National Institute of Standards and Technology
U.S. Department of Commerce

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Standard Reference Material® 3252
Protein Drink Mix
CERTIFICATE OF ANALYSIS

Purpose: This Standard Reference Material (SRM) is intended primarily for validation of methods for determining proximates, fatty acids, cholesterol, vitamins, elements, and amino acids in protein drink mixes and similar materials. This SRM can also be used for quality assurance when assigning values to in-house reference materials.

Description: The SRM is a blend of commercial protein drink mixes. A unit of SRM 3252 consists of five heat-sealed aluminized pouches, each containing approximately 10 g of material.

Certified Values: Certified mass fraction values of cholesterol, elements, and vitamins in SRM 3252, reported on a dry-mass basis, are provided in Tables 1 through 3. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias and variability have been taken into account [1]. Analyses for value assignment were performed by NIST and collaborating laboratories. Certified values were calculated as the mean of the mean values from NIST methods and the median of the mean results provided by collaborating laboratories, where appropriate. The associated uncertainties are expressed at an approximately 95 % level of confidence [2–4].

Non-Certified Values: Non-certified values for additional analytes in SRM 3252 are provided in Appendix A.

Additional Information: Additional information is provided in Appendix B.

Period of Validity: The certified values delivered by **SRM 3252** are valid within the measurement uncertainty specified until **01 July 2025**. The certified values are nullified if the material is stored or used improperly, damaged, contaminated, or otherwise modified.

Maintenance of Certified Values: NIST will monitor this SRM over the period of its validity. If substantive technical changes occur that affect the certification, NIST will issue an amended certificate through the NIST SRM website (https://www.nist.gov/srm) and notify registered users. SRM users can register online from a link available on the NIST SRM website or fill out the user registration form that is supplied with the SRM. Registration will facilitate notification. Before making use of any of the values delivered by this material, users should verify they have the most recent version of this documentation, available through the NIST SRM website (https://www.nist.gov/srm).

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Certificate Revision History on Page 5

Safety: SRM 3252 IS INTENDED FOR RESEARCH USE; NOT FOR HUMAN CONSUMPTION.

Storage: The SRM should be stored at controlled room temperature (20 °C to 25 °C) in the original unopened packets. For elemental analyses, the packet can be resealed, stored at room temperature, and test portions removed and analyzed until the material reaches its expiration date. For organic analyses, the packet can be resealed, stored at room temperature, and test portions removed and analyzed for two weeks after the packet was initially opened.

Use: Before use, the contents of the packet should be mixed thoroughly. The contents should be allowed to settle for one minute prior to opening to minimize the loss of fine particles. Homogeneity of the material has not been evaluated for sample sizes smaller than those used by NIST methods described below. Therefore, the certified and non-certified values may not be valid for test portions smaller than those described in the sections below: 0.2 g to 4 g for elemental analyses, 1 g for fatty acid analyses, 0.5 g for cholesterol analyses, and 1 g to 5 g for vitamin analyses. Results obtained in analyses should include their own estimates of uncertainty and can be compared to the certified values using procedures described in reference 5.

Determination of Moisture: Moisture content of SRM 3252 was determined at NIST by (1) freeze-drying to constant mass over 7 d; (2) drying over magnesium perchlorate in a desiccator at room temperature for 20 d; and (3) drying for 3 h in a forced-air oven at 80 °C. The mean results from all three techniques were averaged to determine a dry mass proportion of (0.9502 ± 0.0038) gram dry mass per gram as-received mass; the uncertainty shown on this value is an expanded uncertainty at an approximately 95 % level of confidence. The conversion factor used to convert data from an as-received to a dry-mass basis is the inverse of the dry mass proportion. A relative uncertainty component for the conversion factor (0.2 %) obtained from the moisture measurements is incorporated in the uncertainties of the certified and non-certified values, reported on a dry-mass basis, that are provided in this certificate.

Certified Mass Fraction Value for Cholesterol: The certified mass fraction value for cholesterol is the mean of results obtained by NIST using ID-GC-MS. Values are expressed as $x \pm U_{95\%}(x)$, where x is the certified value and $U_{95\%}(x)$ is the expanded uncertainty of the certified value. The true value of the analyte lies within the interval $x \pm U_{95\%}(x)$ with 95 % confidence. To propagate this uncertainty, the certified value should be treated as a normally distributed random variable with mean x and standard deviation $U_{95\%}(x)/2$ [2–4]. The measurand is the total mass fraction of cholesterol in protein powder as listed in Table 1 on a dry-mass basis. Metrological traceability is to the International System of Units (SI) measurement unit for chemical mass fraction, expressed as milligrams per gram.

Table 1. Certified Mass Fraction Value for Cholesterol in SRM 3252

	Mass Fraction (mg/g)
Cholesterol	0.5077 ± 0.0056

Certified Mass Fraction Values for Elements: Each certified mass fraction value, reported on a dry-mass basis, is the mean from the combination of means of NIST data sets and the median of the mean results provided by collaborating laboratories, where appropriate. Values are expressed as $x \pm U_{95\%}(x)$, where x is the certified value and $U_{95\%}(x)$ is the expanded uncertainty of the certified value. The true value of the analyte lies within the interval $x \pm U_{95\%}(x)$ with 95 % confidence. To propagate this uncertainty, the certified value should be treated as a normally distributed random variable with mean x and standard deviation $U_{95\%}(x)/2$ [2–4]. The measurands are the total mass fractions of elements in protein powder as listed in Table 2 on a dry-mass basis. Metrological traceability is to the SI measurement unit for chemical mass fraction, expressed as milligrams per kilogram.

Table 2. Certified Mass Fraction Values for Elements in SRM 3252

	Mass Fraction (mg/kg)		
Barium (Ba)[a,b]	3.12	±	0.14
Cadmium (Cd)[c]	0.04078	±	0.00099
Calcium (Ca)[a,d,e,f]	17840	±	970
Copper (Cu)[d,f]	36.36	±	0.75
Iron (Fe)[a,d,e,f]	381	±	13
Lead (Pb)[c]	0.0403	±	0.0042
Magnesium (Mg)[a,e,f]	6337	±	175
Manganese (Mn)[a,d,e,f]	11.12	±	0.46
Molybdenum (Mo)[b,e]	1.19	±	0.19
Phosphorus (P)[a,d,f]	17210	±	720
Potassium (K)[a,d,e,f]	11550	±	550
Selenium (Se)[b,e]	0.596	±	0.037
Sodium (Na)[a,d,e,f]	6820	±	170
Strontium (Sr)[a,b]	13.65	±	0.79
Zinc (Zn)[d,e,f]	235	±	12

[a] NIST ICP-OES
[b] NIST ICP-MS
[c] NIST ID ICP-MS
[d] NIST WDXRF
[e] NIST INAA
[f] Collaborating laboratories. Reported methods included atomic absorption spectroscopy (AAS), ICP optical emission spectroscopy (ICP-OES), ICP-MS, ion chromatography with suppressed conductivity detection, and colorimetry.

Certified Mass Fraction Values for Vitamins: Each certified mass fraction value, reported on a dry-mass basis, is the mean from the combination of means of NIST data sets and the median of the mean results provided by collaborating laboratories, where appropriate. Values are expressed as $x \pm U_{95\%}(x)$, where x is the certified value and $U_{95\%}(x)$ is the expanded uncertainty of the certified value. The true value of the analyte lies within the interval $x \pm U_{95\%}(x)$ with 95 % confidence. To propagate this uncertainty, the certified value should be treated as a normally distributed random variable with mean x and standard deviation $U_{95\%}(x)/2$ [2–4]. The measurands are the total mass fractions of the vitamins in protein powder as listed in Table 3 on a dry-mass basis. Metrological traceability is to the SI measurement unit for chemical mass fraction, expressed as milligrams per kilogram, as realized through the purity determined for the primary chemical standards employed in the NIST methods.

Table 3. Certified Mass Fraction Values for Vitamins in SRM 3252

	Mass Fraction (mg/kg)
Thiamine (Vitamin B_1)[a,b]	12.3 ± 1.6
Riboflavin (Vitamin B_2)[a,b]	28.7 ± 2.8
Niacinamide (Vitamin B_3)[a]	269.7 ± 4.4
Niacin (Vitamin B_3)[a]	7.33 ± 0.26
Total Vitamin B_3 as Niacinamide[a,b,c]	287 ± 21
Pantothenic Acid (Vitamin B_5)[a,b]	150 ± 12
Pyridoxine (Vitamin B_6)[a]	29.2 ± 1.6
Total Vitamin B_6 as Pyridoxine[a,b,d]	29.1 ± 2.7
Ascorbic Acid (Vitamin C)[b,c]	950 ± 110
Biotin[f]	4.43 ± 0.19
Choline[f]	1328 ± 17

[a] NIST ID-LC-MS/MS

[b] Collaborating laboratories. Reported methods included microbiological assay, digestion with fluorescence detection, extraction with LC and fluorescence detection, extraction with LC and electrochemical detection, extraction with LC-MS, an autoanalyzer, and dichloroindophenol titration.

[c] Total vitamin B_3 is the sum of niacin and niacinamide; the mass fraction of niacin was mathematically converted to niacinamide by multiplying by the ratio of the relative molecular masses of niacin and niacinamide.

[d] Total vitamin B_6 is the sum of pyridoxal, pyridoxamine, and pyridoxine; the mass fractions of pyridoxal and pyridoxamine were mathematically converted to pyridoxine by multiplying the mass fraction by the ratio of the relative molecular masses of pyridoxal, pyridoxamine, and pyridoxine.

[e] NIST LC-absorbance

[f] NIST ID-LC-MS

REFERENCES

[1] Beauchamp, C.R.; Camara, J.E.; Carney, J.; Choquette, S.J.; Cole, K.D.; DeRose, P.C.; Duewer, D.L.; Epstein, M.S.; Kline, M.C.; Lippa, K.A.; Lucon, E.; Molloy, J.; Nelson, M.A.; Phinney, K.W.; Polakoski, M.; Possolo, A.; Sander, L.C.; Schiel, J.E.; Sharpless, K.E.; Toman, B.; Winchester, M.R.; Windover, D.; *Metrological Tools for the Reference Materials and Reference Instruments of the NIST Material Measurement Laboratory*; NIST Special Publication 260-136, 2021 edition; National Institute of Standards and Technology, Gaithersburg, MD (2021); available at https://nvlpubs.nist.gov/nistpubs/SpecialPublications/NIST.SP.260-136-2021.pdf (accessed Dec 2023).

[2] JCGM 100:2008; *Evaluation of Measurement Data - Guide to the Expression of Uncertainty in Measurement*; (GUM 1995 with Minor Corrections), Joint Committee for Guides in Metrology (JCGM) (2008); available at https://www.bipm.org/en/committees/jc/jcgm/publications (accessed Dec 2023); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297, U.S. Government Printing Office: Washington, DC (1994); available at https://www.nist.gov/pml/nist-technical-note-1297 (accessed Dec 2023).

[3] JCGM 101:2008; *Evaluation of Measurement Data – Supplement 1 to the "Guide to the Expression of Uncertainty in Measurement" - Propagation of Distributions using a Monte Carlo Method*; JCGM (2008); available at https://www.bipm.org/en/committees/jc/jcgm/publications (accessed Dec 2023).

[4] Efron, B.; Tibshirani, R.J.; *An Introduction to the Bootstrap*; Chapman & Hall, London, UK (1993).

[5] Sharpless, K.E.; Lippa, K.A.; Duewer, D.L.; Rukhin, A.L.; *The ABCs of Using Standard Reference Materials in the Analysis of Foods and Dietary Supplements: A Practical Guide*; NIST Special Publication 260-181; U.S. Government Printing Office: Washington, DC (2014); available at https://nvlpubs.nist.gov/nistpubs/SpecialPublications/NIST.SP.260-181r1.pdf (accessed Dec 2023).

[6] Ellerbe, P.; Meiselman, S.; Sniegoski, L.T.; Welch, M.J.; White, V.E.; *Determination of Serum Cholesterol by a Modification of the Isotope Dilution Mass Spectrometric Definitive Method*; Anal. Chem., Vol. 61, pp. 1718–1723 (1989).

[7] AOAC Official Method 996.06; *Official Methods of Analysis*, 18th ed.; AOAC International, Gaithersburg, MD (2000).

Certificate Revision History: 13 December 2023 (Removed Vitamin B_{12} non-certified value; updated format; editorial changes); 16 October 2019 (Addition of certified values for cadmium and lead; ascorbic acid (vitamin C) reference value updated to certified value and moved from Table 6 to Table 3; fatty acids certified values downgraded to reference values to properly reflect traceability and moved from Table 1 to Table 4; correction of value uncertainties for Ca, Cu, Mg, Mn, Mo, P, K for compliance with NIST uncertainty policy; discussion of uncertainty updated for all values, including removal of *k* values; editorial changes); 10 September 2015 (Original certificate date).

Certain commercial equipment, instruments, or materials may be identified in this Certificate of Analysis to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the Office of Reference Materials 100 Bureau Drive, Stop 2300, Gaithersburg, MD 20899-2300; telephone (301) 975-2200; e-mail srminfo@nist.gov; or the Internet at https://www.nist.gov/srm.

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APPENDIX A

Non-Certified Values: Non-certified values for additional analytes in SRM 3252, reported on a dry-mass basis, are provided in Tables A1 through A6. A non-certified value is the best estimate of the true value based on available data; however, the value does not meet the NIST criteria for certification [1] and is provided with an uncertainty that may reflect only measurement reproducibility, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods. The non-certified values were derived from results reported by NIST and/or collaborating laboratories.

Non-Certified Values for Fatty Acids (as Free Fatty Acids): Each non-certified mass fraction value is the mean from the combination of the mean results from analyses at NIST and the median of the mean of results provided by collaborating laboratories, where appropriate. The method-specific value of the analyte lies within the interval $x \pm U_{95\%}(x)$ with about a 95 % confidence [2–4]. For fatty acids values containing NIST data, the uncertainty incorporates a component for possible inhomogeneity based on the standard deviation. The measurands are the total mass fractions of the fatty acids in the protein powder as listed in Table A1, on a dry-mass basis, as determined by the method indicated.

Table A1. Non-Certified Mass Fraction Values for Fatty Acids (as Free Fatty Acids) in SRM 3252

	Common Name	Mass Fraction (g/100 g)
Butanoic Acid (C4:0)[a]	Butyric Acid	0.0235 ± 0.0098
Hexanoic Acid (C6:0)[a]	Caproic Acid	0.0168 ± 0.0026
Octanoic Acid (C8:0)[a,b]	Caprylic Acid	0.0097 ± 0.0014
Decanoic Acid (C10:0)[a]	Capric Acid	0.0219 ± 0.0025
Dodecanoic Acid (C12:0)[a,b]	Lauric Acid	0.0276 ± 0.0076
Tetradecanoic Acid (C14:0)[a,b]	Myristic Acid	0.093 ± 0.027
(*Z*)-9-Tetradecenoic Acid (C14:1)[a,b]	Myristoleic Acid	0.0081 ± 0.0015
Pentadecanoic Acid (C15:0)[a]		0.0129 ± 0.0011
Hexadecanoic Acid (C16:0)[a]	Palmitic Acid	1.131 ± 0.063
(*Z*)-9-Hexadecenoic Acid (C16:1 n-7)[a,b]	Palmitoleic Acid	0.01649 ± 0.00069
Heptadecanoic Acid (C17:0)[a]	Margaric Acid	0.0126 ± 0.0011
Octadecanoic Acid (C18:0)[a]	Stearic Acid	0.778 ± 0.048
Total *cis*-C18:1 Fatty Acids[a,b]		1.186 ± 0.072
Total *trans*-C18:1 Fatty Acids[a]		0.68 ± 0.20
(*Z*)-9-Octadecenoic Acid (C18:1 n-9)[a]	Oleic Acid	1.026 ± 0.077
(*Z*)-11-Octadecenoic Acid (C18:1 n-7)[a]	Vaccenic Acid	0.0839 ± 0.0077
(*Z,Z*)-9,12-Octadecadienoic Acid (C18:2 n-6)[a]	Linoleic Acid	1.126 ± 0.086
Total *cis*-C18:2 Fatty Acids[a]		1.18 ± 0.14
Total *trans*-C18:2 Fatty Acids[a]		0.030 ± 0.010
Total *trans*-C18:2 Conjugated Fatty Acids[a]		0.0093 ± 0.0021
(*Z,Z,Z*)-9,12,15-Octadecatrienoic Acid (C18:3 n-3)[a]	α-Linolenic Acid	0.122 ± 0.016
Eicosanoic Acid (C20:0)[a]	Arachidic Acid	0.0242 ± 0.0020
(*Z*)-11-Eicosenoic Acid (C20:1 n-9)[b]	Gondoic Acid	0.0070 ± 0.0004
Docosanoic Acid (C22:0)[a]	Behenic Acid	0.0310 ± 0.0048
Tetracosanoic Acid (C24:0)[a]	Lignoceric Acid	0.0203 ± 0.0018
Saturated Fatty Acids[a]		2.173 ± 0.094
cis-Monounsaturated Fatty Acids[a]		1.255 ± 0.063
cis-Polyunsaturated Fatty Acids[a]		1.30 ± 0.10
Total Trans Fatty Acids[a]		0.71 ± 0.22
Total Omega-3 Fatty Acids[a]		0.122 ± 0.011
Total Omega-6 Fatty Acids[a]		1.179 ± 0.062

[a] Collaborating laboratories. Reported methods included GC-FID as well as hydrolysis with derivatization and LC.
[b] NIST GC-FID

Non-Certified Mass Fraction Values for Elements: Each non-certified mass fraction value is the mean result of NIST analyses. Values are expressed as $x \pm U_{95\%}(x)$, where x is the non-certified value and $U_{95\%}(x)$ is the expanded uncertainty of the value. The method-specific value of the analyte lies within the interval $x \pm U_{95\%}(x)$ with about a 95 % confidence [2–4]. The measurands are the total mass fractions of the elements in protein powder as listed in Table A2, on a dry-mass basis, as determined by the methods indicated.

Table A2. Non-Certified Mass Fraction Values for Elements in SRM 3252

	Mass Fraction (mg/kg)
Aluminum (Al)[a]	76.6 ± 4.8
Chlorine (Cl)[a,b]	7160 ± 690
Chromium (Cr)[a]	1.06 ± 0.10
Iodine (I)[a,c]	1.84 ± 0.20
Lanthanum (La)[a]	0.0742 ± 0.0031
Samarium (Sm)[a]	0.0170 ± 0.0014

[a] NIST INAA
[b] NIST WDXRF
[c] Confirmation by NIST ICP-MS

Non-Certified Mass Fraction Values for Vitamins, Carnitine, and myo-Inositol: Each non-certified mass fraction value is the mean result of NIST analyses or the median of the mean of results provided by collaborating laboratories. Values are expressed as $x \pm U_{95\%}(x)$, where x is the estimated value and $U_{95\%}(x)$ is the expanded uncertainty of the value. The method-specific value of the analyte lies within the interval $x \pm U_{95\%}(x)$ with about a 95 % confidence [2–4]. The measurands are the total mass fractions of each analyte in protein powder as listed in Table A3, on a dry-mass basis, as determined by the methods indicated.

Table A3. Non-Certified Mass Fraction Values for Vitamins, Carnitine, and myo-Inositol in SRM 3252

	Mass Fraction (mg/kg)
Pyridoxamine (Vitamin B_6)[a]	0.0605 ± 0.0063
Folic Acid[b]	7.6 ± 1.9
Carnitine[c]	4.76 ± 0.12
myo-Inositol[b]	186 ± 40
Retinol[e]	23.8 ± 1.5
α-Tocopherol[d]	370 ± 86
δ-Tocopherol[d]	6.1 ± 1.5

[a] NIST ID-LC-MS/MS
[b] Collaborating laboratories. Reported methods included microbiological assay.
[c] NIST ID-LC-MS
[d] Collaborating laboratories. No method information was reported.

Non-Certified Mass Fraction Values for Proximates and Calories: Each non-certified mass fraction value is the median of the mean values provided by collaborating laboratories. Values are expressed as $x \pm U_{95\%}(x)$, where x is the estimated value and $U_{95\%}(x)$ is the expanded uncertainty of the value. The method-specific value of the analyte lies within the interval $x \pm U_{95\%}(x)$ with about a 95 % confidence [2–4]. For proximates and fiber, the measurands are the mass fractions in protein powder as listed in Table A4, on a dry-mass basis, as determined by the methods indicated. For calories, the measurand is the caloric content in protein powder as listed in Table A4 on a dry-mass basis as determined by the method indicated.

Table A4. Non-Certified Mass Fraction Values for Proximates and Calories in SRM 3252

	Mass Fraction (g/100 g)
Ash[a]	10.77 ± 0.10
Protein[b]	66.92 ± 0.61
Carbohydrates[c]	15.31 ± 0.99
Total Dietary Fiber	6.22 ± 0.95
Fat (as the sum of fatty acids as triglycerides)	5.81 ± 0.29
	Energy (kcal per 100 g)
Calories[d]	381.2 ± 2.2

[a] Ash was determined by collaborating laboratories using weight loss after ignition in a muffle furnace and thermogravimetric analysis.

[b] Nitrogen was determined by collaborating laboratories using Kjeldahl and combustion (LECO). A factor of 6.25 was used to convert nitrogen results to protein.

[c] Carbohydrates were determined by collaborating laboratories by difference (solids less the sum of protein, fat, and ash).

[d] The non-certified value for calories is the median of lab mean caloric calculations from the interlaboratory comparison exercise. If the mean proximate values above are used for calculation, with caloric equivalents of 9, 4, and 4 for fat (as the sum of fatty acids), protein, and carbohydrate, respectively, the mean caloric content is 381.2 kcal per 100 grams.

Non-Certified Mass Fraction Values for Amino Acids: Each non-certified mass fraction value is the median of the mean results provided by collaborating laboratories. Values are expressed as $x \pm U_{95\%}(x)$, where x is the estimated value and $U_{95\%}(x)$ is the expanded uncertainty of the value. The method-specific value of the analyte lies within the interval $x \pm U_{95\%}(x)$ with about a 95 % confidence [2–4]. The measurands are the mass fractions of the amino acids in protein powder as listed in Table A5, on a dry-mass basis, as determined by the collaborating laboratories.

Table A5. Non-Certified Mass Fraction Values for Amino Acids in SRM 3252

	Mass Fraction (g/100 g)
Alanine	2.87 ± 0.59
Arginine	3.55 ± 0.52
Aspartic Acid	7.0 ± 1.4
Cystine	0.87 ± 0.23
Glutamic Acid	13.4 ± 3.0
Glycine	1.93 ± 0.50
Histidine	1.49 ± 0.18
Isoleucine	3.42 ± 0.82
Leucine	6.2 ± 1.2
Lysine	4.84 ± 0.78
Methionine	1.426 ± 0.058
Phenylalanine	3.19 ± 0.47
Serine	3.85 ± 0.94
Threonine	3.38 ± 0.67
Tyrosine	2.66 ± 0.32
Valine	3.7 ± 1.1

Non-Certified Mass Fraction Values for Additional Measurands: Each non-certified mass fraction value is the median of the mean results provided by collaborating laboratories using LC-absorbance or Folin-Ciocalteu. Values are expressed as $x \pm U_{95\%}(x)$, where x is the estimated value and $U_{95\%}(x)$ is the expanded uncertainty of the value. The method-specific value of the analyte lies within the interval $x \pm U_{95\%}(x)$ with about a 95 % confidence [2–4]. The measurands are the mass fractions of the analyte in protein powder as listed in Table A6, on a dry-mass basis, as determined by the collaborating laboratories.

Table A6. Non-Certified Mass Fraction Values for Additional Measurands in SRM 3252

	Mass Fraction (mg/kg)
Caffeine	174.4 ± 2.5
Theobromine	2315 ± 93
Total Polyphenols (Gallic Acid Equivalents)	7800 ± 1500

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APPENDIX B

Source and Preparation: The SRM is a blend of commercially available protein drink mixes. The commercial products were transferred to High-Purity Standards (Charleston, SC) where the material was blended and packaged. The protein drink mix was heat-sealed in approximately 10 g aliquots inside nitrogen-flushed 4-mil polyethylene bags, which were then sealed inside nitrogen-flushed Mylar bags along with two packets of silica gel each. Following packaging, SRM 3252 was irradiated (Neutron Products, Inc., Dickerson, MD) to an absorbed dose of 6.3 kGy to 8.4 kGy.

Analytical Approach for Determination of Cholesterol: Value assignment of the cholesterol mass fraction was based on measurements made by NIST using an isotope dilution (ID) gas chromatography (GC) method with mass spectrometry (MS).

NIST Analyses for Cholesterol: The mass fraction of cholesterol was measured using the ID-GC-MS method developed at NIST for serum cholesterol [6] and modified for the determination of cholesterol in food matrices using AOAC International Official Method 996.06 for hydrolysis [7]. Three sets of samples were prepared, each consisting of duplicate 0.5 g test portions from each of three packets of SRM 3252 weighed into Pyrex test tubes. An aliquot of a solution containing a known mass of the internal standard, cholesterol-$^{13}C_3$, was added to each tube. Cholesterol esters were hydrolyzed by heating the samples in an alcohol-KOH solution for 1 h at 100 °C. Cholesterol was extracted into hexane, and a portion of the hexane extract was evaporated to dryness prior to addition of N,O-bis(trimethylsilyl)acetamide to convert cholesterol to the trimethylsilyl (TMS) derivative. GC-MS was performed using a 30 m (phenyl/methyl polysiloxane, 5/95 mole fraction) non-polar fused silica column directly interfaced to the ion source. Cholesterol was determined in the electron ionization mode with selected ion monitoring at *m/z* 458 and *m/z* 461 for the unlabeled and labeled cholesterol-TMS, respectively. Calibrants were prepared gravimetrically from SRM 911c *Cholesterol*, at levels intended to approximate the level of the cholesterol in the SRM following extraction. A single internal standard solution was used for the calibrants and samples. Calculations are based on linear regression analysis for the calibrants.

Analytical Approach for Determination of Elements: Value assignment of the mass fractions of elements in SRM 3252 was based on the combination of measurements from analytical methods at NIST and collaborating laboratories, where appropriate. NIST provided measurements by using inductively coupled plasma optical emission spectrometry (ICP-OES), inductively coupled plasma mass spectrometry (ICP-MS), isotope dilution inductively coupled plasma mass spectrometry (ID ICP-MS), wavelength dispersive X-ray fluorescence spectrometry (WDXRF), and instrumental neutron activation analysis (INAA).

NIST Analyses for Ba, Ca, Fe, I, K, Mg, Mn, Mo, Na, P, Se, and Sr using ICP-OES and/or ICP-MS: Mass fractions of barium, calcium, iron, magnesium, manganese, phosphorus, potassium, sodium, and strontium were measured by ICP-OES. The mass fractions of barium, iodine, molybdenum, selenium, and strontium were measured by ICP-MS. For each technique, duplicate 0.4 g test portions were taken from each of 10 packets of SRM 3252. Samples were digested in a microwave sample preparation system using nitric acid. For the determination of iodine by ICP-MS, samples were neutralized using NH_4OH. Quantification for ICP-OES and ICP-MS was based on the method of standard additions using the SRM 3100 series single element standard solutions.

NIST Analyses for Cd and Pb by ID ICP-MS: Mass fractions of cadmium and lead were determined by ID ICP-MS using duplicate, nominal 0.5 g test portions taken from each of six packages of SRM 3252. Samples were spiked with isotopically enriched ^{111}Cd and ^{206}Pb and were digested in nitric acid using a microwave sample preparation system. Sample digests were evaporated to near dryness and a portion was reconstituted in dilute nitric acid for analysis. Lead was measured in standard mode, whereas cadmium was measured in collision cell/kinetic energy discrimination mode. Quantification was based on calibration by reverse isotope dilution ICP-MS using primary standard solutions prepared from SRM 746 *Cadmium-Vapor Pressure*, SRM 3108 *Cadmium (Cd) Standard Solution*, SRM 3128 *Lead (Pb) Standard Solution*, and from Pb metal (Johnson Matthey, 0.99999 % pure).

NIST Analyses for Ca, Cu, Fe, K, Mn, Na, P, and Zn using WDXRF: Mass fractions of calcium, copper, iron, potassium, manganese, sodium, phosphorus, and zinc were measured by WDXRF in duplicate 4.0 g test portions taken from each of six packets of SRM 3252. Briquettes were prepared for each sample, and the K-$L_{2,3}$ characteristic X-ray lines of all elements were used for quantification. Calibration standards included SRM 1515 *Apple Leaves*, SRM 1547 *Peach Leaves*, SRM 1549 N*on-Fat Milk Powder*, SRM 1566b *Oyster Tissue*, SRM 1570a *Trace Elements in Spinach*, SRM 1571 *Orchard Leaves*, SRM 1573a *Tomato Leaves*, SRM 1575a *Trace Elements in Pine Needles*, and SRM 1577c *Bovine Liver*.

NIST Analyses for Al, Ca, Cl, Cr, Fe, I, K, La, Mg, Mn, Mo, Na, Se, Sm, and Zn using INAA: Mass fractions of aluminum, calcium, chlorine, chromium, iodine, iron, lanthanum, magnesium, manganese, molybdenum, potassium, samarium, selenium, sodium, and zinc were measured by INAA in individual disks that were prepared from 0.2 g test portions taken from each of ten packets of SRM 3252. Samples, standards, and controls were packaged individually in clean polyethylene bags and irradiated individually. For determination of aluminum, calcium, chlorine, iodine, magnesium, manganese, potassium, and sodium, samples were irradiated at 20 MW for 60 s and nuclides were counted for 15 min after a 10 min decay. For determination of chromium, iron, lanthanum, molybdenum, samarium, selenium, and zinc, samples were irradiated at 20 MW for 8 h and nuclides were counted for 5 d following a 2 h decay or for 8 h following more than a 14 d decay. Quantification was based on standard solutions prepared from SRM 918b *Potassium Chloride*, SRM 919a *Sodium Chloride*, SRM 3109a *Calcium (Ca) Standard Solution*, SRM 3131a *Magnesium (Mg) Standard Solution*, SRM 3134 *Molybdenum (Mo) Standard Solution*, validated pure metal foils, or validated pure compounds.

Analytical Approach for Determination of Vitamins: Value assignment of the mass fractions of the vitamins in SRM 3252 was based on the combination of results provided by various analytical methods at NIST and collaborating laboratories. NIST provided measurements by using liquid chromatography (LC) with absorbance detection, or isotope dilution (ID) with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) detection.

NIST Analyses for Thiamine, Riboflavin, Niacinamide, Niacin, Pantothenic Acid, Pyridoxamine, and Pyridoxine: Mass fractions of thiamine, riboflavin, niacinamide, niacin, pantothenic acid, pyridoxamine, and pyridoxine were measured by ID-LC-MS/MS in duplicate 5 g test portions taken from each of ten packets of SRM 3252. The analytes and internal standards were extracted into ammonium acetate at pH 2.6 by stirring at 100 °C for 30 min. Samples were centrifuged following extraction and an aliquot of the supernatant was analyzed by positive ion-mode ID-LC-MS/MS. A gradient method with an ammonium formate buffer/methanol mobile phase and a C18 column were used for ID-LC-MS/MS determination of the vitamins. Calibrants were prepared gravimetrically, at levels intended to approximate the levels of the vitamins in the SRM following extraction. The purity of neat calibrant materials was determined at NIST using LC-absorbance, Karl Fischer titration, thermogravimetric analysis, and differential scanning calorimetry. A single internal standard solution was used for the calibrants and samples.

NIST Analyses for Ascorbic Acid: The mass fraction of ascorbic acid was measured by LC-absorbance in two or more 2 g test portions from each of 10 packets of SRM 3252. Samples were dissolved in 0.1 mol/L HCl and an internal standard, 4-pyridoxic acid, was added. Metaphosphoric acid was added as a stabilizing agent, and dithiothreitol was added to convert dihydroascorbic acid to total ascorbic acid. The ascorbic acid was extracted by room-temperature sonication, and following centrifugation, an aliquot of the supernatant was removed and filtered prior to analysis by LC-absorbance. Separations were performed on a C18 column using a gradient LC method with potassium phosphate (dibasic)/acetonitrile mobile phase. The separation was monitored using an absorbance detector at 243 nm for ascorbic acid and 260 nm for the internal standard. Calibrants were prepared gravimetrically, at levels intended to approximate the level of ascorbic acid in the SRM following extraction. The purity of the neat calibrant material was determined at NIST using LC-absorbance at 243 nm. A single internal standard solution was used for the calibrants and samples.

NIST Analyses for Biotin: The mass fraction of biotin was measured in two 1.0 g test portions taken from each of ten packets of SRM 3252. $^{2}H_{2}$-biotin was added as an internal standard. An aqueous solution of formic acid was added to the samples, which were then subjected to mechanical shaking for 30 min. Samples were centrifuged, and biotin and $^{2}H_{2}$-biotin were extracted on solid-phase extraction cartridges and eluted with a water/methanol solution containing formic acid for positive ion mode ID-LC-MS analysis. An isocratic LC method with a water/methanol/formic acid mobile phase and a C18 reversed-phase column were used for the determination of biotin. Biotin and $^{2}H_{2}$-biotin were monitored at *m/z* 245 and *m/z* 247, respectively. Calibrants were prepared gravimetrically, at levels intended to approximate the levels of the vitamins in the SRM following extraction. The purity of the neat calibrant material was determined at NIST using quantitative proton nuclear magnetic resonance spectroscopy (^{1}H-qNMR). A single internal standard solution was used for the calibrants and samples.

NIST Analyses for Choline and Carnitine: Mass fractions of choline and carnitine were measured in duplicate 1.0 g test portions taken from each of ten packets of SRM 3252. $^{2}H_{9}$-choline chloride and $^{2}H_{9}$-carnitine hydrochloride were added as internal standards. The analytes and internal standards were extracted and hydrolyzed by microwave digestion in dilute hydrochloric acid for analysis by positive-ion mode LC-MS. A gradient method with an ammonium formate/acetonitrile mobile phase and a mixed-mode C18 column were used for LC-MS determination. Choline and $^{2}H_{9}$-choline were measured at *m/z* 104 and *m/z* 113, respectively. Carnitine and $^{2}H_{9}$-carnitine were measured at *m/z* 162 and *m/z* 171, respectively. Calibrants were prepared gravimetrically, at levels intended to approximate the levels of the vitamins in the SRM following extraction. The purity of neat calibrant materials was determined at NIST using ^{1}H-qNMR. A single internal standard solution was used for the calibrants and samples.

Analytical Approach for Determination of Fatty Acids: Value assignment of the mass fractions of fatty acids in SRM 3252 was based on the combination of measurements made at NIST and collaborating laboratories, where appropriate. NIST provided measurements by using gas chromatography (GC) with flame ionization detection (FID).

NIST Analyses for Fatty Acids: Mass fractions of fatty acids were determined by GC-FID from two 1.0 g test portions from each of ten packets of SRM 3252. The analytes and internal standards were extracted into a mixture of methanol and toluene containing butylated hydroxytoluene (BHT, 1 g/L) as an antioxidant by sonication for 30 min. After centrifuging, the solvent was removed and fresh toluene containing BHT was added. The extraction was repeated for a total of three cycles, and all supernatants were combined and concentrated to approximately 2 mL under nitrogen. The concentrated extract was combined with 1 mL of MethPrep II (0.2 N methanolic [m-trifluoromethylphenyl] trimethylammonium hydroxide, Alltech, Deerfield, IL). Samples were mixed for 1 min and allowed to equilibrate for at least 1 h prior to analysis by GC-FID. GC-FID was performed using a 0.25 mm × 100 m biscyanopropyl polysiloxane fused silica capillary column. Calibrants were prepared gravimetrically from SRM 2377 *Fatty Acid Methyl Esters in 2,2,4-Trimethylpentane*, at levels intended to approximate the levels of the fatty acids in the SRM following extraction. A single internal standard solution was used for the calibrants and samples. Calculations are based on average response factors for the calibrants.

Collaborating Laboratories' Analyses: The GMA FIACC laboratories were asked to use their usual methods to make single measurements of proximates, calories, vitamins, elements, fatty acids, and amino acids on test portions taken from each of two packets of SRM 3252. Because of variability among data provided by laboratories participating in an interlaboratory comparison exercise, the median of laboratory means is used, with the uncertainty estimated using a bootstrap procedure [3–4].

Homogeneity Assessment: The homogeneity of fatty acids, cholesterol, elements, and vitamins was assessed at NIST using the methods and test portion sizes described above. Analysis of the variance showed statistically significant heterogeneity in some cases, and the uncertainties for ascorbic acid (vitamin C), cadmium, lead, lauric acid, and myristic acid incorporate an additional uncertainty component for possible inhomogeneity. Homogeneity of constituents measured solely by collaborating laboratories (e.g., proximates, amino acids) was not assessed, although the data were treated as though these analytes were homogeneously distributed.

Value Assignment: For calculation of assigned values for analytes that were measured only by NIST, the mean of the mean values from NIST results were used. For calculation of assigned values for analytes that were measured only by the collaborating laboratories, the median of the laboratory means was used. For analytes that were also measured by NIST, the mean of the individual sets of NIST data were averaged with the median of the individual collaborating laboratory means, as appropriate. For biotin, the calculation of assigned values is the mean of the NIST results with confirmation provided by collaborating laboratories. For niacin, niacinamide, and pyridoxine, the calculation of assigned values is the mean of the NIST results with confirmation provided by the determined total vitamin value, based on the combination of data from NIST and collaborating laboratories.

Table B1. ID-LC-MS/MS Transitions Monitored for Vitamins

Compound	Precursor Ion (*m/z*)	→ Product Ion (*m/z*)	Internal Standard	IS Precursor Ion (*m/z*)	→ IS Product Ion (*m/z*)
Thiamine	266	42	$^{13}C_3$-Thiamine	269	42
		123			123
Riboflavin	377	43	$^{13}C_4$,$^{15}N_2$-Riboflavin	383	43
		172			175
		198			202
		243			249
Niacinamide	123	53	$^{2}H_4$-Niacinamide	127	56
		78			81
		80			84
Niacin	124	52	$^{2}H_4$-Niacinamide	127	53
		53			56
		78			81
		80			84
Pantothenic Acid	220	41	$^{13}C_3$,^{15}N-Pantothenic Acid	224	41
		43			43
		72			76
		90			94
Pyridoxamine	169	77	$^{2}H_3$-Pyridoxamine	172	79
		134			136
		152			155
Pyridoxine	170	77	$^{13}C_4$-Pyridoxine	174	81
		80			83
		134			138
		152			156

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