	1.02
16	Last	14	0.329	5.25	1.09
10	First	15	0.334	5.15	1.04
9	First	16	0.325	5.33	1.04
8	First	17	0.336	5.30	1.10
7	First	18	0.328	5.14	1.14
6	Last	19	0.321	5.20	1.15
5	Center	20	0.325	5.28	1.09

SRM 968f Level 2

Box	Vial Location	Analysis Order	Total retinol µg/mL	α-Tocopherol µg/mL	γ+β-tocopherol µg/mL
20	Last	67	0.621	11.59	2.63
2	Center	1	0.691	12.31	2.79
19	Last	2	0.661	12.25	2.65
3	Center	3	0.637	11.40	2.77
18	Last	4	0.589	11.12	2.63
4	Center	5	0.596	11.15	2.65
17	Last	6	0.687	12.37	2.68
16	Last	8	0.664	11.64	2.85
6	Center	9	0.662	11.62	2.77
15	Last	10	0.645	11.14	2.55
7	First	11	0.656	11.51	2.68
14	Last	12	0.676	11.45	2.85
8	First	13	0.667	11.62	2.69
13	Last	14	0.633	11.28	2.42
9	First	15	0.605	10.53	2.53
12	Last	16	0.669	11.70	2.70
10	First	17	0.683	12.28	2.53
11	Last	18	0.595	10.42	2.74
1	Center	19	0.639	11.15	2.45
5	Center	20	0.642	11.16	2.58

Table A.2: MMQAP Certification Data for SRM 968f Level 1
Source: NISTIR 7880-47 and 7880-48

	Total Retinol µg/mL			α-Tocopherol µg/mL			γ+β-Tocopherol µg/mL		
Lab	#428	#433	#438	#428	#433	#438	#428	#433	#438
FSV-BA	0.309	0.330	0.320						
FSV-BD	0.288	0.298	0.284	5.00	5.30	5.30			
FSV-BE	0.370	0.330	0.320	5.16	4.90	6.10	1.09		1.10
FSV-BFa	0.300			4.24					
FSV-BG	0.363			4.68			1.13		
FSV-BH	0.368	0.265	0.283	5.00	4.67	4.46	0.91	1.02	0.98
FSV-BJ	0.314	0.309	0.310	4.65	4.47	5.09	1.14	0.99	1.04
FSV-BK	0.335			5.02					
FSV-BL	0.320	0.340	0.370	5.20	5.20	4.70			
FSV-BM	0.435	0.270	0.342	4.66	5.30	5.50			
FSV-BN	0.291	0.364	0.217	3.93	5.41	3.34			
FSV-BR	0.360	0.331	0.340	5.42	5.17	4.83			
FSV-BS	0.366			5.10	7.17	4.98			
FSV-BT	0.355	0.292	0.335	5.34	5.22	5.12	1.26	1.22	1.20
FSV-BU	0.306	0.293	0.357	5.22	4.56	3.87	1.05	0.97	0.97
FSV-BUa				5.51			1.44		
FSV-BV	0.326			3.76			0.89		
FSV-BW	0.340	0.336	0.336	5.00	5.07	4.58			
FSV-CD	0.290	0.380	0.360	4.99	5.94	5.65	1.04	1.25	1.19
FSV-CE	0.320	0.337	0.310	5.16	5.23	4.81			
FSV-CF	0.317	0.339	0.341	4.80	5.40	4.00			
FSV-CG	0.315	0.297	0.285	4.49	5.69	6.11	0.88	1.09	1.11
FSV-CI	0.290	0.312	0.290	4.82	5.42	5.10	1.04	1.16	1.10
FSV-CO	0.345			5.28			1.15		
FSV-CZ	0.374	0.355	0.353	4.29	5.14	4.11	1.23	1.05	1.07
FSV-DV	0.316	0.322	0.344	4.70	4.50	4.60			
FSV-EZ	0.299			4.72			1.00		
FSV-FK	0.353			5.30					
FSV-FZ	0.290	0.290	0.290	5.10	5.00	4.90	1.06	1.10	1.10
FSV-GD	0.323	0.330	0.320	5.38	5.25	5.09	1.12	1.14	1.08
FSV-GE		0.352	0.270		4.90	7.49			
FSV-GF				5.10	5.90	6.10			
FSV-GG			0.300			5.03			
FSV-GJ		0.326	0.352		5.00	4.80			
FSV-GK		1.589			11.49			1.88	
FSV-GL		0.341	0.333		4.88	4.49		0.70	1.00

Table A.3: MMQAP Certification Data for SRM 968f Level 2
Source: NISTIR 7880-47 and 7880-48

	Total Retinol µg/mL			α-Tocopherol µg/mL			γ+β-Tocopherol µg/mL		
Lab	#428	#433	#438	#428	#433	#438	#428	#433	#438
FSV-BA	0.627	0.660	0.630						
FSV-BD	0.654	0.672	0.649	12.40	13.10	12.80			
FSV-BE	0.670	0.670	0.680	11.88	12.20	15.80	2.38	2.30	2.60
FSV-BFa	0.580			9.97					
FSV-BG	0.695			11.86			2.71		
FSV-BH	0.705	0.586	0.583	14.10	12.02	10.66	2.22	2.54	2.39
FSV-BJ	0.676	0.657	0.664	12.59	11.90	13.40	2.86	2.38	2.62
FSV-BK	0.661			11.85					
FSV-BL	0.660	0.690	0.720	12.10	12.10	12.10			
FSV-BM	0.671	0.560	0.659	13.10	13.60	13.10			
FSV-BN	0.689	0.736	0.589	11.46	12.93	10.43			
FSV-BR	0.701	0.660	0.670	13.56	13.14	12.56			
FSV-BS	0.783			14.53	12.78	12.43			
FSV-BT	0.679	0.687	0.700	12.29	11.93	11.63	2.61	2.59	2.52
FSV-BU	0.641	0.622	0.689	12.56	12.31	12.32	2.45	2.46	2.47
FSV-BUa				13.47			3.54		
FSV-BV	0.625			8.83			2.05		
FSV-BW	0.640	0.674	0.703	11.22	12.33	11.88			
FSV-CD	0.600	0.570	0.620	12.57	11.31	11.88	2.58	2.36	2.55
FSV-CE	0.690	0.667	0.650	10.77	12.40	11.95			
FSV-CF	0.596	0.701	0.734	13.50	12.00	10.90			
FSV-CG	0.610	0.553	0.558	11.36	13.91	14.99	2.32	2.61	2.58
FSV-CI	0.606	0.673	0.630	11.26	12.72	11.40	2.43	2.70	2.50
FSV-CO	0.675			12.64			2.77		
FSV-CZ	0.642	0.722	0.637	10.94	12.59	9.97	2.96	2.40	1.98
FSV-DV	0.684	0.594	0.795	10.40	10.80	12.40			
FSV-EZ	0.618			11.82			2.40		
FSV-FK	0.704			13.50					
FSV-FZ	0.700	0.650	0.660	12.60	12.30	11.80	2.60	2.60	2.50
FSV-GD	0.658	0.682	0.671	12.70	12.78	12.96	2.59	2.69	2.65
FSV-GE		0.779	0.616		11.45	18.07			
FSV-GF				13.50	15.20	15.40			
FSV-GG			0.669			12.51			
FSV-GJ		0.649	0.660		10.80	13.30			
FSV-GK		3.656			26.20			3.66	
FSV-GL		0.686	0.679		11.37	11.64		1.69	2.69

Table A.4: MMQAP Consensus Data for SRM 968f Level 1
Source: NISTIR 7880-47 and 7880-48

	Total β-Carotene µg/mL			*trans*-β-Carotene µg/mL			Total *cis*-β-Carotene µg/mL			Total α-Carotene µg/mL		
Lab	#428	#433	#439	#428	#433	#439	#428	#433	#439	#428	#433	#439
FSV-BE	0.120	0.160	0.131									
FSV-BG	0.120									0.023		
FSV-BH	0.102	0.088	0.096		0.093	0.096		0.005	0.005	0.019	0.019	0.018
FSV-BJ	0.101	0.097	0.049							0.025	0.027	0.026
FSV-BN	0.065	0.144	0.115			0.089			0.026	0.016	0.052	0.031
FSV-BS	0.067	0.116	0.076	0.067	0.116					0.031	0.052	0.037
FSV-BT	0.130	0.126	0.118	0.122	0.108	0.110	0.008	0.018	0.008	0.028	0.024	0.022
FSV-BU	0.088		0.115							0.020		0.026
FSV-BV	0.111									0.017		
FSV-BW	0.128	0.103	0.085							0.046	0.020	0.062
FSV-CD	0.120	0.130	0.120									
FSV-CE	0.090	0.100	0.130									
FSV-CG	0.104	0.101	0.144	0.098	0.094	0.134	0.006	0.005	0.010	0.023	0.024	0.035
FSV-CI												
FSV-CO	0.118									0.022		
FSV-CZ	0.095	0.126	0.100									
FSV-EE												
FSV-EZ	0.092			0.092								
FSV-FK	0.150			0.150								
FSV-FZ												
FSV-GD	0.115	0.111	0.108	0.101	0.097	0.095	0.014	0.014	0.013	0.025	0.026	0.024
FSV-GE		0.070	0.109									
FSV-GF												
FSV-GG												
FSV-GJ												
FSV-GK		0.139			0.139						0.032	
FSV-GL		0.141	0.131								0.065	0.024

Table A.4 (Continued): MMQAP Consensus Data for SRM 968f Level 1

	Total Lycopene µg/mL			*trans*-Lycopene µg/mL			Total β-Cryptoxanthin, µg/mL			Total Lutein µg/mL		
Lab	#428	#433	#439	#428	#433	#439	#428	#433	#439	#428	#433	#439
FSV-BE												
FSV-BG	0.156			0.084			0.030					
FSV-BH	0.182	0.162	0.170				0.031	0.032	0.024	0.045	0.024	0.030
FSV-BJ	0.142	0.156	0.125				0.020	0.016	0.017	0.045	0.037	0.032
FSV-BN	0.075	0.188	0.126			0.070	0.020	0.044	0.043			0.023
FSV-BS	0.065	0.160	0.180	0.051	0.108	0.084	0.028	0.033	0.035	0.034	0.053	0.037
FSV-BT	0.112	0.103	0.102	0.099	0.091	0.091	0.027	0.025	0.024	0.047	0.037	0.037
FSV-BU	0.152		0.140				0.020		0.032			
FSV-BV	0.108						0.013					
FSV-BW	0.135	0.260	0.107									
FSV-CD	0.180	0.280	0.200									
FSV-CE												
FSV-CG	0.136	0.142	0.210	0.068	0.076	0.114	0.026	0.030	0.042			
FSV-CI												
FSV-CO	0.164						0.033					
FSV-CZ												
FSV-EE												
FSV-EZ												
FSV-FK												
FSV-FZ												
FSV-GD	0.160	0.151	0.159									
FSV-GE		0.113	0.181									
FSV-GF												
FSV-GG												
FSV-GJ												
FSV-GK		0.179			0.075			0.061			0.029	
FSV-GL		0.401	0.183									

Table A.4 (Continued): MMQAP Consensus Data for SRM 968f Level 1

	Total Zeaxanthin µg/mL			Total Lutein + Zeaxanthin µg/mL			Retinyl Palmitate µg/mL			Phylloquinone (K1) ng/mL		
Lab	#428	#433	#439	#428	#433	#439	#428	#433	#439	#428	#433	#439
FSV-BE										0.216	0.227	0.243
FSV-BG				0.050			0.019					
FSV-BH	0.014	0.011	0.008	0.059	0.035	0.038						
FSV-BJ												
FSV-BN			0.095	0.039	0.054	0.033						
FSV-BS	0.010	0.008	0.024	0.044	0.061	0.061						
FSV-BT	0.017	0.016	0.012	0.064	0.053	0.049						
FSV-BU				0.050		0.047						
FSV-BV				0.050								
FSV-BW							0.006		0.032			
FSV-CD				0.050	0.070	0.060						
FSV-CE												
FSV-CG				0.055	0.053	0.048						
FSV-CI										0.242	0.304	0.302
FSV-CO				0.058								
FSV-CZ												
FSV-EE												
FSV-EZ												
FSV-FK										0.200		
FSV-FZ							0.015	0.018	0.016			
FSV-GD												
FSV-GE												
FSV-GF												
FSV-GG												
FSV-GJ											0.230	0.220
FSV-GK		0.027			0.056			0.001				
FSV-GL												

Table A.4 (Continued): MMQAP Consensus Data for SRM 968f Level 1

	25-(OH_2)D ng/mL			Coenzyme Q10 µg/mL		
Lab	#428	#433	#439	#428	#433	#439
FSV-BE				0.620	0.610	0.500
FSV-BG						
FSV-BH	12.9	15.6	15.2			
FSV-BJ				0.540	0.514	0.545
FSV-BN			8.3			
FSV-BS						
FSV-BT				0.451	0.436	0.730
FSV-BU						
FSV-BV						
FSV-BW				0.370	0.460	0.430
FSV-CD						
FSV-CE					0.483	0.446
FSV-CG						
FSV-CI				0.480	0.490	0.500
FSV-CO						
FSV-CZ				0.521	0.564	0.413
FSV-EE				0.463	0.469	0.501
FSV-EZ						
FSV-FK						
FSV-FZ						
FSV-GD				0.503	0.517	0.553
FSV-GE		20.2				
FSV-GF				0.480	0.440	0.400
FSV-GG		13.4	13.7	0.500	0.500	0.500
FSV-GJ						
FSV-GK						
FSV-GL		14.0			0.483	0.583

Table A.5: MMQAP Consensus Data for SRM 968f Level 2
Source: NISTIR 7880-47 and 7880-48

	Total β-Carotene µg/mL			*trans*-β-Carotene µg/mL			Total *cis*-β-Carotene µg/mL			Total α-Carotene µg/mL		
Lab	#428	#433	#439	#428	#433	#439	#428	#433	#439	#428	#433	#439
FSV-BE	0.210	0.280	0.230									
FSV-BG	0.218									0.014		
FSV-BH	0.171	0.166	0.164		0.176	0.164		0.010	0.008		0.008	0.008
FSV-BJ	0.193	0.245	0.193							0.009	0.007	0.008
FSV-BN	0.164	0.236	0.184			0.153			0.031	0.016	0.044	0.035
FSV-BS	0.184	0.164	0.145	0.184	0.164					0.076	0.056	0.050
FSV-BT	0.210	0.208	0.194	0.196	0.189	0.183	0.014	0.019	0.011	0.015	0.012	0.013
FSV-BU	0.166	0.194	0.191							0.005		0.006
FSV-BV	0.166									0.004		
FSV-BW	0.171	0.178	0.163							0.121		0.006
FSV-CD	0.210	0.190	0.190									
FSV-CE	0.120	0.166	0.200									
FSV-CG	0.177	0.205	0.233	0.168	0.192	0.218	0.009	0.013	0.015	0.009	0.011	0.017
FSV-CI												
FSV-CO	0.198									0.010		
FSV-CZ	0.147	0.134	0.153									
FSV-EE												
FSV-EZ	0.174			0.174								
FSV-FK	0.280			0.280								
FSV-FZ												
FSV-GD	0.187	0.189	0.192	0.165	0.166	0.169	0.022	0.023	0.023	0.011	0.012	0.013
FSV-GE		0.086	0.138									
FSV-GF												
FSV-GG												
FSV-GJ												
FSV-GK		0.337			0.337						0.021	
FSV-GL		0.205	0.200								0.022	0.014

Table A.5 (Continued): MMQAP Consensus Data for SRM 968f Level 5

	Total Lycopene µg/mL			*trans*-Lycopene µg/mL			Total β-Cryptoxanthin, µg/mL			Total Lutein µg/mL		
Lab	#428	#433	#439	#428	#433	#439	#428	#433	#439	#428	#433	#439
FSV-BE												
FSV-BG	0.602			0.290			0.045					
FSV-BH	0.625	0.719	0.613				0.039	0.057	0.035	0.077	0.068	0.077
FSV-BJ	0.638	0.993	0.717				0.033	0.042	0.032	0.134	0.113	0.105
FSV-BN	0.516	0.700	0.368			0.211	0.062	0.050	0.043			0.044
FSV-BS	0.430	0.430	0.806	0.337	0.233	0.268	0.096	0.028	0.044	0.090	0.082	0.083
FSV-BT	0.380	0.361	0.364	0.332	0.317	0.321	0.042	0.041	0.035	0.094	0.090	0.085
FSV-BU	0.574	0.592	0.549				0.022	0.047	0.039			
FSV-BV	0.371						0.019					
FSV-BW	0.657	1.336	0.575									
FSV-CD	0.710	0.820	0.700									
FSV-CE												
FSV-CG	0.480	0.690	0.735	0.221	0.362	0.349	0.033	0.053	0.059			
FSV-CI												
FSV-CO	0.585						0.039					
FSV-CZ												
FSV-EE												
FSV-EZ												
FSV-FK												
FSV-FZ												
FSV-GD	0.529	0.541	0.629									
FSV-GE		0.274	0.405									
FSV-GF												
FSV-GG												
FSV-GJ												
FSV-GK		0.617			0.296			0.139			0.151	
FSV-GL		1.429	0.721									

Table A.5 (Continued): MMQAP Consensus Data for SRM 968f Level 2

	Total Zeaxanthin µg/mL			Total Lutein + Zeaxanthin µg/mL			Retinyl Palmitate µg/mL			Phylloquinone (K1) ng/mL		
Lab	#428	#433	#439	#428	#433	#439	#428	#433	#439	#428	#433	#439
FSV-BE										0.549	0.693	0.670
FSV-BG				0.108			0.084					
FSV-BH	0.031	0.040	0.027	0.108	0.107	0.104						
FSV-BJ												
FSV-BN			0.028	0.122	0.136	0.072						
FSV-BS	0.027	0.016	0.034	0.116	0.098	0.117						
FSV-BT	0.032	0.031	0.025	0.126	0.121	0.110						
FSV-BU				0.114	0.096	0.094						
FSV-BV				0.115								
FSV-BW							0.025		0.027			
FSV-CD				0.140	0.130	0.120						
FSV-CE												
FSV-CG				0.124	0.125	0.109						
FSV-CI							0.030	0.030	0.030	0.576	0.877	0.765
FSV-CO				0.130								
FSV-CZ												
FSV-EE												
FSV-EZ												
FSV-FK										0.610		
FSV-FZ							0.046	0.057	0.053			
FSV-GD												
FSV-GE												
FSV-GF												
FSV-GG												
FSV-GJ											0.780	0.700
FSV-GK		0.079			0.230			0.024				
FSV-GL												

Table A.5 (Continued): MMQAP Consensus Data for SRM 968f Level 2

	25-(OH_2)D ng/mL			Coenzyme Q10 µg/mL		
Lab	#428	#433	#439	#428	#433	#439
FSV-BE				1.420	1.450	1.310
FSV-BG						
FSV-BH	17.2	22.1	19.6			
FSV-BJ				1.000	1.178	1.049
FSV-BN			11.1			
FSV-BS						
FSV-BT				1.169	1.204	0.705
FSV-BU						
FSV-BV						
FSV-BW				0.940	1.310	1.090
FSV-CD						
FSV-CE					1.159	1.184
FSV-CG						
FSV-CI				1.210	1.240	1.200
FSV-CO						
FSV-CZ				1.409	1.447	1.130
FSV-EE				1.169	1.188	1.244
FSV-EZ						
FSV-FK						
FSV-FZ						
FSV-GD				1.260	1.320	1.510
FSV-GE		16.6				
FSV-GF				1.110	1.140	0.950
FSV-GG		16.9	16.9	1.300	1.400	1.200
FSV-GJ						
FSV-GK						
FSV-GL		18.8			1.302	1.639

Table A.6: NIST Vitamin D Metabolite Data
Source: ROA 646.02-16-064

Analyte	Prep	Level 1 Rep1	Level 1 Rep2	Level 2 Rep1	Level 2 Rep2
$25(OH)D_2$	A	0.820	0.811	0.165	0.164
ng/g	B	0.825	0.880	0.153	0.175
		Rep1	Rep2	Rep1	Rep2
$25(OH)D_3$	A	12.263	12.095	14.936	15.429
ng/g	B	12.185	11.938	15.410	15.458
	C	12.303	11.822	15.421	15.314
		Rep1	Rep2	Rep1	Rep2
3-*epi*-$25(OH)D_3$	A	0.714	0.718	1.195	1.111
ng/g	B	0.675	0.759	0.830	1.056
	C	0.678	0.698	0.962	1.145

Table A.7. Participants in the 2016 Summer and 2017 MMQAP Studies

Participating Organization	Location
ARUP Laboratories	Salt Lake City, UT, USA
Bio-Reference Laboratories	Elmwood Park, NJ, USA
Biochemical Genetics Laboratory, Duke University Medical Center	Durham, NC, USA
Biochemical Genetics Laboratory, Mayo Clinic	Rochester, MN, USA
Bumrungrad Hospital Public Company Limited	Wattana, Bangkok, Thailand
Cancer Research Center of Hawaii, University of Hawaii at Manoa	Honolulu, HI, USA
Centers for Disease Control and Prevention	Atlanta, GA, USA
Children's Hospital and Regional Medical Center	Seattle, WA, USA
Children's Hospital National Medical Center	Washington, DC, USA
Children's Nutrition Research Center, USDA/ARS, Baylor College of Medicine	Houston, TX, USA
Chromatography and Mass Spectrometry Lab, National Healthcare Systems Co., Ltd.	Bangkok, Thailand
Clinical Mass Spectrometry Laboratory, Mayo Clinic Rochester	Rochester, MN, USA
Department of Laboratory Medicine and Pathology, University of Alberta Hospital	Alberta, Canada
Department of Nutrition, Harvard School of Public Health	Boston, MA, USA
Division of Nutritional Sciences, University of Illinois at Urbana-Champaign	Urbana, IL, USA
Fred Hutchinson Cancer Research Center	Seattle, WA, USA
Genova Diagnostics-ATL	Duluth, GA, USA
Global Central Laboratory	Highland Heights, KY, USA
Harborview Medical Center, University of Washington	Seattle, WA, USA
International Centre for Diarrhoeal Diseases Research	Dhaka, Bangladesh
Institut Fédératif de Biologie, Hôpital Purpan	Toulouse, France
Life Sciences Group, Wyle Laboratories, Inc	Houston, TX, USA
Mayo Medical Laboratories New England	Andover, MA, USA
MRC Laboratory for Human Nutrition Research	Cambridge, England
MedPace Reference Laboratories LLC	Cincinnati, OH, USA
Neonatal Nutrition Research Laboratory, University of Louisville	Louisville, KY, USA
Nutrition Research Laboratory, University of California at San Diego	La Jolla, CA, USA
Pathology Associates Medical Laboratories LLC	Spokane, WA, USA
Pediatric CTRC CORE Laboratory, Children's Hospital Colorado	Denver, CO, USA
Quest Diagnostics, Inc.	Chantilly, VA, USA
R&D Analytical Research Center, DSM Nutritional Products, Ltd.	Kaiseraugst, Switzerland
Rowett Institute of Nutrition and Health	Aberdeen, Scotland
SEAMEO RECFON	Central Jakarta, Indonesia
Servicio de Bioquímica Clínica, Hospital Universitario Puerta de Hierro	Madrid, Spain
True Health Diagnostics LLC	Richmond, VA, USA

Appendix B

Extracted from
Report of Statistical Analysis for
SRM 968f Fat-Soluble Vitamins in Frozen Human Serum

November 28, 2017

SRM 968f Fat-Soluble Vitamins in Frozen Human Serum is the latest in a series of related SRMs. It includes two materials that are named as Levels 1 and 2. Total retinol, alpha-tocopherol, and gamma+beta tocopherol were measured by both NIST and an Interlaboratory study. Three Vitamin D analytes were measured by NIST only. A large number of nutrients were measured only by the interlaboratory study.

The original analysis in July 2017 used interlaboratory data from a worksheet provided by Jeanice Brown Thomas of MML where each laboratory had at most two measurements per analyte. In November 2017, David Duewer of MML provided a revised worksheet where each laboratory had at most three measurements per analyte. (In the interim, the interlaboratory study achieved a third round.) The results in this report incorporate these latest results.

Measurement Methods

NIST methods. For an analyte, the method means for a NIST method is the mean of the measurements available for that analyte. The uncertainty of each NIST mean is the standard error of that mean.

Interlab. These are results from a NIST Micronutrients Measurement Quality Assurance Program collaborative study. There are often very marked differences between the results from the different collaborative laboratories. Hence the Interlab method estimate for a certain analyte is the weighted median of the individual laboratory means for that analyte, where the weights are based on a Laplace random effects model [5]. For most of the results for this SRM, the weighted median is equal to the unweighted median of laboratory means, with the rest only slightly different. The uncertainty of the weighted median is estimated using a bootstrap procedure based on a Laplace random effects model for the between-lab and within-lab effects [1-5].

The weights of the weighted median are based in part on the uncertainties of the individual laboratory means. Here, the uncertainty assigned to each laboratory mean is the standard error of that mean. If a laboratory has only one measurement for an analyte, then for the purposes of the computation it is assigned an uncertainty equal to the maximum of the uncertainties reported by the other laboratories for that analyte.

One laboratory had measurements from two different chemical methods; those measurements were treated in the computations as if coming from separate laboratories.

Assignment of Values and Uncertainties

For each analyte, the certified or reference value is the mean of the method estimates available for that analyte.

When the certified/reference value is based on more than one method, the uncertainty of the combined mean is estimated using a bootstrap procedure based on a Gaussian random effects model for the between-method effects [1-4].

When the value is based on only one method, the uncertainty is that of the single method estimate used, consistent with the GUM [1].

Note on significant digits

Some of the numbers in the tables of results are purposely listed with perhaps more significant digits than is scientifically warranted. It is presumed that the relevant chemical experts will trim any estimates and uncertainties to the number of significant digits that are scientifically warranted before placement in any ensuing Certificate of Analysis or other document.

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SRM 968f Fat-Soluble Vitamins in Frozen Human Serum Results Nov 20, 2017

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Based on NIST and Interlab:

Analyte	Result	U=expanded uncertainty	Unit
Level.1.Totalretinol	0.327	0.013	µg/mL
Level.2.Totalretinol	0.658	0.028	µg/mL
Level.1.gbtocopherol	1.094	0.049	µg/mL
Level.2.gbtocopherol	2.587	0.143	µg/mL
Level.1.aTocopherol	5.145	0.210	µg/mL
Level.2.aTocopherol	11.847	0.726	µg/mL

==========

Based on Interlab only:

Level 1

ANALYTE	VALUE	U=expanded uncertainty	Unit
Total.Beta.Carotene	0.111	0.011	µg/mL
trans.Beta.Carotene	0.098	0.021	µg/mL
Total.cis.Beta.Carotene	0.011	0.010	µg/mL
Total.alpha.Carotene	0.026	0.007	µg/mL
Total.Lycopene	0.154	0.028	µg/mL
trans.Lycopene	0.084	0.015	µg/mL
Total.Beta.Cryptoxanthin	0.030	0.008	µg/mL
Total.Lutein	0.036	0.010	µg/mL
Total.Zeaxanthin	0.015	0.033 #	µg/mL
Total.Lutein.Zeaxanthin	0.052	0.006	µg/mL
Retinyl.Palmitate	0.017	0.015	µg/mL
Coenzyme.Q10	0.500	0.034	µg/mL
Phylloquinone..K1.	0.227	0.047	ng/mL
25..OH2.D	14.0	4.6	ng/mL

Listed for information only and not recommended for use. Since the expanded uncertainty exceeds the estimate, any resulting interval should be bounded below at 0.

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Level 2

ANALYTE	VALUE	U=expanded uncertainty	Unit
Total.Beta.Carotene	0.195	0.021	µg/mL
trans.Beta.Carotene	0.174	0.039	µg/mL
Total.cis.Beta.Carotene	0.015	0.011	µg/mL
Total.alpha.Carotene	0.012	0.012	µg/mL
Total.Lycopene	0.584	0.119	µg/mL
trans.Lycopene	0.293	0.047	µg/mL
Total.Beta.Cryptoxanthin	0.044	0.017	µg/mL
Total.Lutein	0.087	0.037	µg/mL
Total.Zeaxanthin	0.029	0.021	µg/mL
Total.Lutein.Zeaxanthin	0.115	0.019	µg/mL
Retinyl.Palmitate	0.030	0.029	µg/mL
Coenzyme.Q10	1.208	0.113	µg/mL
Phylloquinone..K1.	0.688	0.137	ng/mL
X25..OH2.D	16.9	4.0	ng/mL

==========

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Based on NIST only:

Analyte                    Result       U=expanded uncertainty     k      Unit

Level.1.25(OH)D2             0.834              0.050             3.18    ng/g
Level.2.25(OH)D2             0.164              0.014             3.18    ng/g

Level.1.25(OH)D3            12.101              0.198             2.57    ng/g
Level.2.25(OH)D3            15.328              0.208             2.57    ng/g

Level.1.3-epi-25(OH)D3       0.707              0.033             2.57    ng/g
Level.2.3-epi-25(OH)D3       1.050              0.141             2.57    ng/g

================================================================================
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References

[1] JCGM 100:2008 (2008) *Evaluation of measurement data –Guide to the expression of uncertainty in measurement.* Joint Committee for Guides in Metrology. Sèvres, France.
http://www.bipm.org/en/publications/guides/#gum
See also: Taylor BN, Kuyatt CE (1994) *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*. NIST Technical Note 1297, NIST, Gaithersburg, MD, USA.
http://www.nist.gov/pml/pubs/tn1297/index.cfm

[2] JCGM 101:2008 (2008) *Evaluation of measurement data – Supplement 1 to the "Guide to the expression of uncertainty in measurement" – Propagation of distributions using a Monte Carlo method*. Joint Committee for Guides in Metrology. Sèvres, France.
http://www.bipm.org/en/publications/guides/#gum
See also: Lafarge T, Possolo A (2016) *The NIST Uncertainty Machine*.
NCSLI Measure 10(3):20-27.
http://www.tandfonline.com/doi/abs/10.1080/19315775.2015.11721732
Software freely available at: https://uncertainty.nist.gov/

[3] Efron B, Tibshirani RJ (1993) *An Introduction to the Bootstrap*, Chapman & Hall, UK.

[4] Searle S, Casella G, McCulloch C (1992) *Variance Components*; John Wiley, Hoboken, NJ.

[5] Rukhin AL, Possolo A (2011) Laplace random effects models for interlaboratory studies. *Computational Statistics and Data Analysis* 55, 1815-1827.
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Appendix C

Extracts from the SRM 968f Reports of Analysis

A NIST Report of Analysis (ROA) is defined as:

"Document containing the certification of the material and including such information as the base material used, how the SRM was manufactured, the certification method(s) and description of procedures, outside collaborators, instructions for use, special instructions for packaging, handling, and storage, and plan for stability testing. The ROA is intended for internal NIST use only." [https://www.nist.gov/srm/srm-definitions]

The following pages have been extracted from the NIST ROAs that are directly related to the production and certification of NIST SRM 968f Fat-Soluble Vitamins in Frozen Human Serum. All information pertinent to the evaluation and use of the SRM has been retained.

Extracted from
NIST REPORT OF ANALYSIS 646.02-16-041

Preparation of SRM 968f Fat-Soluble Vitamins in Frozen Human Serum

May 4, 2016

INTRODUCTION

The National Institute of Standards and Technology (NIST) is producing Standard Reference Material (SRM) 968f Fat-Soluble Vitamins, in Frozen Human Serum to replace SRM 968e.

MATERIAL DESCRIPTION

Solomon Park Research Laboratories (Kirkland, WA) was awarded the contract to produce 3,000 ± 300 vials of each of two levels of serum (candidate SRM 968f-Level 1 and candidate SRM 968f-Level 2) using plasma that NIST had previously acquired from Interstate Blood Bank (Memphis, TN and Chicago, IL). NIST provided approximately eight liters of citrated plasma for this project. These materials were shipped in various containers having volumes of 100 to 800 mL each. These containers were labeled with an arbitrary code; no identifiers were present on these bottles to enable determination of the identity of the donors linked to these containers. The contractor was instructed to store the plasma received from NIST in -70 °C freezers from the time of receipt.

NIST provided Solomon Park with the labels appropriate for use at -70 °C and the blending protocols provided in Table 1 to reach the indicated target levels for retinol, γ- and α-tocopherol (in **bold** typeface). The scheme was based upon previously established measurement results available to NIST. Because the contractor was not required to measure analytes in these units and because there was no way for the contractor to identify the source of the plasma, no Institutional Review Board approval was needed or sought by the contractor for this project.

Source plasma concentrations used to generate the blending protocols were previously reported along with documentation that all source plasma used was negative for hepatitis B, human immunodeficiency virus, and hepatitis C. Once received by Solomon Park, the serum was pooled, blended, bottled in 1.1-mL aliquots, and stored at –70 °C. Prior to blending and bottling, the plasma was frozen, thawed, and filtered through Whatman 541 filter paper twice to convert to serum. Details regarding the protocol used for the conversion of plasma to serum are specified in the Attached Statement of Work (Appendix 646.02-16-041-A) and Solomon Parks' Technical Proposal (Appendix 646.02-16-041-B).

Solomon Park shipped the candidate material on dry ice, much of which remained when the SRM arrived at NIST. The SRM was stored at –80 °C upon receipt at NIST. Slightly more than 3,000 vials of each level were received (3298 vials of Level 1 and 3313 vials of Level 2).

Table 1. Blending Schema for SRM 968f

	Nominal Analyte[a] Concentrations, µg/mL										
Identifier	TR	aT	gT	bC	aC	Tly	t-Ly	TbX	Tlu	TZ	mL[b]
HP169859	0.50	9.11	2.29	0.32	0.04	0.23	0.19	0.02	0.19	0.06	500
HZ085536	0.48	9.88	1.94	0.23	0.07	0.27	0.21	0.08	0.12	0.04	200
KP52416	0.47	9.90	1.48	0.34	0.03	0.10	0.10	0.04	0.07	0.04	800
KP52685	0.44	14.72	0.83	0.35	0.15	0.10	0.21	0.03	0.11	0.04	450
KP47840	0.43	4.35	2.09	0.06	0.01	0.16	0.09	0.03	0.05	0.02	824
B7119[c]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1250
Level I	**0.32**	**6.13**	**1.20**	0.17	0.03	0.11	0.10	0.02	0.07	0.02	4024[d]
HP188021	1.60	21.18	7.42	0.14	?	0.60	0.51	0.03	0.13	0.02	880
HZ086935	0.99	18.22	3.78	0.25	?	0.70	0.56	0.02	0.10	0.03	824
HZ087262	0.91	16.79	2.94	0.26	?	0.69	0.59	0.02	0.10	0.01	824
HP187814	0.79	17.38	2.90	0.22	?	0.58	0.46	0.03	0.04	0.02	824
HZ085864	0.86	19.31	3.22	0.15	?	0.17	0.14	0.05	0.14	0.06	691
Level II	**1.04**	**18.59**	**4.13**	0.20	?	0.56	0.46	0.03	0.10	0.03	4043[d]

a TR = Total retinol, aT = α-Tocopherol, gT = γ+β-Tocopherol, bC = Total β-Carotene, aC = Total α –Carotene, Tly = Total Lycopene, t-Ly = *trans*-lycopene, TbX = Total β-Cryptoxanthin, Tlu = Total Lutein, TZ = Total Zeaxanthin

b volume of each plasma to be combined; in most cases, the nominal volume listed on the container

c delipidized plasma, supplied in two containers with the same identification code

d nominal total volume of pool

Appendix 646.02-16-041-A

STATEMENT OF WORK

TITLE: Blending and Vialing of Serum for Candidate SRM 968f Fat-Soluble Vitamins in Frozen Human Serum

LAB REQUESTING SERVICE: Material Measurement Laboratory, Chemical Sciences Division

I. BACKGROUND INFORMATION

Standard Reference Material (SRM) 968 and its reissues are human serum based materials used as validation standards by clinical, epidemiological, and nutrition laboratories. Historically, about 300 units of this SRM have been sold annually. Based on this annual sales projection, as of November 1, 2015 there are about 16 months of inventory of the current issue: SRM 968e Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum. To avoid a gap in service to our customers, it is necessary to have the next issue of SRM 968 available for use in interlaboratory comparison studies beginning in late February, 2016.

This next SRM 968 version, Candidate SRM 968f Fat-Soluble Vitamins in Human Serum, shall be prepared as a two-component reference material with concentration values certified by the National Institute of Standards and Technology (NIST) for retinol, alpha- and beta-/gamma-tocopherol. These two component materials will be labeled “SRM 968f Level 1” and “SRM 968f Level 2”; hereafter, in this document they are referred to as “Level 1” and “Level 2”.

II. SCOPE OF WORK

The contractor shall combine plasma from multiple individual containers into Level 1 and Level 2 pools, thoroughly blend each pool, convert to serum, filter, deliver 1.1 mL of clear serum into 2-ml amber borosilicate vials, stopper and cap the vials, attach the Level 1 and Level 2 labels provided by NIST to vials produced from the respective pools, store the completed vials overnight at -70 °C, and ship them to NIST on dry-ice. The volume of the Level 1 and Level 2 pools will be approximately 4 L each. The individual containers of plasma provided by NIST will be clearly labeled “for Level 1” or “for Level 2”.

III. DELIVERABLES AND DUE DATES

The contractor shall provide the following deliverables: 3000 ± 300 matched sets of vials of the two serum pools will be delivered to NIST by March 7, 2016. A matched set consists of one vial of the Level 1 pool and one vial of the Level 2 pool.

Status updates shall be provided within two business days via telephone or e-mail by the contractor as requested by NIST.

IV. PERIOD OF PERFORMANCE

The deliverables must be provided to NIST by March 7, 2016.

V. GOVERNMENT-FURNISHED PROPERTY, DATA AND/OR INFORMATION

The NIST will provide approximately eight liters of citrated plasma (and spiking solutions, if NIST chooses to provide). These materials will be shipped in various containers having volumes of 100 mL to 800 mL each. These containers will be labeled with an arbitrary code. NIST will provide the contractor appropriate documents that show that all serum pools have been tested and confirmed negative for: human immuno-deficiency virus (HIV 1/2 Ab), human immunodeficiency virus 1 antigen (HIV-1 RNA), hepatitis B surface antigen (HBsAg), hepatitis C virus (HCV Ab and HCV RNA), and sexually transmitted diseases (STDs).

NIST will designate which containers to combine into the Level 1 and Level 2 pools. The volume of each pool will be approximately four liters. The plasma in each container will be used in only one of the two pools.

NIST will provide the contractor with labels appropriate for use at the required storage temperature, -70 °C or below.

All necessary Government furnished property will be provided to the Contractor by February 15, 2016. Should there be a delay in providing the necessary materials to the Contractor, the required delivery date will be adjusted accordingly to three (3) weeks from the receipt of materials.

VI. TASKS

General requirements

The contractor will at all times handle the plasma and serum pools in accord with standard practice for Biosafety Level 2 materials as defined in: CDC/NIH; Biosafety in Microbiological and Biomedical Laboratories, 5th ed.; Richardson, J.; Barkley, W.E.; Richmond, J.; McKinney, R.W., Eds.; U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention and National Institutes of Health; US Government Printing Office: Washington, DC (2007); available at http://www.cdc.gov/biosafety/publications/bmbl5/

The plasma and serum must at all times be handled hygienically, never exposed to temperatures above 25 °C, and with minimal exposure to ultraviolet light.

The contractor shall follow best practices throughout, but must perform the following specific tasks.

Specific tasks

1) Contractor must acquire specified materials and quantities before vialing. Each material must be as specified, or equivalent. To be considered for award, offers of "equal" products must meet the salient characteristics specified below, clearly identify the item by brand name and make/model number, and include descriptive literature demonstrating that the salient characteristics are met. Salient characteristics are listed below each material description.
    a. Quantity 6000 (nominal), 2-mL amber borosilicate vials, Wheaton® product #223693
        i. 2-mL
        ii. amber color
        iii. borosilicate vials,
    b. Quantity 6000 (nominal), 13 mm bromobutyl ultra pure rubber stoppers, Wheaton® product #W224100-402
        i. 13 mm diameter
        ii. Ultra-pure bromobutyl rubber
    c. Quantity 3000 (nominal), 13 mm-colored (green) aluminum complete tear-off seals, Wheaton® product #W000581J.
        i. 13 mm
        ii. aluminum complete tear-off
        iii. green color
    d. Quantity 3000 (nominal), 13 mm-colored (pink) aluminum complete tear-off seals, Wheaton® product #W015598J.
        i. 13 mm
        ii. aluminum complete tear-off
        iii. pink color
2) Notification: NIST reserves the right to visit contractor's facility during the blending and vialing. NIST will provide the Contractor with at least 48 hours advance notice should it determine that a site visit is necessary. Contractor shall alert the NIST technical point of contact (TPOC) five business days before blending either pool.
3) Conversion of plasma to serum: Plasma in the containers designated by NIST shall be thawed, pooled, centrifuged, and filtered by vacuum through Whatman 541 filter paper and stirred overnight at 4 °C. On the following day, each pool shall be filtered a second time using the same process. At this time each pool shall be tested for clot formation. If there is evidence of clotting, the serum shall be re-frozen overnight at -20 °C or below and the process repeated until there is no evidence of clotting.
4) If NIST chooses to provide spiking solutions, contractor will add a specified volume (on the order of 0.2 mL) of an ethanoic spiking solution to each plasma pool just prior to centrifuging.

There will be at most two such solutions, one for each of the two pools. NIST will provide details on the volume and solution identification along with the spiking solutions.

5) Dispensing and labelling: 1.1 (± 0.05) mL aliquots of each serum pool shall be dispensed into 2-mL amber borosilicate vials. The serum pools shall be continuously stirred during dispensing. The vials shall be stoppered with 13 mm bromobutyl rubber stoppers. The vials for the Level 1 pool shall be capped with 13 mm-colored (green) aluminum tear-off seals. The vials for the Level 2 pool shall be capped with 13 mm-colored (pink) aluminum tear-off seals. All vials of the Level 1 pool shall be labeled with the Level 1 labels supplied by NIST. All vials of the Level 2 pool shall be labeled with the Level 2 labels supplied by NIST.
6) Packaging: The vials for each of the pools shall be separately packaged for storage and shipped in a manner that reflects the fill-order of the vials. The supplier shall provide NIST with information needed to determine the fill-order and approximate date and time each vial was filled.
7) Storage and shipping: Once packaged, all vials shall be stored at -70 °C until they are shipped. All vials shall be shipped overnight on dry ice to NIST in appropriate containers. To ensure timely receipt of the package(s) by the end user and minimize the risk of environmental damage incurred during transit, the Contractor shall ensure the package is not delivered to NIST on a Friday, Saturday, Sunday, or on a Federal Holiday.

VII. ACCEPTANCE TESTING

NIST requires a two-week acceptance testing period. A minimum of 10 vials of each of the two pools will be tested by NIST to ensure suitable homogeneity (coefficient of variation of less than 5% for retinol and α-tocopherol concentration measurements made under repeatability conditions using NIST's established analytical method) and adequate volume (all vials containing at least 1.05 mL as determined by calibrated pipette) for use as a NIST SRM. Due to the limited supply of input materials, the Contractor will not have the opportunity to repair, replace, or re-perform in the event that the deliverables fail acceptance testing, and will not be reimbursed for performance. Should the Contractor wish to retrieve the vials from NIST in the event of a failure of the acceptance testing, the Contractor shall contact the NIST TPOC within 15 days of notification of performance failure to arrange for the Contractor to retrieve the vials from NIST Gaithersburg. If the Contractor does not contact NIST within the 15 day timeframe, the vials will be disposed of by NIST.

VIII. PERFORMANCE SUMMARY

Contractor has to	How does NIST check	Contractor has to do it by
Alert TPOC	TPOC acknowledges alert via email	Five business days before dispensing
Provide TPOC with shipment tracking information	TPOC acknowledges alert via email	When vials are shipped
Contractor delivers 3000 ± 300 matched sets of satisfactory vials	acceptance testing	March 7, 2016

IX. CONTRACTOR'S MINIMUM QUALIFICATIONS

The contractor must:

1) be able to customize orders according to the exact specifications stated in this SOW.
2) have and show proof of experience in blending and vialing **reference materials** as defined by JCGM 200:2012, definition 5.13 (document freely available at http://www.bipm.org/en/publications/guides/#vim or online at http://jcgm.bipm.org/vim/en/index.html)

Additional consideration will be given for experience in blending and vialing certified reference materials as defined by JCGM 200:2012, definition 5.14 (document freely available at http://www.bipm.org/en/publications/guides/#vim or on-line at http://jcgm.bipm.org/vim/en/index.html)

Appendix 646.02-16-041-B

Solomon Park Research Laboratories Technical Proposal for National Institute of Standards and Technology
Standard Reference Materials 968f Solicitation Number SB1341-16-RQ-0066-B

Introduction:

Solomon Park Research Laboratories is submitting the following technical proposal for the production of one, two Level standard reference material (SRM) aliquoted into six thousand 1.0 mL samples in amber borosilicate vials to measure the presence of retinol, alpha- and beta-/gamma-tocopherol in serum derived from plasma to be furnished by the National Institute of Standards and Technology (NIST). No tests are required to be performed by the contractor on the plasma received from the NIST.

Background:

The National Institute of Standards and Technology, known between 1901 and 1988 as the National Bureau of Standards (NBS), is a non-regulatory agency of the United States Department of Commerce. The institute's mission is to promote U.S. innovation and industrial competitiveness by advancing measurement science, standards, and technology in ways that enhance economic security and improve quality of life,

The Institute is working to produce a Standard Reference Material (SRM) for the fat-soluble vitamins retinol and alpha- and beta-/gamma-tocopherol in serum produced from human plasma supplied by the NIST. Because of the nature and number of potential analytes, every attempt to avoid additives, or procedures that might affect the serum matrix should be avoided where possible. The accompanying Performance Work Statement (PWS) describes the requirements for preparation of SRM 968f which will consist of two levels (levels I and II) identified with labels provided by the government. The NIST will assign values for the analytes of interest in the source material based on a number of independent analytical techniques. It is not the Contractor's responsibility to assign values for these analytes. Solomon Park Research Laboratories (SPRL) was founded in February 1984. The laboratory is located in Kirkland Washington and has been at the same address since December 1986 (12815 NE 124th St. Ste. L). The Laboratory has produced fresh and frozen serum pools for quality controls, calibrators and proficiency surveys and occasionally urine samples and other products (for example whole blood or blood plasma) for over 30 years. The laboratory was instrumental in developing the protocol for and in the manufacture of the current and previous standard reference material for cholesterol SRM 1951a and SRM 1951b and for the Creatinine standard reference materials SRM 967 used at the NIST. The Laboratory has also been tasked with a large number of standard reference materials including the following:

SRM 3667, 2973, 2922, 2378, 2922, 2378, 1950, 972, 936e, 967, 965a, 956d, 909 and most recently the vitamin D commutability materials.

The serum cholesterol project was originally organized jointly through the NIST, National Committee for Clinical Laboratory Standards (NCCLS), the Centers for Disease Control and Prevention (CDC) and the College of American Pathologists (CAP) and was completed in the fall of 1995. The serum creatinine project was organized in conjunction with the CAP in 2005.

The laboratory also produces fresh serum controls for lipid certification for Northwest Lipid Reference Laboratory and fresh frozen proficiency testing materials for the Lipid Standardization Program. The following may be contacted for references as to our ability to produce these materials:

References redacted

SPECIFIC TASKS

Bottling: 3,000 bottles (+/- 10%) of each of Levels I and II of SRM 968f must be prepared. Each bottle must contain 1 mL of serum. Bottles must be amber glass, and be capable of withstanding ultra-cold temperatures. The bottles must be labeled by the contractor, prior to filling, using labels that will be provided by the NIST. Bottles must be filled with an accuracy of 0.1 mL (1.1 mL is acceptable, 0.90 mL is not acceptable). Bottles will be sealed with a bromobutyl ultra pure rubber stoppers, and a color-coded aluminum crimp cap will be applied. Bottles must be transferred, in fill order, from the bottling equipment to a box in a "Z" pattern, filling each row left to right. The location of the first bottle in each box must be noted on each side of the outside corner of the box, and boxes must be numbered sequentially. Boxes must be labeled to indicate their contents. Materials must be stored frozen (-80° C.) prior to overnight shipment on dry ice to NIST. Delivery is expected 90 days following receipt of labels by the contractor.

DELIVERABLE

Progress reports will be provided via telephone or e-mail by the contractor as requested by NIST.

The contractor will provide NIST with 3,000 bottles (+/- 10%) of each of the three materials as described above. Each bottle must contain 1 mL of serum.

Plasma received from the NIST will be stored in -70° freezers provided by Solomon Park. It is anticipated that 45-60 individual bottles of whole citrated plasma will be furnished by the NIST for this project. It is also anticipated that these bottles will be labeled with an arbitrary code and that no

identifiers will be present on these bottles to indicate the patient or donor linked to these units. Finally, it is anticipated that the NIST will provide a scheme to segregate the units received into the two levels desired based on their measurements of the analytes of interest. Because Solomon Park will not be measuring any analytes in these units and because it is anticipated that there is no way for Solomon Park to identify the source of the plasma, no institutional review board approval will be needed or sought by Solomon Park for this project.

The plasma samples will be held segregated on separate shelves designated SRM 968f I and SRM 968f II. One level will be thawed and processed at a time to avoid mixing the levels.

DELIVERABLE DUE DATES AND/OR PERIOD OF PERFORMANCE

Delivery is due at the NIST by March 7, 2016.

This schedule is agreeable to Solomon Park.

GOVERNMENT-FURNISHED PROPERTY, DATA AND/OR INFORMATION

NIST will provide the plasma and spiking material used for preparation of SRM 968f. A NIST staff member will have the option of visiting the contractor's facilities and being responsible for spiking the serum.

Solomon Park has performed many similar materials for various government agencies and encourages site visits and observation of our procedures at the government agency's decision.

NIST will provide the contractor with labels appropriate for use at the low temperature at which SPRL will store the material upon receipt.

Acceptable Quality Level

The material must be suitable for use as an SRM. If deficiencies or inconsistencies between the material and the documentation are found, or if less than the stated number of bottles are received intact, the contractor has 30 days to correct the deficiency.

This is standard operating procedure in our laboratory and we have performed numerous similar projects with all to date being filled. Since the government is providing the source materials, this would be the only source of shortage as the number of bottles for aliquoting will be delivered to our site in advance. Three notes should be made concerning the amount of material we receive from the NIST.

1. **We intend to set and deliver 1.1 mL to each final bottle (unless specifically instructed to do otherwise) as this will ensure that each sample bottle will contain at least 1.0 mL of the final SRM**
2. **We will run a test run to be certain that the procedure produces a clear serum that will not continue to clot in the final SRM.**

3. **Finally, it is our experience that all processing will result in a volume loss of at least 10% no matter how uncomplicated or straightforward. Because filtering and processing are prone to loss of materials, it is recommended that the NIST provide excess plasma for each of the levels of interest.**

All of these factors calculated in, we would need to receive at a minimum of 4070 mL source plasma for each proposed pool:

1.	**Needed to fill 3000 X 1.1**	**3,300 mL**
2.	**Needed to compensate for loss**	**330 mL**
3.	**Total needed for each pool**	**3,630 mL**
4.	**Total needed for all pools**	**7,260 mL**

Monitoring Method

The NIST Technical Information Contact (TIC) will know that the SRMs were successfully prepared when they are analyzed as part of the value assignment process.

****** Award shall be made to the quoter whose quote offers the best value to the Government, technical capability, past performance, past experience and price shall be considered. The Government will evaluate information based on the following evaluation criteria:***

1) Technical Capability factor "Meeting or Exceeding the Requirement,"

2) Past Performance,

3) Past Experience, and

4) Price.

Technical capability, Past Performance, and Past Experience, when combined, shall be approximately equal in importance to price. If Technical Capability and Past Performance are equivalent, price shall be the determining factor. Technical Evaluation:

The contractor will demonstrate their understanding of the project and their ability to successfully complete the project. Technical capability shall be demonstrated through the propose

d methodology, including a description of the contractor's plan for processing and bottling the serum.

Normally, Solomon Park would draw donors with known lipid levels to fill the required levels of an SRM of this nature from the pool of donors with known lipid values that regularly give units of blood at the laboratory. In such a case, serum would be produced directly from whole units of blood which were processed according to the procedure for commutable serum described in the Clinical and Laboratory Standards Institute (CLSI) document C37-A.

However, since the NIST either has an existing supply of citrated plasma or is able to procure citrated plasma for approximately $0.50/mL or less, which is less than SPRL's standard cost to produce serum by our in house method, SPRL will produce serum from the plasma supplied. According to the NIST when 968e was produced from similar materials, clotting was (will be) defeated by the addition of 4% citrate (0.137 M) to the whole blood producing plasma.

Although the normal method for reversing the action of citrate is to add an adequate amount of calcium and thrombin, this was apparently used as a technique for SRM 968d which has exhibited commutability problems (although the final product was also reported to have been dialyzed and salts added which may have contributed to the lack of commutability more than the addition of calcium and thrombin).

The three areas that should be carefully monitored for this SRM are:

1. **Volume control as described above**
2. **Efficacy of clotting**
3. **Homogeneity of the final SRM**

The monitoring of the volume delivered to each vial well be as described above.

The efficacy of clotting will be tested as follows:

a. **5 mL of processed serum (that is serum that has been thawed and twice filtered) will be pipetted into a clean clear glass test tube.**

b. **To this 0.5 mL of 1.4 M calcium chloride will be added and the contents mixed.**

c. **Finally, 25 units of thrombin (Sigma cat # T1063) will be added to the test material.**

d. **The OD 710 will be read on this initial sample.**

e. **The contents of this sample will be held at 37° C. for 30 minutes**

f. **The OD 710 will be read on the incubated sample and recorded. Pre and post OD 710 readings must be less than OD 0.5 at 710nm.**

We would recommend using the exact procedure initially used by the NIST for SRMs 968 and 968a-c, which is to thaw the frozen units of plasma and vacuum filter through Whatman 541 filter paper (or Gelman P1 QUAL MED FLW – part no. 61000), mix and add any required materials provided by the NIST needed to enhance the existing analyte levels of interest, continue to mix overnight at 4-8° C. and filter a second time and while continuing to mix aliquot into 3000 one mL aliquots into 5 mL amber vials pre-labeled with NIST provided labels.

A trial pool containing at least three different samples of plasma will be pre-tested before committing the entire pool(s) to this procedure and the presence of clotting tested by the method described above. This test should also be done on the final pools before aliquoting into the end product.

The serum will be aliquoted into the 2 mL amber vials while these vials are placed in 2" Revco boxes (64 vials per box) using a wheaton Unispense. The Unispense will be calibrated before aliquoting begins and the volume adjusted and rechecked until 1.1 mL is delivered to the final vials. The accuracy of the aliquoting will be tested at approximately one hour intervals during the entire aliquoting procedure. The vials will be filled in a "Z" pattern and the boxes will have been labeled sequentially as follows:

SRM968f[I|II]### where ### begins at 001 and continues to 47 for each level. The SRM968f is obvious. The I or II signifies the level and the numbers are the sequential numbers for the boxes. The label will also have a bar code which will allow easy reading of the box number. The upper right hand corner of each box (with the label being forward) will be marked in order to track the order of fill.

Past Performance:

Past Performance evaluation shall be conducted to determine the overall quality of the products and services provided by the Contractor. Evaluation of Past Performance shall be based on information provided by references and/or the Contractor's recent and relevant procurement history with NIST or its affiliates. Provide contact information for three references with similar requirements. Timeliness, quality, and customer service will be evaluated.

Solomon Park Research Laboratories is the manufacturer of record for the following SRMs:

SRM 1951a	**cholesterol**
SRM 1951b	**cholesterol**
SRM 967	**creatinine**

Solomon Park is the manufacturer of record for these similar products:

Certification/Relabs	**cholesterol**
Certification	**cholesterol**
Lipid Standardization	**cholesterol**
Lipid reference	**cholesterol**

Past Experience:

The Contractor shall describe past experience performing similar work and explain how this experience is relevant to this project and how the experience will ensure successful completion of the project.

See above.

The Contractor must also acquire amber glass serum bottles of an appropriate volume (2 mL) capable of withstanding ultra-cold temperatures (min of -80 degrees C), butyl rubber stoppers, and color-coded aluminum crimp caps (one color for each of the three materials); label the bottles prior to filling; and ship the material on dry ice overnight to NIST.

Each of the serum pools must be individually blended, filtered, and dispensed into nominally 3000 bottles of 1.0 mL each

STATEMENT OF WORK

As stated above, the plasma units provided by the NIST will be held on separate shelves in ultralow freezers until sufficient plasma has been delivered and until the 5 mL amber vials have been delivered, tested for ultralow temperature use and the labels provided by the NIST have been affixed to these vials.

Plasma designated by the NIST will be pooled and filtered by vacuum through a Whatman 541 filter paper and allowed to mix overnight and filtered a second time through the same filters. At this time each pool (and the test pool in advance of the regular pools) will be tested for further clot formation by the procedure outlined above. If there is evidence of clotting, the serum will be filtered through a 0.22u filter and the clot test repeated.Aliquoting of pooled serum will be performed with a Wheaton Unispense into 2 mL amber borosilicate vials (Wheaton cat# 223693 or equivalent) which have been pre-labeled with the appropriate labels provided by the NIST for either SRM 968f I or II. The lots representing these vials will have been pre-tested for non-breakage under freeze thaw conditions with at least five cycles of freezing and thawing with 1.1 mL of serum at -70°C before labels are affixed. After The Unispense has equilibrated (by diverting serum through the system back into the source pool for a minimum of 50 rounds or 55 mL, it will be calibrated by dispensing 1.1 mL X 20 into a 25 mL cylinder to check for accuracy. Accuracy checks on the delivery volume will be repeated at approximately one hour intervals during the aliquoting. The check volume serum will be returned into the source pool. As is our normal procedure with serum pools, the vials will be filled in a "Z"

pattern in boxes that have been pre-numbered and with the upper left corner or start area of filling marked with black marker and stoppered (grey butyl 20 mm from West cat #10144257) and finally capped with colored aluminum caps (20 mm tear off VWR 16171-851 or equivalent). The source pool will be constantly stirred during aliquoting and the entire pool will be held in an ice cooled environment. The samples will also be held on cooled gel packs to keep the contents refrigerated.

Freezing of aliquoted samples will be performed as follows. Layers of boxes (Revco 2") containing the aliquoted vials will be laid out on pre cooled steel plates or layers of dry ice in-70o C freezers . These in turn will be covered with slabs of dry ice and held in this manner until frozen. The frozen aliquoted samples still in their original Revco boxes will finally be transferred to racks and held at -70o C. until ready for shipment to the NIST. Because of the large number of samples in each SRM, some boxes will necessarily be stacked onto slabs of dry ice in the freezers and then overlaid with a second, third, fourth and so on layer of dry ice.

Homogeneity of the serum pools can be assured by measuring any arbitrarily chosen analyte which can be measured with sufficient accuracy. Cholesterol is the assay of preference for this procedure for our laboratory as the test for homogeneity as this assay has been performed in our facility for other projects. Note, this analysis is to be performed only on pooled serum specimens and therefore does not constitute testing on the individual donor's serum specimens. The procedure is as follows:

Select one vial from each of a minimum of 15 different periods spaced in time equally throughout the dispensing run. For each pool, perform in a single run, quadruplicate (4) cholesterol analyses on each of the 15 sample diluted vials. This design will allow an analysis of variance to be run to check for significant vial-to-vial variability within periods of the dispensing run and to check for significant vial-to-vial variability over the entire dispensing run.

Data Analysis is performed on the data using a one-way analysis of variance (ANOVA). The analysis of variance table is as follows:

Source of Variation	Degrees of Freedom	Expected Mean Square
Among vials	14	(Sa)2 + (4)(Sv)2 (A)
Within vials	45	(Sa)2 (B)

Where (Sa)2 is the analytic variance, (Sv)2 is the vial-to-vial variance [i.e., the heterogeneity of the pool]. Degrees of freedom associated with the mean square estimate are 14 for number of vials and 45 for number of assays. Four (4) is the number of replicate determinations per vial.

Calculate the F-ratio formed by (Mean Square A)/(Mean Square B) with 14, 45 degrees of freedom. The critical value of F (probability = 0.05) for this comparison is 1.918. If the calculated F-ratio is greater than 1.918, the vial-to-vial variation for the pool may be too large and therefore the pool shall be evaluated further to substantiate the homogeneity of the pool before considering rejection.

NOTE: This procedure provides a test of the null hypothesis that the vial-to-vial variance is zero (i.e., the pool is perfectly homogenous) versus the alternative hypothesis that the vial-to-vial variance is greater than zero (i.e., the pool is not perfectly homogeneous). The sample size (i.e., the number of vials and the number of measurements per vial) was chosen so that the F-test would have probability of 0.80 or greater of rejecting a pool with a vial-to-vial coefficient of variation greater than or equal to 1%. In order for the F-test to have the stated power, it is important that the within run coefficient of variation be no larger than 1.5%.

Microbial contaminates will be tested on standard agar cultures (Remel 061572 or equivalent) which must yield growth of <10 CFUs/ml of pooled serum . If positive, additional microbial testing on blood/MAC biplates (Remel 02050 or equivalent) must be performed to specifically rule out the presence of the following pathogenic organisms: E. coli, Salmonella, Staph aureus, Pseudomonas aeruginosa." Pools testing positive for any bacterial growth will not be used.

Cholesterol testing will be performed on our Hitachi 704 using the enzymatic method reagents from Pointe Scientific.

Shipping to the NIST in Styrofoam containers capable of holding at least 18 2" Revco boxes and 50 pounds of dry ice will be performed when the pools are completed. All shipments will be by overnight carrier and will be insured for the cost of replacement of the contents.

Certificates of Analyses will be prepared for each pool and at the NIST's direction will be either included with the shipped materials or electronically delivered to the NIST.

Extracted from
NIST REPORT OF ANALYSIS 646.02-16-064

Screening of
25-Hydroxyvitamin D2, 25-Hydroxyvitamin D3, and 3-epi-25-Hydroxyvitamin D3 in
Candidate SRM 968f Fat-Soluble Vitamins in Frozen Human Serum
Using Isotope-Dilution Liquid Chromatography-Tandem Mass Spectrometry
for the use by the Vitamin D Metabolites Quality Assurance Program

August 4, 2016

INTRODUCTION

The 2016 Vitamin D Metabolites Quality Assurance Program (VitDQAP) sample set contained samples of NIST candidate SRM 968f Fat-Soluble Vitamins in Frozen Human Serum Level 1 (L1) and Level 2 (L2) for the determination of 25(OH)D levels. The concentrations of 25(OH)D2, 25(OH)D3, and 3-epi-25(OH)D3 in 968f L1 and L2 were determined using isotope-dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) (1) and are detailed in this report.

EXPERIMENTAL

Traceability
Traceability to amount-of-substance units of the SI is based on masses of 25(OH)D2, 25(OH)D3, and 3-epi-25(OH)D3 reference compounds, their purity assessments, and appropriate uncertainties.

Materials
The 25(OH)D3 (as monohydrate) reference compound (lot # G1E064) was obtained from United States Pharmacopeia (USP, Rockville, MD). The impurities in this material were evaluated by LC/UV, TGA, and qNMR, and moisture content for 25(OH)D3 was determined by Karl Fischer titration. The combined purity of 25(OH)D3 was determined to be 95.26 % ± 0.63 %. Ampoules of SRM 2972a 25-Hydroxyvitamin D2 and D3 Calibration Solutions (stored in Freezer # 12 in 227, A134) with a certified value of 293.6 ± 9.4 ng/g for 25(OH)D2 were used as a reference compound to prepare calibrants for 25(OH)D2. The 3-epi-25(OH)D3 (lot # RT3-2011-051A1) reference compound was obtained from IsoSciences (King of Prussia, PA). The impurities in this material were evaluated by LC/UV, TGA, and qNMR. No Karl Fischer analysis for3-epi-25(OH)D3 was performed due to a limited quantity of material available. The purity of 3-epi-25(OH)D3 was determined to be 96.48 % ± 3.07 %.

The isotopically labeled compound 25(OH)D3-*d*6 (lot # FN051410-01) was obtained from Cerilliant (Round Rock, TX). Isotopically labeled compounds 25(OH)D2-*d*3 (lot # SL3-2005-141A1) and 3-epi-25(OH)D3-*d3* (lot # RT3-2011-122A2) were obtained from IsoSciences (King of Prussia, PA). No purity assessments were performed for these compounds.

Sample Preparation
For 25(OH)D3 and 3-epi-25(OH)D3, three standard stock solutions for each of the two 25(OH)D species were gravimetrically prepared for the LC-MS/MS measurements. Approximately 1 to 2 mg of each reference compound for each stock solution for 25(OH)D3 and 3-epi-25(OH)D3 was accurately weighed (Mettler Toledo UMX5 balance) in an aluminum foil cup. The cup was placed in a 100 mL volumetric flask, the flask was stoppered and tared, then approximately 100 mL of anhydrous ethanol was added to the flask and weighed (Mettler Toledo XP205 balance). The concentrations for each of three stock solutions ranged from 11 µg/g to 16 µg/g and 11 µg/g to 13 µg/g for 25(OH)D3 and 3-epi-25(OH)D3, respectively. A working solution was gravimetrically prepared from each stock solution by diluting 1.5 mL to 3.0 mL of the stock solution with approximately 120 mL of anhydrous ethanol. The concentrations for each of the three working standard solutions ranged from 208 ng/g to 220 ng/g and 208 ng/g to 230 ng/g for 25(OH)D3 and 3-epi-25(OH)D3, respectively. Concentrated stock solutions for 25(OH)D3 and 3-epi-25(OH)D3 were prepared on 4/15/2013. Working stock solutions for 25(OH)D3 were prepared on 4/18/2016 and for 3-epi-25(OH)D3 on 4/15/2013.

A solution of an isotopically labeled internal standard (WS 1) at a concentration of 343.31 ng/g, 177.44 ng/g, and 273.95 ng/g for 25(OH)D3-*d*6, 25(OH)D2-*d*3, and 3-epi-25(OH)D3-*d*3, respectively, was prepared gravimetrically in the same way as the unlabeled 25(OH)D3. The 25(OH)D2-*d*3 solution (WS 1) was further gravimetrically diluted to a concentration of 22.83 ng/g (WS 2) for use as an internal standard for low level 25(OH)D2 samples. The 3-epi-25(OH)D3-*d*3 solution (WS 1) was further diluted to a concentration of 36.22 ng/g (WS 2) for use as an internal standard for low level 3-epi-25(OH)D3 samples. Internal standard solutions for 25(OH)D3-*d*6 and 25(OH)D2-*d*3 were prepared on 1/27/2014 and 4/10/2013 respectively. Internal standard solutions for 3-epi-25(OH)D3-*d*3 were prepared on 4/20/2015.

Eight calibrants with mass ratios of unlabeled to labeled compound ranging from 0.3 to 2.0 were gravimetrically prepared for 25(OH)D3. Two aliquots (281 µL - 482 µL) from one 25(OH)D3 working solution and three aliquots (149 µL - 1004 µL) from two working solutions were spiked with 300 µL of 25(OH)D3-*d*6 (WS 1). For 3-epi-25(OH)D3 samples, eight calibrants with mass ratios of unlabeled to labeled compound ranging from 0.3-2.0 were prepared. Two aliquots (250 µL - 429 µL) from one 3-epi-25(OH)D3 working solution and three aliquots (118 µL to 747 µL) from two working solutions were spiked with 300 µL of 3-epi-25(OH)D3-*d3* (WS 1). The mixtures were dried under nitrogen at approximately 45 °C and reconstituted with 300 µL of methanol, and transferred to two separate autosampler vials (150 µL each) for LC-MS/MS analysis.

For 25(OH)D2, three ampoules of SRM 2972a were used to gravimetrically prepare eight calibrants. Three aliquots (127 µL - 218 µL) from two of the ampoules and two aliquots (54 µL - 363 µL) from the remaining ampoule of SRM 2972a were spiked with 300 µL of 25(OH)D2-*d3* (WS 1), yielding eight calibrants with mass ratios of unlabeled to labeled compound ranging from 0.3 to 2.0. The mixtures were dried under nitrogen at approximately 45 °C and reconstituted with 300 µL of methanol, and transferred to two separate autosampler vials (150 µL each) for LC-MS/MS analysis.

One set of samples was prepared for analysis of 25(OH)D3 and 25(OH)D2, consisting of triplicate preparations of each level of 968f and a single preparation of SRM 972a Level 3 as a control. Each sample (approximately 2 g from combined contents of two vials of each sample) was accurately weighed into a 50 mL glass centrifuge tube. Each sample was spiked gravimetrically with amounts of 25(OH)D3-d_6 (WS 1) and 25(OH)D2-d_3 (WS 1 for SRM 972a Level 3; WS 2 for 968f) to get an approximately 1:1 mass ratio of analyte to internal standard. Target values for 25(OH)D3 were 15 ng/g for 968f L1 and 20 ng/g for 968f L2, and target values for 25(OH)D2 were 1 ng/g for 968f L1 and L2. After equilibration at room temperature for 1 h, the pH of each sample was adjusted to pH 9.8 ± 0.2 with 0.1 g/mL pH 9.8 carbonate buffer (approximately 200 µL buffer per mL of liquid). The 25(OH)D3 and 25(OH)D2 were simultaneously extracted from the serum matrix with 8 mL of hexane-ethyl acetate (50:50, volume fraction). Each sample was shaken vigorously for 10 min using a mechanical shaker to allow complete mixing. The upper hexane-ethyl acetate layer was transferred to another 50 mL centrifuge tube. The hexane-ethyl acetate extraction was repeated once more with another 8 mL of solvent by shaking for 3 min. The combined extract was dried under nitrogen at 45 °C and the residue was reconstituted with 170 µL of methanol for LC-MS/MS analysis.

One set of serum samples was prepared for 3-epi-25(OH)D3 analysis, consisting of triplicate preparations of each level of 968f and a single preparation of SRM 972a Level 4 as a control. Each sample (approximately 2 g from combined contents of two vials of each sample) was accurately weighed into a 50 mL glass centrifuge tube. Each sample was spiked gravimetrically with amounts of 3-epi-25(OH)D3-d_3 (WS 1 for SRM 972a Level 4; WS 2 for SRM 986f) to get an approximately 1:1 mass ratio of analyte to internal standard. The target values were 1.5 ng for 968f L1 and 2 ng/g for 968f L2. 3-epi-25(OH)D3 samples were processed in the same manner as described for 25(OH)D3 and 25(OH)D2 samples. The final combined extract was dried under nitrogen at 45 °C and the residue was reconstituted with 170 µL of methanol for LC-MS/MS analysis.

Instrumental method
LC-MS/MS Analysis for 25(OH)D3 and 3-epi-25(OH)D3:

The analyses were performed on an Applied Biosystems API 5000 LC/MS/MS system equipped with an Agilent 1260 Series LC system. An isocratic method was used with a Zorbax SB CN column (15 cm X 4.6 mm, 3.5 µm particle diameter, serial # USLA012060, from Agilent) at 30 ºC with 32:68 (volume fraction) water-methanol, operated at 0.75 mL/min. At the completion of each run, the column was rinsed with 100 % methanol for 10 min and then equilibrated at the initial condition for 12 min. The injection volume was 5 µL to 10 µL. The autosampler tray temperature was set at 5 ºC. Atmospheric pressure chemical ionization (APCI) in the positive ion mode and multiple reaction monitoring (MRM) mode were used for LC-MS/MS. The transitions at *m/z* 401 → *m/z* 383 and at *m/z* 407 → *m/z* 389 were monitored for 25(OH)D3 and 25(OH)D3-*d*6, respectively. The transitions at *m/z* 401 → *m/z* 383 and at *m/z* 404 → *m/z* 386 were monitored for 3-epi-25(OH)D3 and 3-epi-25(OH)D3-*d*3, respectively. An additional transition *m/z* 419 → *m/z* 401 was monitored along with the 3-epi-25(OH)D3-*d*3 analyses to detect the presence of a previously observed interferent. For 25(OH)D3 measurements, the dwell times were 0.25 s for each MRM. The curtain gas and collision gas were nitrogen at settings of 30 psi and 5 psi, respectively. The ion source gas 1 was air at a setting of 40 psi. The needle current was set at 5 µA and the temperature was maintained at 325 ºC. The declustering potential, entrance potential,

collision energy, and collision exit potential were set at 90 V, 12 V, 10 V, and 16 V, respectively. For 3-epi-25(OH)D3 measurements, the dwell times were 0.25 s for each MRM. The curtain gas and collision gas were nitrogen at settings of 10 psi and 3 psi, respectively. The ion source gas 1 was air at a setting of 60 psi. The needle current was set at 4 μA and the temperature was maintained at 300 ºC. The declustering potential, entrance potential, collision energy, and collision exit potential were set at 90 V, 12 V, 10 V, and 15 V, respectively.
LC-MS/MS Analysis for 25(OH)D2:

The analyses were performed on an Applied Biosystems API 5000 LC-MS/MS system equipped with an Agilent 1260 Series LC system. An isocratic method was used with an Ascentis Express F5 column (15 cm X 4.6 mm, 2.7 μm particle diameter, serial # USBK001541) at 30 ºC with 27:73 (volume fraction) water-methanol, operated at 0.75 mL/min. At the completion of each run (24 min), the column was rinsed with 100 % methanol for 10 min and then equilibrated at the initial condition for 15 min. The injection volume was 5 μL to 10 μL. The autosampler tray temperature was set at 5 ºC. APCI in the positive ion mode and MRM mode were used for LC-MS/MS. The transitions at *m/z* 413 → *m/z* 395 and at *m/z* 416 → *m/z* 398 for 25(OH)D2 and 25(OH)D2-*d*3, respectively, were monitored. The dwell times were 0.25 s for each MRM. The curtain gas and collision gas were nitrogen at settings of 12 psi and 6 psi, respectively. The ion source gas 1 was air at a setting of 40 psi. The needle current was set at 5 μA and the temperature was maintained at 300 ºC. The declustering potential, entrance potential, collision energy, and collision exit potential were set at 90 V, 5 V, 10 V, and 30 V, respectively.

Quantitation
The measurement protocol used for LC-MS/MS analysis was combined with the DEQAS July 2016 sample set. The control, the first preparation of the five DEQAS samples, 968f L1 triplicate preparations, 968f L2 triplicate preparations, and eight calibrants were run first. Subsequently, the entire analysis order was run again in reverse order. By combining the data of calibrants run before and after the samples, a linear regression was calculated using a slope-intercept model (y = mx + b), which was used to convert the measured intensity ratios of analyte to mass ratios. The mass ratios were then used along with the amounts of the internal standard added to calculate analyte concentrations. All samples were analyzed between 7/27/2016 – 7/28/2016.

RESULTS AND DISCUSSION

The results of the LC-MS/MS measurements of 25(OH)D in SRM 968f L1 and L2 are shown in Table 1. The 25(OH)D3 and 3-epi-25(OH)D3 results are corrected for the purity (which includes the water content) of the reference compound. Selected ion chromatograms for samples are shown in Figures 1-3, for 25(OH)D2, 25(OH)D3, and 3-epi-25(OH)D3, respectively.

It is important to note that during the extraction of 25(OH)D2, the second extraction volume of 968f L1 Preparation C was incorrectly combined with 968f L2 Preparation C extraction volumes. The 25(OH)D2 values for these samples are denoted in grey in Table 1 and should be used with caution as the result could be biased low for L1 (did not have 2 extraction volumes) and high for L2 (had 2 extraction volumes for L2 plus the 2nd extraction volume from L1).

The correlation coefficients of the linear regression lines were 0.9999, 0.9999, and 0.9948 for 25(OH)D2, 25(OH)D3, and 3-epi-25(OH)D3 respectively. The results of the controls (SRM 972a level 3 and level 4) as shown in Table 2 were within the certified values for all three 25(OH)D species indicating the measurements were under control.

CONCLUSIONS

The values of controls are consistent with certified values. These data are suitable for the use in the VitDQAP assessment of 25(OH)D2, 25(OH)D3, and 3-epi-25(OH)D3 in SRM 968f L1 and L2.

REFERENCE

1. Tai SSC, Bedner M, Phinney KW. Development of a Candidate Reference Measurement Procedure for the Determination of 25-Hydroxyvitamin D3 and 25-Hydroxyvitamin D2 in Human Serum Using Isotope-Dilution Liquid Chromatography/Tandem Mass Spectrometry. Anal Chem 2010;82:1942-8.

Table 1. LC-MS/MS results for 25(OH)D2, 25(OH)D3, and 3-epi-25(OH)D3 in 968f L1 and L2. Results for D3 and epi-D3 are corrected for the purity of the reference standards. D2 was calibrated using SRM 2972a. During sample preparation, the 2nd extraction of 968f L1 Preparation C was combined with 968f L2 Preparation C. The 25(OH)D2 values for these samples are denoted in grey and should be used with caution as the result could be biased low for L1 (did not have 2 extraction volumes) and high for L2 (had 2 extraction volumes for L2 plus the 2nd extraction volume from L1). The average values and standard deviations for 25(OH)D2 do not include Preparation C.

25(OH)D2 (ng/g)

	Prep A		Prep B		Prep C	
Sample	**Injection 1**	**Injection 2**	**Injection 1**	**Injection 2**	**Injection 1**	**Injection 2**
968f L1	0.820	0.811	0.825	0.880	0.782	0.763
	Avg Prep A	0.815	Avg Prep B	0.852		
	Avg L1	**0.83 ± 0.03**				
968f L2	0.165	0.164	0.153	0.175	0.269	0.255
	Avg Prep A	0.165	Avg Prep B	0.164		
	Avg L2	**0.164 ± 0.009**				

25(OH)D3 (ng/g)

	Prep A		Prep B		Prep C	
	Injection 1	**Injection 2**	**Injection 1**	**Injection 2**	**Injection 1**	**Injection 2**
968f L1	12.263	12.095	12.185	11.938	12.303	11.822
	Avg Prep A	12.179	Avg Prep B	12.061	Avg Prep C	12.062
	Avg L1	**12.1 ± 0.2**				
968f L2	14.936	15.429	15.410	15.458	15.421	15.314
	Avg Prep A	15.183	Avg Prep B	15.434	Avg Prep C	15.367
	Avg L2	**15.33 ± 0.2**				

3-epi-25(OH)D3 (ng/g)

	Prep A		Prep B		Prep C	
	Injection 1	**Injection 1**	**Injection 1**	**Injection 2**	**Injection 1**	**Injection 2**
968f L1	0.714	0.718	0.675	0.759	0.678	0.698
	Avg Prep A	0.716	Avg Prep B	0.717	Avg Prep C	0.688
	Avg L1	**0.71 ± 0.03**				
968f L2	1.195	1.111	0.830	1.056	0.962	1.145
	Avg Prep A	1.153	Avg Prep B	0.943	Avg Prep C	1.054
	Avg L2	**1.1 ± 0.1**				

Table 2. LC-MS/MS measurements of 25(OH)D in SRMs 972a controls

	SRM 972a Level 3		SRM 972a Level 4	
Analyte	**Measured**	**Certified**	**Measured**	**Certified**
25(OH)D3	19.4 ± 0.3	19.4 ± 0.4	25.6 ± 0.3	25.8 ± 2.0
25(OH)D2	12.70 ± 0.01	13.0 ± 0.3		

Figure 1a. Selected ion chromatograms by LC-MS/MS for 25(OH)D2 (top) and 25(OH)D2-*d3* (bottom) at a concentration of 0.82 ng/g from 968e L1.

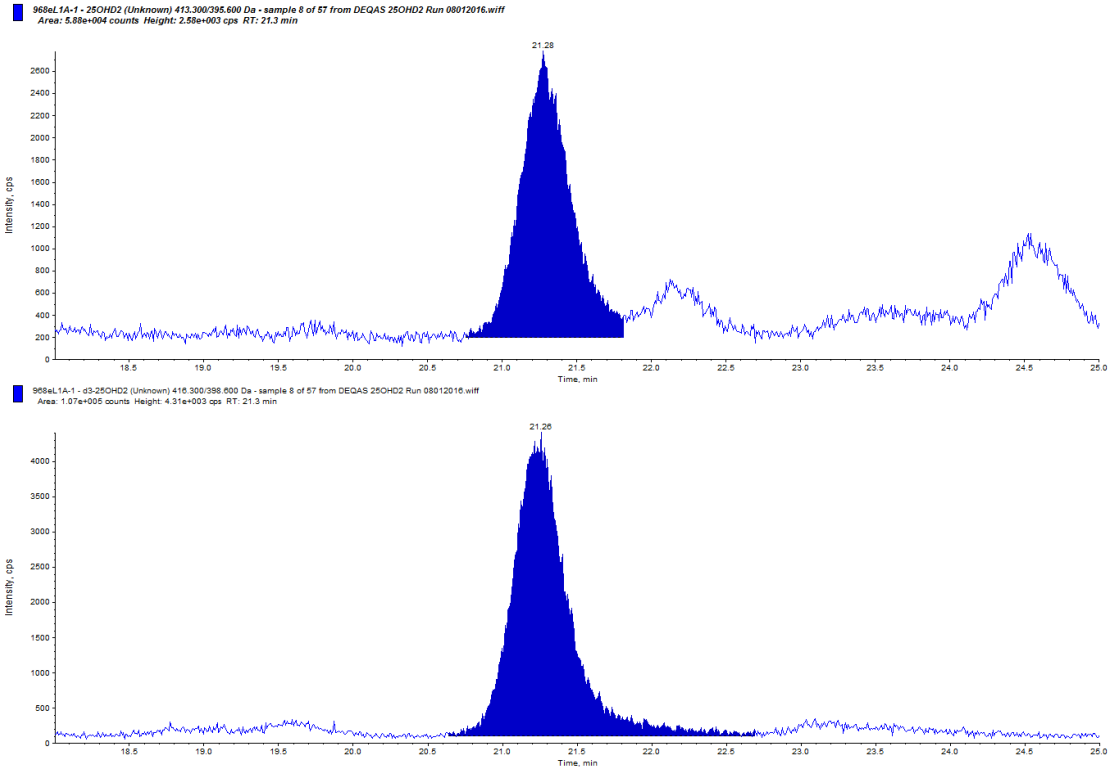


Figure 1b. Selected ion chromatograms by LC-MS/MS for 25(OH)D2 (top) and 25(OH)D2-*d3* (bottom) at a concentration of 0.16 ng/g from 968e L2.

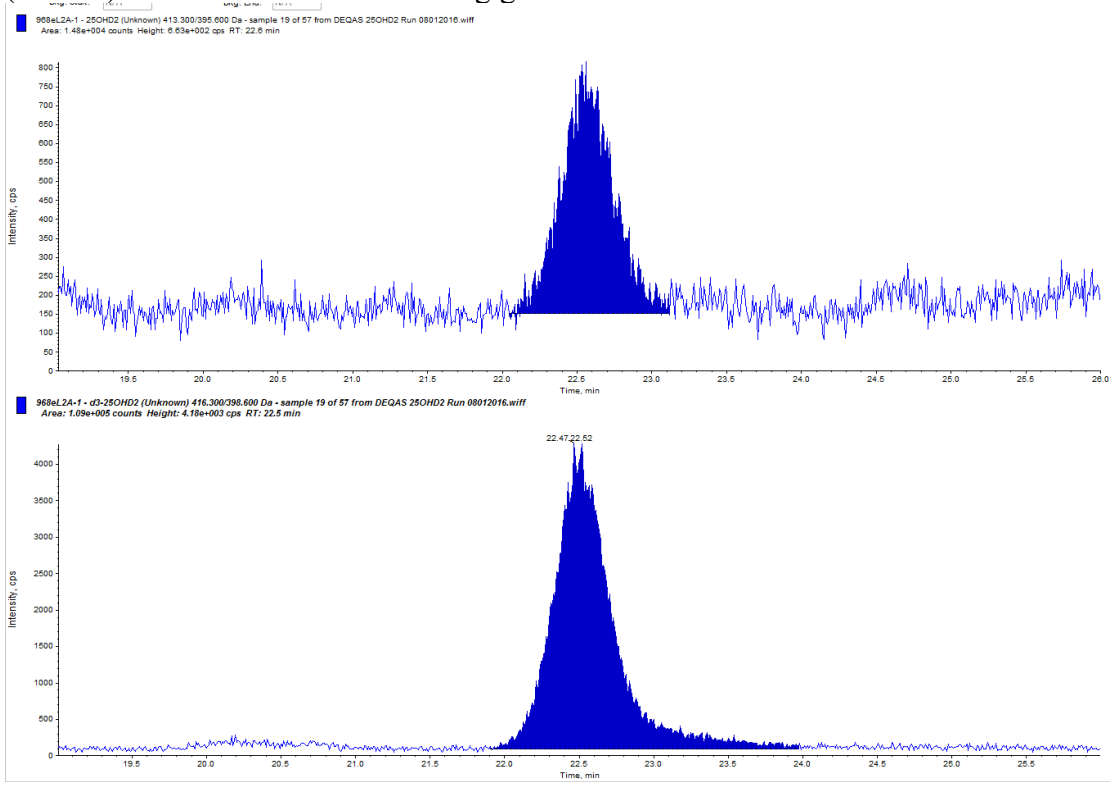


Figure 2a. Selected ion chromatograms by LC-MS/MS for 25(OH)D3 (top) and 25(OH)D3-*d6* (bottom) at a concentration of 12.26 ng/g from 968e L1.

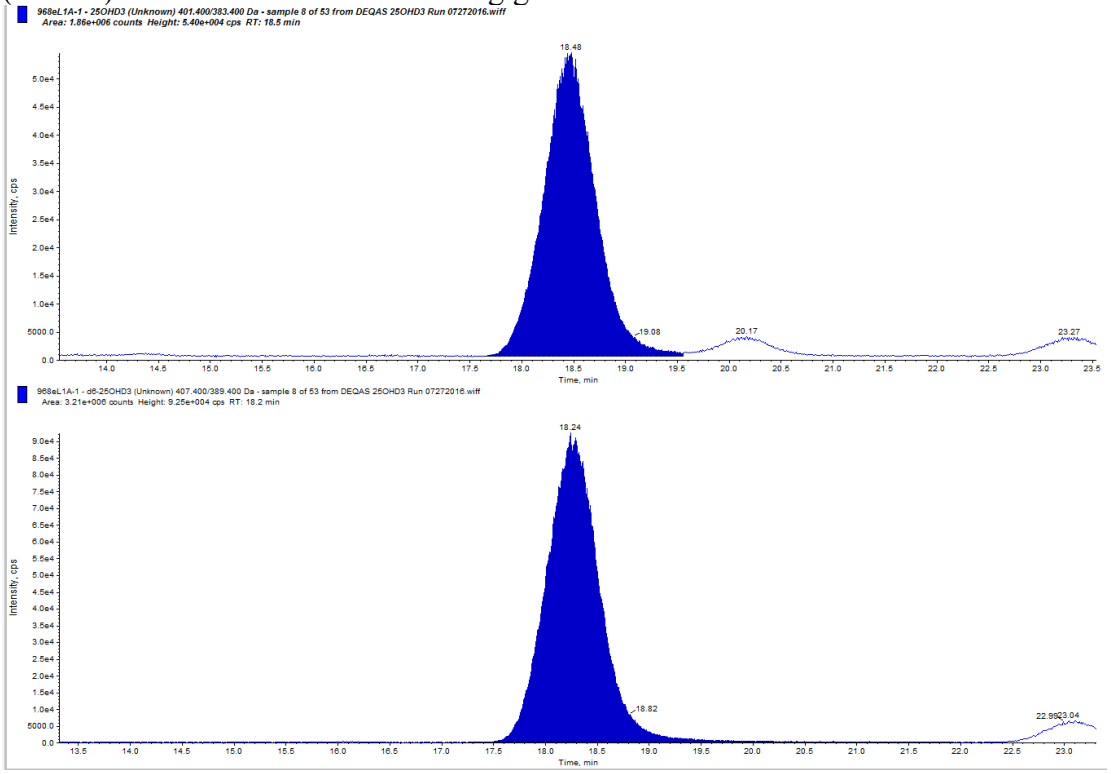


Figure 2b. Selected ion chromatograms by LC-MS/MS for 25(OH)D3 (top) and 25(OH)D3-*d6* (bottom) at a concentration of 14.94 ng/g from 968e L2.

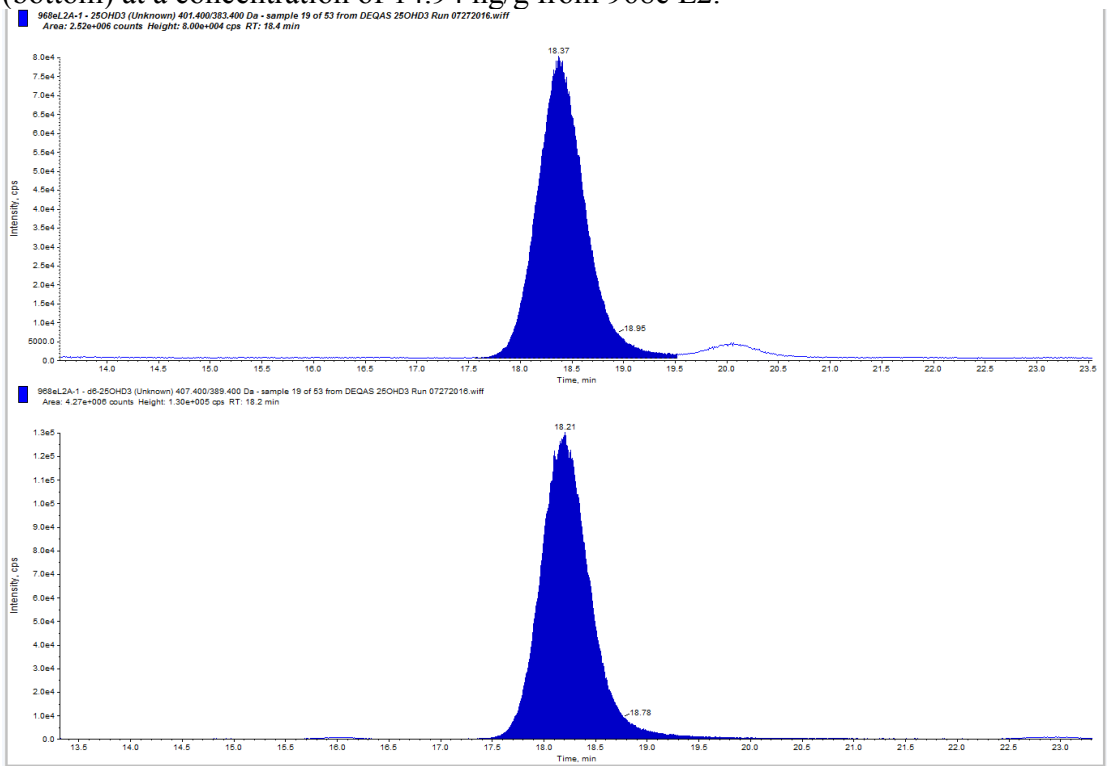


Figure 3a. Selected ion chromatograms by LC-MS/MS for 3-epi-25(OH)D3 (top) and 3-epi-25(OH)D3-*d3* (bottom) at a concentration of 0.71 ng/g from 968e L1.

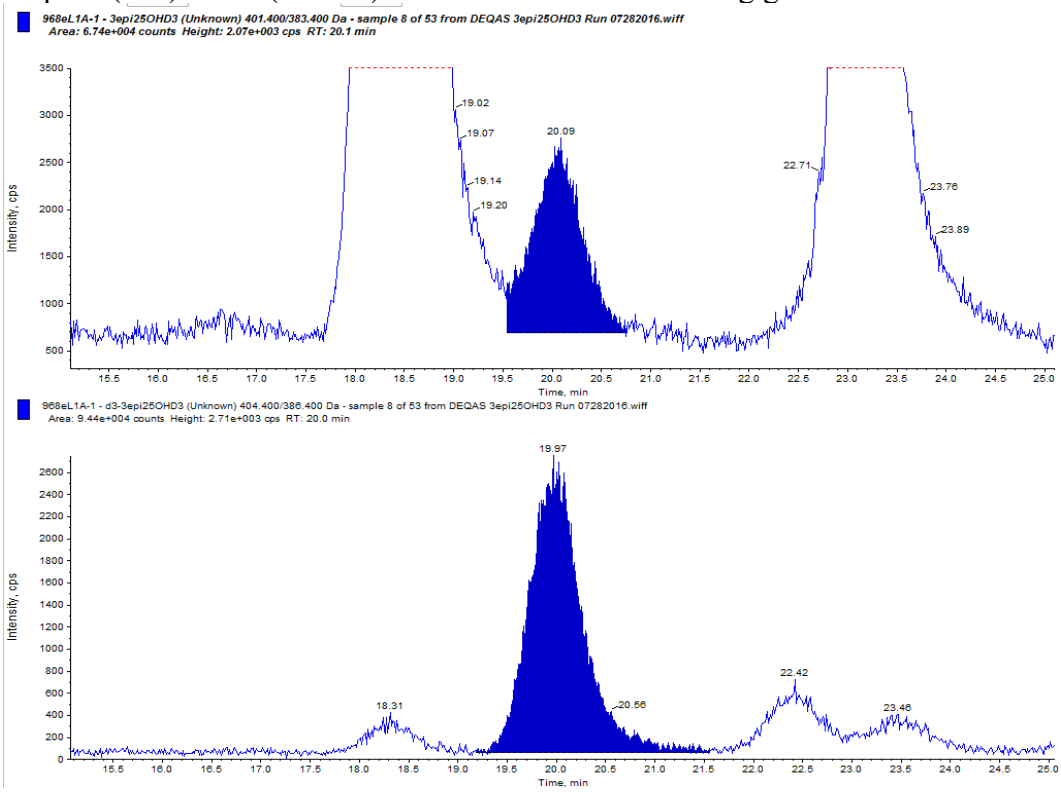
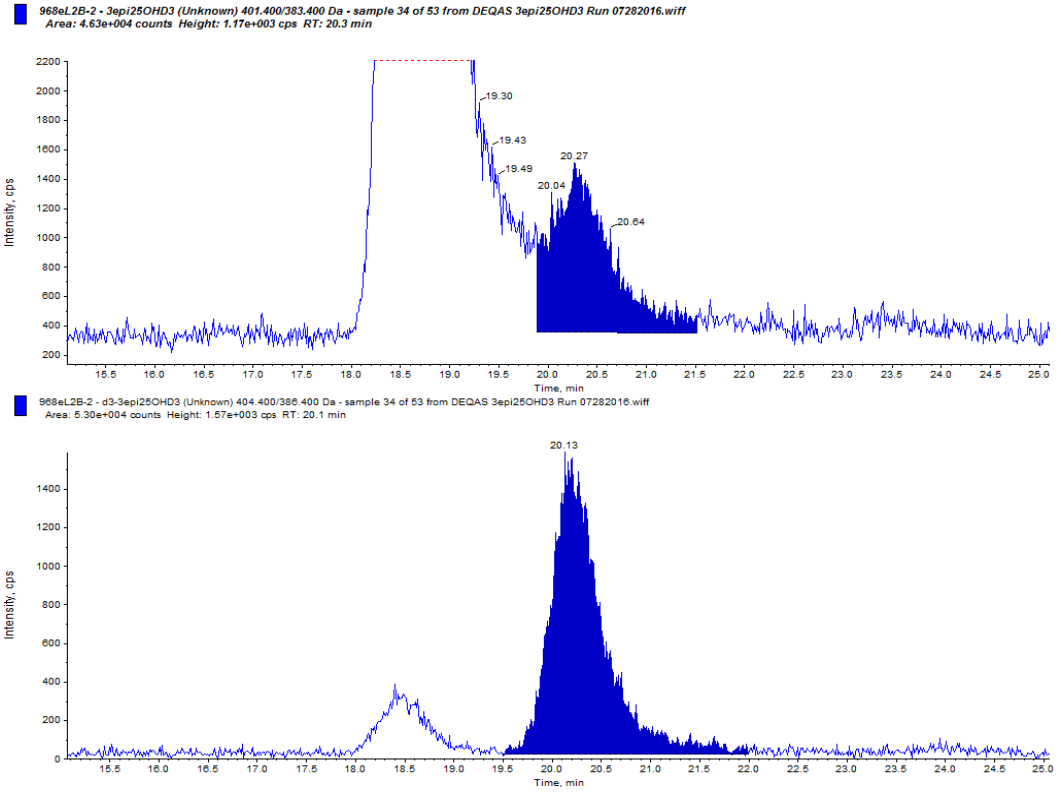


Figure 3b. Selected ion chromatograms by LC-MS/MS for 3-epi-25(OH)D3 (top) and 3-epi-25(OH)D3-*d3* (bottom) at a concentration of 1.2 ng/g from 968e L2.



Extracted from
NIST REPORT OF ANALYSIS 646.02-17-044

The Determination of the Density of SRM 968f, Fat-Soluble Vitamins in Frozen Human Serum, Levels 1 and 2

June 28, 2017

INTRODUCTION

Standard Reference Material (SRM) 968f, Fat-Soluble Vitamins in Frozen Human Serum, consists of two levels, and each vial contains 1 mL of frozen human serum. Density measurements would be useful for converting mass/mass to mass/volume units. The density of each level was determined by the Lang-Levy pipet method[1], in which small (one mL or less) quantities of serum are used.

EXPERIMENTAL

Technical Procedures: TP 646.02.01 Calibration and Use of Analytical Balances

A one mL Lang-Levy pipet had been calibrated previously with water at ambient balance room temperature (22.6°C). In this calibration, the dry pipet was wiped with a damp lint-free cloth and weighed on a metal stand on the AT201 semi-micro balance. The balance was zeroed, the pipet was filled to the mark with distilled water, wiped with a damp lint-free cloth, and weighed. The pipet was then rinsed with methanol, dried, and the procedure repeated. The volume of the pipet was calculated from the weight of water and the density of water at the temperature used. Three vials of each level of the SRM were thawed at room temperature and pooled. The weighing procedure was repeated with the pooled samples. Between each weighing the pipet was rinsed with water, then methanol, and dried.

RESULTS

The results for the density measurements of the two levels of SRM 968f are given in table 1.

CONCLUSIONS

The values for the density will allow the conversion of the mass/mass units for the concentration of fat-soluble vitamins to mass/volume units.

REFERENCE

1 L.T. Sniegoski and J.R. Moody, Determination of Serum and Blood Densities, Anal. Chem. 1979, 1577-8.

Table 1.
Determination of the Density of SRM 968f, Fat-Soluble Vitamins in Frozen Human Serum

		Readings, g					
		# 1	# 2	# 3	Mean, g	Density	
Level 1	Fill 1	1.01728	1.01729		1.01729	1.01798	
	Fill 2	1.01739	1.01738		1.01739	1.01808	
						1.01803	Mean, g/mL
						0.00007	SD, g/mL
						0.00695	CV, %
Level 2	Fill 1	1.01954	1.01954	1.01961	1.01956	1.02026	
	Fill 2	1.01949	1.01946	1.01948	1.01948	1.02017	
						1.02021	Mean, g/mL
						0.00006	SD, g/mL
						0.00601	CV, %

Volume of pipet at 22.6 C: 0.999319 mL

Extracted from
NIST REPORT OF ANALYSIS 646.02-17-045

Determination of Retinol, γ+β- and α-Tocopherol in
SRM 968f Fat-Soluble Vitamins in Frozen Human Serum

July 10, 2017

INTRODUCTION

The National Institute of Standards and Technology (NIST) has produced Standard Reference Material (SRM) 968f Fat-Soluble Vitamins in Frozen Human Serum to replace SRM 968e. This SRM consists of two levels of serum (SRM 968f-Level 1 and SRM 968f-Level 2) using material that NIST had previously acquired from Interstate Blood Bank (Memphis, TN and Chicago, IL).

Measurements for retinol, γ/β-tocopherol and α-tocopherol in SRM 968f have been made by reversed-phase liquid chromatography (LC) with wavelength programmed ultraviolet (UV)/visible absorbance detection. These measurements will be used for value assigning the analytes in this material in conjunction with results obtained from the laboratories that participate in the Micronutrients Measurement Quality Assurance Program (MMQAP). Data from this study will also be used to determine homogeneity of the SRM.

EXPERIMENTAL SECTION

Measurement Traceability
The measurements documented in this report are metrologically traceable to the International System of Units (SI) through the molar absorptivities listed in the literature [1,2].

Calibration Solutions
Stock solutions of *trans*-retinol (CAS 68-26-8), γ-tocopherol (CAS 7616-22-0), and α-tocopherol (CAS 59.02-9) were prepared by dissolving each compound in absolute ethanol that contained 30 mg/L butylated hydroxytoluene (BHT; added to prevent analyte oxidation). Retinol (Lot #BCBR9941V) and α-tocopherol (Lot #44238/1) were purchased from Sigma-Aldrich (St. Louis, MO); γ-tocopherol (Lot #20313-304) was purchased from Chromadex (Irvine, CA). Calibration solutions were independently prepared from the stock solutions. A 1:1 mass ratio (g/g) of each calibration solution to the internal standard solution (42.91 μg/mL tocol in ethanol) was prepared and used to determine detector responses for each analyte. See Appendix 646.02-17-045-A. Tocol was purchased from Eisai Inc., Tokyo, Japan).

The concentrations of the analytes in the calibration solutions were determined by spectrophotometry based on the following extinction coefficients in absolute ethanol (dL/g/cm): 1843 for retinol at 325 nm, 75.8 for α-tocopherol at 292 nm, 91.4 for γ-tocopherol at 298 nm [1,2]. Corrections for purity (mass fraction) were made based on the LC analysis of the stock solutions at the wavelength at which the concentration was determined. The following purities were used in this study: *trans*-retinol (97.68 %; 0.11 SD), γ-tocopherol (98.99 %; 0.02 SD), α-tocopherol (98.56 %; 0.05 SD). The uncertainties for the LC purity measurements represent the standard deviation of a single measurement and is less than one percent. The LC purity values for these solutions can be found in Appendix 646.02-17-045-B.

Sample Preparation
An aliquot (250 μL) from each of 20 different vials/boxes from two levels of SRM 968f were extracted and prepared for analysis using the following protocol.[1] Prior to extraction, the serum samples were equilibrated to room temperature and sonicated for approximately 3 min. Each aliquot was combined

with an equal volume of ethanol containing tocol (internal standard; 42.91 μg/mL) and BHT (antioxidant; 30 μg/mL) to precipitate the proteins from the serum matrix. About 1 mL of hexane was added to each mixture to extract the analyte. The mixture was subsequently vortex mixed for about 1 min and centrifuged (1000 x G) at room temperature for 10 min. The hexane layer was removed and the extraction process was repeated. The supernatants from the two extractions were combined. The extracts were then evaporated to dryness under a stream of nitrogen and reconstituted with 250 μL of ethanol containing 30 μg/mL BHT. The reconstituted extracts were placed in amber autosampler vials and vortex mixed for about 30 s to ensure dissolution prior to HPLC analysis. All 20 samples from each level of SRM 968f were prepared and analyzed on the same day. Level 3 of SRM 968e Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum was prepared using the above extraction protocol and analyzed for quality control.

The serum density for each level of SRM 968f was determined using the Lang-Levy pipet method in which 1 mL or less quantities of serum are used.[3]

Instrumental Method
The LC system used for these measurements consisted of a Varian 9012 LC, Agilent 1100 Series absorbance detector (Serial # JP24020446), and an Agilent 1100 Series autosampler.

The following chromatographic conditions were used for the analyses.
Column: Develosil RP aqueous (4.6 x 250 mm; serial #2701661; batch #310812)
Column temperature: 25 °C
Auto-sampler temperature: 10 °C
Mobile phase: methanol:water (96:4 volume fraction) with isocratic elution
Flow rate: 0.6 mL/min
UV-visible absorbance detection: Retinol at 325 nm; γ+β-tocopherol, and α-tocopherol at 292 nm
Injection volume: 30 μL

Quantitation
Samples from 20 different boxes/vials of each level of SRM 968f were randomly selected for analysis. Quantitation was based on the internal standard approach using averaged response factors. Concentrations (expressed in μg/mL*)* were calculated from the ratio of peak areas and the detector response factors. The uncertainty of the mean represents the standard deviation of single measurements of samples from 20 different boxes/vials of each level of SRM 968f.

RESULTS

The results for the analysis of the control material SRM 968e (Level 3) are summarized in Table 1. The means provided are the results from at least two LC injections from two extracts of the SRM. Concentrations (expressed in μg/mL*)* of the analytes were calculated from the ratio of peak areas and the internal standard and the (averaged) detector response factors. Results from these analyses are comparable to the assigned values for the SRM.[4]

A summary of the results for the measurement of retinol, γ/β- tocopherol, and α-tocopherol in Levels 1 and 2 of SRM 968f is provided in Table 2 and Table 3, respectively. Samples are listed in the order in which they were analyzed. Samples for level 1 were prepared on April 13, 2016 and analyzed from April 13 through April 14, 2016. Samples for level 2 were prepared on April 18, 2016 and analyzed from April 18 through April 19, 2016. Data from samples from boxes 1 and 5 for level 2 (highlighted data in Table 3) were questionable due to possible sample preparation technicalities and were not included in the mean. To investigate these findings, measurements from box 1 and box 5 were repeated on February 16, 2017 and March 21, 2017, respectively. Repeat measurements (indicated by an asterisk) are from the average of two LC injections of a single vial from each sample. Data from the repeat measurements for box 1 and box 5 are found in Appendix 646.02-17-045-C. Data from box 1 (file 399) and box 5 (file 408) in Table 3 were replaced with data from the repeat measurements.

Representative chromatograms from the LC separation of the analytes in Level 1 and Level 2 of SRM 968f are provided in Figure 1. The concentration (μg/mL) of each analyte in both levels of SRM 968f versus the box number/sample is shown in Figure 2 through Figure 4. The concentration of each analyte in both levels of SRM 968f versus run order is presented in Figure 5 through Figure 7. In the figures, the solid diamonds represent the individual concentrations per sample; the mean is indicated by the solid line. The dashed lines in each figure represent the associated uncertainties expressed as the 95 % level of confidence (U95).

Representative chromatograms from LC purity measurements for retinol and tocopherol reference standards are provided in Appendix 646.02-17-045-D.

CONCLUSIONS

Based on the data generated from these measurements, SRM 968f appears to be homogeneous. The results from the measurements present no analytical concerns regarding the data being used to value assign retinol and the tocopherols in this material. These data will be sent to the Statistical Engineering Division for evaluation.

REFERENCES

1. Thomas, J.B; Duewer, D.L.; Mugenya, I.O.; Phinney K.W.; Sander, L.C.; Sharpless, K.E.; Sniegoski, L.T.; Tai, S.S; Welch, M.J.; Yen, J.H. *Preparation and Value Assignment of Standard Reference Material 968e Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum*; Anal Bioanal Chem, Vol. 402, pp. 749-762 (2012).
2. Sharpless, K.E.; Thomas, J.B.; Duewer, D.L. NIST IR 7880-40 *Handout of the Fat-Soluble Vitamin and Carotenoid Analysis Tutorial October 27, 1997* (2016). http://dx.doi.org/10.6028/NIST.IR.7880-40
3. Sniegoski, L.T.; Moody, J.R. Determination of Serum and Blood Densities, Anal. Chem., Vol. 51(9), pp. 1577−1578 (1979).
4. Certificate of Analysis, Standard Reference Material SRM 968e Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum, NIST, Gaithersburg, MD (2015).

Table 1. Summary of Quality Control Data from the Analysis of SRM 968e (Level 3) Fat-Soluble Vitamins and Carotenoids in Human Serum[a]

	Response factor	0.03572		0.7123		0.9279		
4/13/2016								
File No.	**Sample Description**	**Total retinol**	**(µg/mL)**	**γ/β-Tocopherol**	**(µg/mL)**	**α-Tocopherol**	**(µg/mL)**	
		Peak area		**Peak area**		**Peak area**		
369	Serum 968e-Level 3-A	3.634	**0.662**	0.629	**2.30**	4.054	**19.24**	
370	Serum 968e-Level 3-A	3.640	**0.639**	0.627	**2.21**	4.055	**18.56**	
393	Serum 968e-Level 3-B	5.515	**0.618**	0.943	**2.11**	6.383	**18.59**	
407	Serum 968e-Level 3-B	3.212	**0.637**	0.581	**2.30**	3.723	**19.18**	
		Mean	**0.639**		**2.23**		**18.89**	
		SD	**0.018**		**0.09**		**0.37**	
		% SD	**2.794**		**4.08**		**1.96**	
2/1/2017								
437	Serum 968e-Level 3-B	4.942	**0.653**	0.925	2.43	5.488	18.82	
438	Serum 968e-Level 3-B	4.902	**0.645**	0.855	2.24	5.502	18.80	
439	Serum 968e-Level 3-B	4.919	**0.649**	0.874	2.30	5.536	18.98	
440	Serum 968e-Level 3-B	4.902	**0.647**	0.913	2.41	5.451	18.78	
441	Serum 968e-Level 3-A	4.746	**0.641**	0.871	2.35	5.427	19.05	
442	Serum 968e-Level 3-A	4.757	**0.646**	0.865	2.34	5.460	19.26	
443	Serum 968e-Level 3-A	4.776	**0.646**	0.819	2.21	5.465	19.21	
		Mean	**0.647**		**2.33**		**18.99**	
		SD	**0.004**		**0.08**		**0.20**	
		% SD	**0.542**		**3.59**		**1.04**	
		Certified Value	**0.647 +/- 0.021**		**2.27 +/- 0.17**		**19.37 +/- 0.63**	

a The mean is from at least two LC injections from two extracts of the SRM. Concentrations are corrected for the purity of the reference standard.

Table 2. Summary of Measurements for Retinol and Tocopherols in SRM 968f (Level 1)[a]

	Response factor	0.03572		0.7123		0.9279	
File No.	**Sample Description**	**Total retinol peak area**	**(µg/mL)**	**γ/β-Tocopherol peak area**	**(µg/mL)**	**α-Tocopherol peak area**	**(µg/mL)**
371	SRM 968f-Level 1-Box 11-First Vial	1.762	**0.331**	0.301	**1.13**	1.065	**5.20**
372	SRM 968f-Level 1-Box 12-Center Vial	1.750	**0.325**	0.296	**1.10**	1.072	**5.17**
373	SRM 968f-Level 1-Box 13-Last Vial	1.711	**0.325**	0.291	**1.10**	1.081	**5.33**
374	SRM 968f-Level 1-Box 14-Last Vial	1.698	**0.328**	0.270	**1.04**	1.049	**5.26**
375	SRM 968f-Level 1-Box 15-Last Vial	1.760	**0.334**	0.284	**1.07**	1.078	**5.31**
376	SRM 968f-Level 1-Box 1-Center Vial	1.800	**0.321**	0.306	**1.09**	1.109	**5.14**
377	SRM 968f-Level 1-Box 2-Center Vial	1.637	**0.325**	0.270	**1.07**	1.006	**5.19**
378	SRM 968f-Level 1-Box 3-Center Vial	1.840	**0.325**	0.293	**1.03**	1.162	**5.33**
379	SRM 968f-Level 1-Box 4-First Vial	1.796	**0.322**	0.293	**1.05**	1.106	**5.16**
380	SRM 968f-Level 1-Box 20-Center Vial	1.628	**0.317**	0.290	**1.12**	1.014	**5.12**
381	SRM 968f-Level 1-Box 19-Last Vial	1.738	**0.319**	0.296	**1.08**	1.090	**5.20**
382	SRM 968f-Level 1-Box 18-First Vial	1.881	**0.324**	0.330	**1.13**	1.161	**5.19**
383	SRM 968f-Level 1-Box 17-Last Vial	1.938	**0.335**	0.292	**1.01**	1.173	**5.27**
384	SRM 968f-Level 1-Box 16-Last Vial	1.866	**0.328**	0.309	**1.08**	1.143	**5.21**
385	SRM 968f-Level 1-Box 10-First Vial	1.988	**0.333**	0.307	**1.03**	1.174	**5.11**
386	SRM 968f-Level 1-Box 9-First Vial	2.032	**0.324**	0.323	**1.03**	1.276	**5.29**
387	SRM 968f-Level 1-Box 8-First Vial	2.130	**0.335**	0.347	**1.09**	1.288	**5.26**
388	SRM 968f-Level 1-Box 7-First Vial	2.090	**0.327**	0.363	**1.13**	1.258	**5.11**
389	SRM 968f-Level 1-Box 6-Last Vial	2.107	**0.320**	0.378	**1.14**	1.310	**5.17**
390	SRM 968f-Level 1-Box 5-Center Vial	2.051	**0.324**	0.344	**1.08**	1.278	**5.24**
		Mean	**0.326**		**1.08**		**5.21**
		SD	**0.005**		**0.04**		**0.07**
		% SD	**1.62**		**3.71**		**1.36**

a Concentrations are corrected for purity of reference standard. Samples are listed in the order in which they were analyzed. One sample was prepared from each box. A single LC injection was made for each sample.

Table 3. Summary of Measurements for Retinol and Tocopherols in SRM 968f (Level 2)[a]

	Response factor	0.03572		0.7123		0.9279	
File No.	**Sample Description**	**Total retinol peak area**	**(µg/mL)**	**γ/β-Tocopherol peak area**	**(µg/mL)**	**α-Tocopherol peak area**	**(µg/mL)**
399	SRM 968f-Level 2-Box 1-Center Vial	4.582	**0.798**	0.737	**2.56**	3.230	**14.62**
*466/467	SRM 968f-Level 2-Box 1-Center Vial	5.4858	**0.6387**	1.055	**2.45**	3.685	**11.15**
400	SRM 968f-Level 2-Box 20-Last Vial	4.290	**0.621**	0.910	**2.63**	3.081	**11.59**
401	SRM 968f-Level 2-Box 2-Center Vial	4.664	**0.691**	0.946	**2.79**	3.200	**12.31**
402	SRM 968f-Level 2-Box 19-Last Vial	4.068	**0.661**	0.818	**2.65**	2.904	**12.25**
403	SRM 968f-Level 2-Box 3-Center Vial	4.500	**0.637**	0.980	**2.77**	3.101	**11.40**
404	SRM 968f-Level 2-Box 18-Last Vial	3.887	**0.589**	0.872	**2.63**	2.825	**11.12**
405	SRM 968f-Level 2-Box 4-Center Vial	4.272	**0.596**	0.954	**2.65**	3.078	**11.15**
406	SRM 968f-Level 2-Box 17-Last Vial	5.225	**0.687**	1.022	**2.68**	3.620	**12.37**
408	SRM 968f-Level 2-Box 5-Center Vial	5.671	**0.785**	1.134	**3.22**	3.819	**14.12**
*485/486	SRM 968f-Level 2-Box 5-Center Vial	5.609	**0.6421**	1.130	**2.579**	3.752	**11.16**
409	SRM 968f-Level 2-Box 16-Last Vial	4.844	**0.664**	1.043	**2.85**	3.267	**11.64**
410	SRM 968f-Level 2-Box 6-Center Vial	4.380	**0.662**	0.919	**2.77**	2.961	**11.62**
411	SRM 968f-Level 2-Box 15-Last Vial	4.733	**0.645**	0.938	**2.55**	3.145	**11.14**
412	SRM 968f-Level 2-Box 7-First Vial	4.771	**0.656**	0.976	**2.68**	3.223	**11.51**
413	SRM 968f-Level 2-Box 14-Last Vial	4.798	**0.676**	1.014	**2.85**	3.130	**11.45**
414	SRM 968f-Level 2-Box 8-First Vial	4.669	**0.667**	0.943	**2.69**	3.133	**11.62**
415	SRM 968f-Level 2-Box 13-Last Vial	4.532	**0.633**	0.870	**2.42**	3.108	**11.28**
416	SRM 968f-Level 2-Box 9-First Vial	4.119	**0.605**	0.862	**2.53**	2.759	**10.53**
417	SRM 968f-Level 2-Box 12-Last Vial	4.805	**0.669**	0.972	**2.70**	3.237	**11.70**
418	SRM 968f-Level 2-Box 10-First Vial	4.561	**0.683**	0.847	**2.53**	3.158	**12.28**
419	SRM 968f-Level 2-Box 11-Last Vial	4.286	**0.595**	0.990	**2.74**	2.889	**10.42**
		Mean	**0.646**		**2.66**		**11.49**
		SD	**0.031**		**0.12**		**0.54**
		% SD	**4.86**		**4.56**		**4.68**

a Concentrations are corrected for purity of reference standard. Samples are listed in the order in which they were analyzed. One sample was prepared from each box. Except for box 1 and box 5, one LC injection was made for each sample. Results from box 1 and box 5 are from the average of two LC injections of a single vial from each sample.

Highlighted data are questionable due to possible sample preparation technicalities and were not included in the mean. Repeat measurements for the highlighted samples were made. Highlighted data were replaced with data from repeat measurements denoted by an asterisk.

Figure 1. Representative Chromatograms for SRM 968f (Level 1 and Level 2)

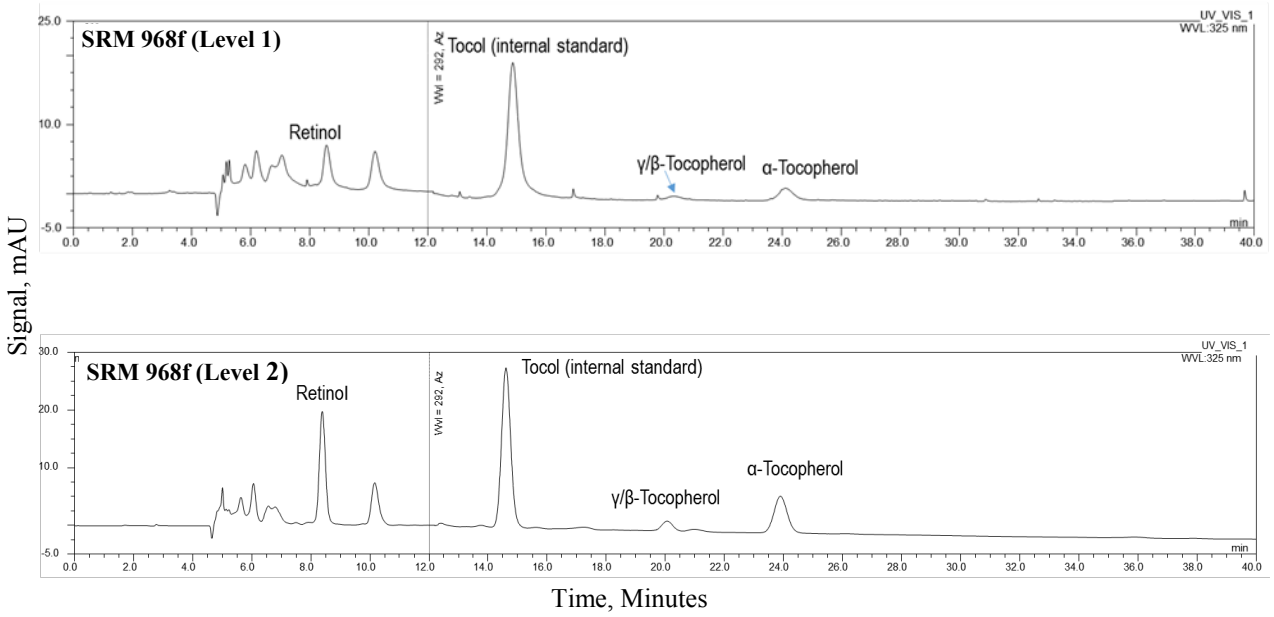


Figure 2. Retinol Concentrations in SRM 968f (Level 1 and Level 2) versus Box Number.

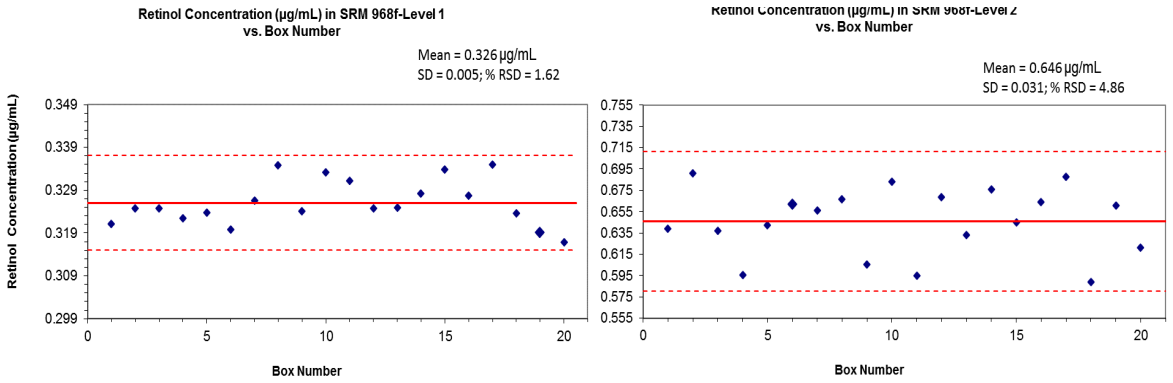


Figure 3. γ+β-Tocopherol Concentrations in SRM 968f (Level 1 and Level 2) versus Box Number.

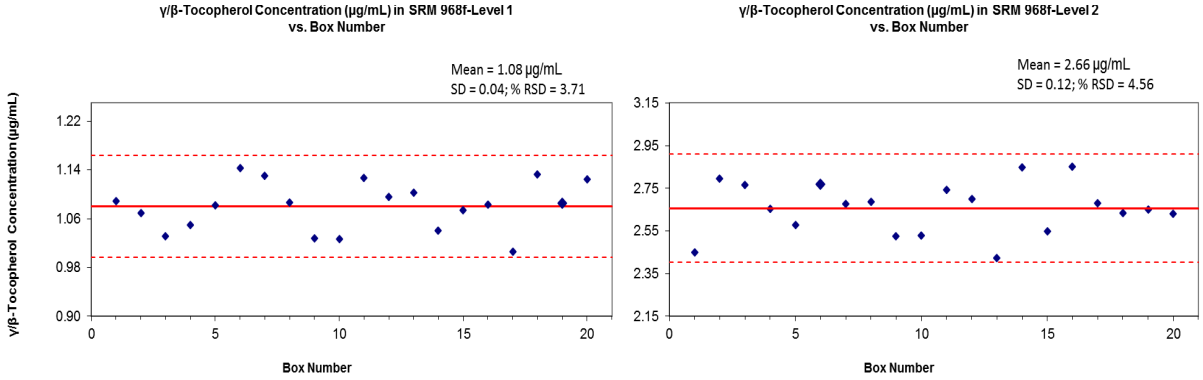
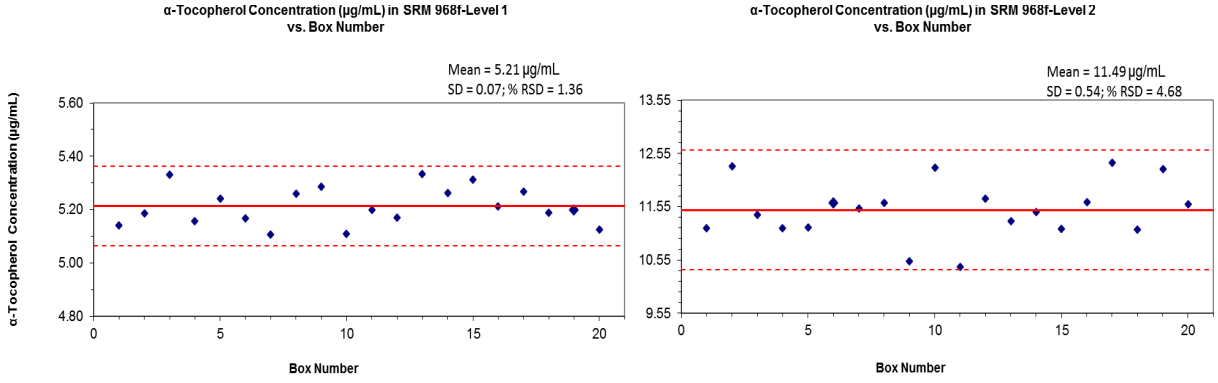


Figure 4. α-Tocopherol Concentrations in SRM 968f (Level 1 and Level 2) versus Box Number.



Solid diamonds represent the individual concentrations per sample; the mean is indicated by the solid line. The dashed lines in each figure represent the associated uncertainties expressed as the 95 % level of confidence (U95).

Figure 5. Retinol Concentrations in SRM 968f (Level 1 and Level 2) versus Run Order.

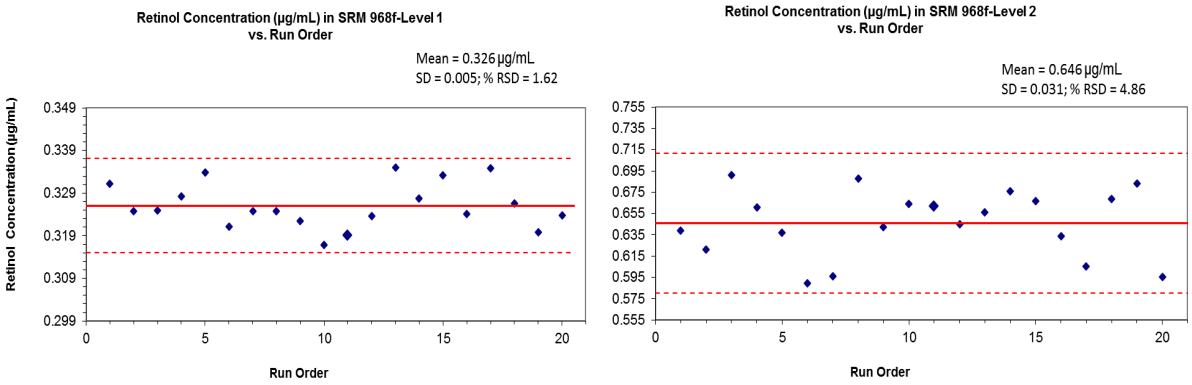


Figure 6. γ+β-Tocopherol Concentrations in SRM 968f (Level 1 and Level 2) versus Run Order.

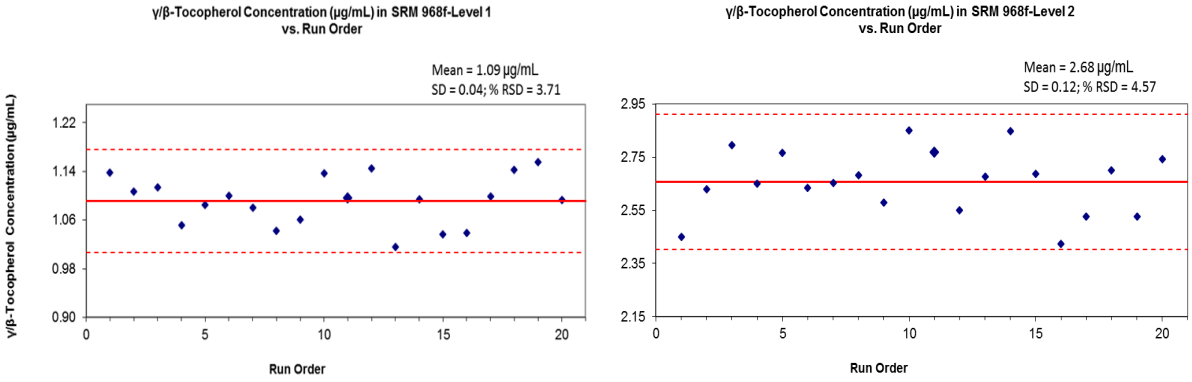
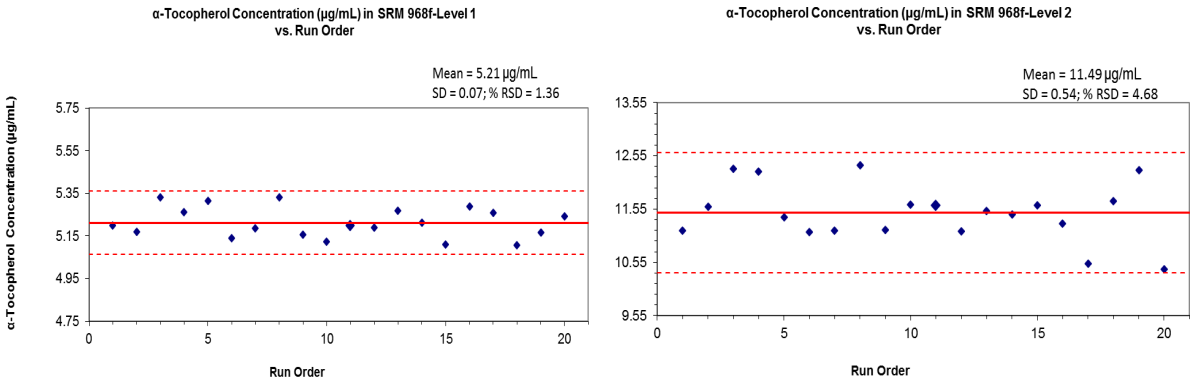


Figure 7. α-Tocopherol Concentrations in SRM 968f (Level 1 and Level 2) versus Run Order.



Solid diamonds represent the individual concentrations per sample; the mean is indicated by the solid line. The dashed lines in each figure represent the associated uncertainties expressed as the 95 % level of confidence (U95).

Appendix 646.02-17-045-A
Relative Response Factors for Retinol and Tocopherols Reference Standards

Gamma-Tocopherol Calibrants	Conc of Gamma-Tocopherol mg/g	Area of IS	Area of gamma-tocopherol	Conc (mg/g) of Internal Std added to cal soln	Relative Response factor
Cal soln 1	0.1229	0.993	8.170	0.0213	0.7031
Cal soln 2	0.1716	1.008	10.189	0.0233	0.7276
Cal soln 3	0.2901	1.066	16.138	0.0271	0.7063
				Averaged Relative Response Factors	**0.7123**
				SD	**0.0133**
				% SD	**1.87**

Alpha-Tocopherol Calibrants	Conc of alpha-Tocopherol mg/g	Area of IS	Area of alpha-tocopherol	Conc (mg/g) of Internal Std added to cal soln	Relative Response Factor
Cal soln 2	0.0476	2.010	4.918	0.0214	0.9232
Cal soln 3	0.0396	1.965	4.368	0.0212	0.8492
Cal soln 4	0.0293	1.984	2.578	0.0215	1.0502
Cal soln 5	0.0096	1.949	0.997	0.0211	0.8888
				Averaged Relative Response Factors	**0.9279**
				SD	**0.0870**
				% SD	**9.38**

Retinol Calibrants	Conc of Retinol stock soln mg/g	Area of IS	Area of Retinol in cal soln	Conc (mg/g) of Internal Std added to soln	Relative Response Factor
Cal soln 1	0.606	5.263	12.241	7.235	0.03602
Cal soln 2	0.365	8.597	12.288	6.919	0.03695
Cal soln 3	0.389	8.700	12.325	8.021	0.03420
				Averaged Relative Response Factors	**0.03572**
				SD	**0.0014**
				% SD	**3.92**

Appendix 646.02-17-045-B.
LC purity measurements for retinol and tocopherols used for the certification of SRM 968f

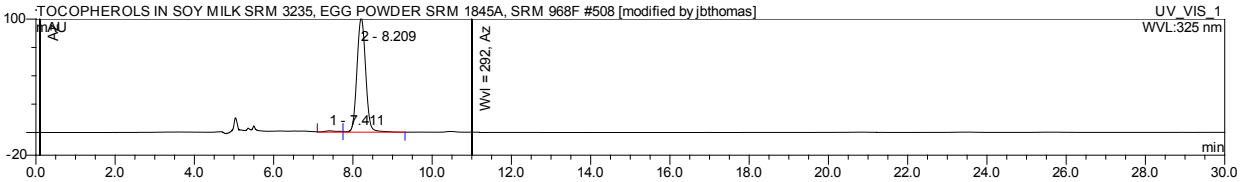
LC Purity Measurements for Retinol and Tocopherols			
Retinol (Sigma-Aldrich; Lot BCBR9941V)			
File #	Anayte Peak Area		Analyte Relative % Purity at 325 nm
506	25.520		97.56
507	25.491		97.75
508	25.599		97.60
		Average	97.68
		SD	0.11
		% SD	0.11
Gamma/beta-Tocopherol (Chromadex; Lot # 20313-304)			
File #	Anayte Peak Area		Analyte Relative % Purity at 298 nm
532	3.707		99.01
533	1.926		98.98
534	1.725		98.99
		Average	98.99
		SD	0.02
		% SD	0.02
alpha-Tocopherol (Fluka/BioChemica;Lot #442328/1)			
File #	Anayte Peak Area		Analyte Relative % Purity at 292 nm
535	2.891		98.56
536	2.821		98.51
537	2.535		98.61
		Average	98.56
		SD	0.05
		% SD	0.05

Appendix 646.02-17-045-C.
Data from repeat measurements for SRM 968f (Level 2)

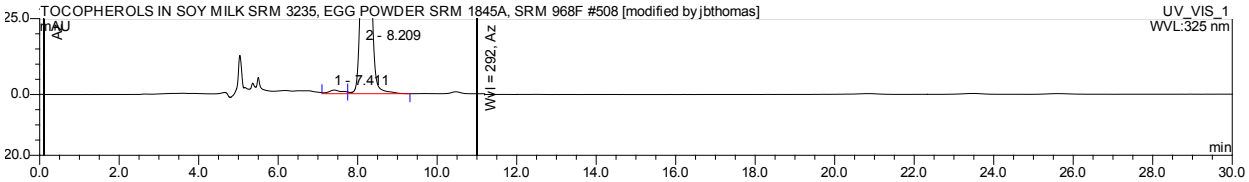
	Response factor	0.03572		0.7123		0.9279	
File No.	Box number/vial position	**Total retinol peak area**	**(µg/mL)**	**γ-Tocopherol peak area**	**(µg/mL)**	**α-Tocoherol peak area**	**(µg/mL)**
466	Box 1-First	5.454	**0.637**	1.046	**2.44**	3.693	**11.20**
467	Box 1-First	5.517	**0.641**	1.064	**2.46**	3.677	**11.09**
	Average		**0.639**		**2.45**		**11.15**
	SD		**0.003**		**0.02**		**0.08**
	% SD		**0.42**		**0.78**		**0.71**
485	Box 5-Center	5.627	**0.643**	1.136	**2.59**	3.755	**11.15**
486	Box 5-Center	5.591	**0.641**	1.124	**2.57**	3.748	**11.17**
	Average		**0.642**		**2.58**		**11.16**
	SD		**0.001**		**0.01**		**0.02**
	% SD		**0.16**		**0.49**		**0.14**

Appendix 646.02-17-045-D
Representative chromatograms from LC purity measurements for retinol and tocopherols reference standards used for the certification of SRM 968f

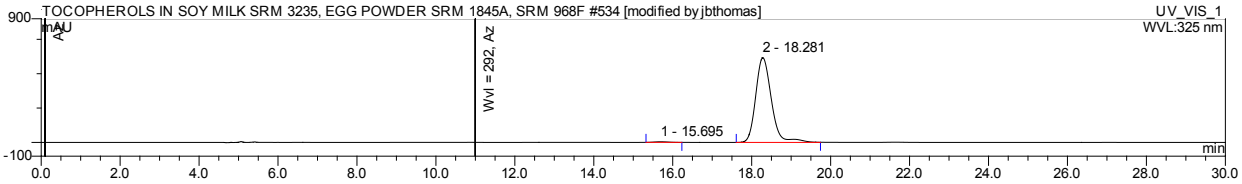
Retinol purity measurement



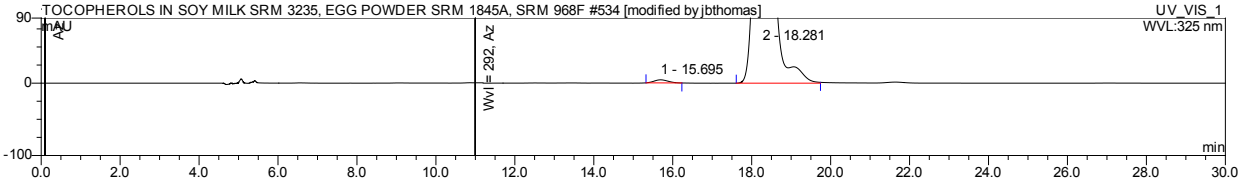
at 4x attenuation



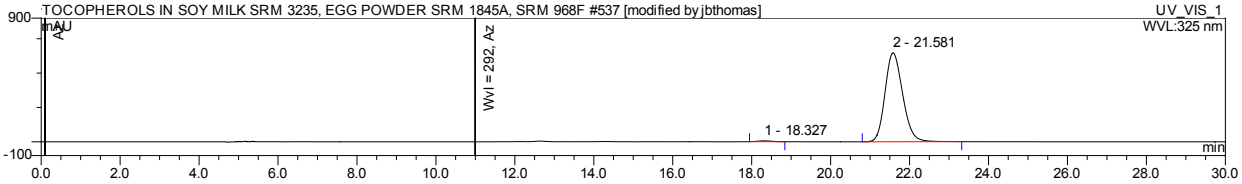
γ+β-Tocopherol purity measurement



at 100x attenuation



α-Tocopherol purity measurement



at 100x attenuation