Certification of Vitamins and Carotenoids in SRM 3280 Multivitamin/Multielement Tablets

ARTICLE *in* ANALYTICAL CHEMISTRY · JANUARY 2011

Impact Factor: 5.64 · DOI: 10.1021/ac101953u · Source: PubMed

17 AUTHORS, INCLUDING:

Bryant C Nelson
National Institute of Science & Technology
59 PUBLICATIONS **1,481** CITATIONS
SEE PROFILE

Jeanice B Thomas
National Institute of Standards and Techn…
36 PUBLICATIONS **744** CITATIONS
SEE PROFILE

Laura J Wood
National Institute of Standards and Techn…
26 PUBLICATIONS **250** CITATIONS
SEE PROFILE

James H Yen
National Institute of Standards and Techn…
49 PUBLICATIONS **678** CITATIONS
SEE PROFILE

Anal. Chem. **2011,** *83,* 99–108

Certification of Vitamins and Carotenoids in SRM 3280 Multivitamin/Multielement Tablets

L. C. Sander,*,† K. E. Sharpless,† S. A. Wise,† B. C. Nelson,‡ K. W. Phinney,† B. J. Porter,† C. A. Rimmer,† J. B. Thomas,† L. J. Wood,† J. H. Yen,§ D. L. Duewer,† R. Atkinson,|| P. Chen,|| R. Goldschmidt,|| W. R. Wolf,|| I.-P. Ho,⊥ and J. M. Betz▽

Analytical Chemistry Division, National Institute of Standards and Technology (NIST), 100 Bureau Drive, MS 8311, Gaithersburg, Maryland 20899-8392, United States; Biochemical Science Division, National Institute of Standards and Technology (NIST), 100 Bureau Drive, MS 8311, Gaithersburg, Maryland 20899-8392, United States; Statistical Engineering Division, National Institute of Standards and Technology (NIST), 100 Bureau Drive, MS 8980, Gaithersburg, Maryland 20899-8980, United States; Food Composition and Methods Development Laboratory, U.S. Department of Agriculture (USDA), 10300 Baltimore Blvd, Beltsville, Maryland 20705, United States; Grocery Manufacturers Association (GMA), 1350 I Street, NW, Suite 300, Washington, District of Columbia 20005, United States; and Office of Dietary Supplements, National Institutes of Health, 6100 Executive Blvd., Room 3B01, Bethesda, Maryland 20892, United States

A new multivitamin/multielement dietary supplement Standard Reference Material (SRM) has been issued by the National Institute of Standards and Technology (NIST), with certified and reference concentration values for 13 vitamins, 24 elements, and 2 carotenoids. The constituents have been measured by multiple analytical methods with data contributed by NIST and by collaborating laboratories. This effort included the first use of isotope dilution mass spectrometry for value assignment of both fat-soluble vitamins (FSVs) and water-soluble vitamins (WSVs). Excellent agreement was obtained among the methods, with relative expanded uncertainties for the certified concentration values typically ranging from <2% to 15% for vitamins.

Micronutrients are a group of vitamins and elements that are essential to human health and development. These compounds and elements perform catalytic functions to regulate metabolic reactions (e.g., as coenzymes). Deficiencies and excesses of micronutrients typically produce symptoms that are alleviated when adjusted to "normal" physiological levels. In most cases, vitamins must be obtained through the diet, since these compounds are not biosynthesized at significant levels within the body, with the exception of vitamin D, which is produced with the skin's exposure to sunlight, and pantothenic acid, which is produced by intestinal flora. Recommended and reference intakes and allowances for micronutrients are periodically assessed by national and international organizations (e.g., Food and Nutrition Board of the Institute of Medicine and the World Health Organization); dietary supplements may be utilized by individuals to ensure that these recommended levels are achieved.

The Dietary Supplement Health and Education Act of 1994 specifically includes "vitamins" and "minerals" in the definition of a dietary supplement, and further defines dietary supplements as "a dietary substance for use by man to supplement the diet by increasing the total dietary intake".[1] Multivitamin/multielement formulations are among the most widely consumed dietary supplements in the marketplace; their use may be prescribed by physicians or elected by individuals to promote good health. Multivitamin/multielement formulations often include vitamins, carotenoids (see Figure 1), and nutrient elements, many of which have established recommended intakes. Vitamins are often categorized as water-soluble vitamins (WSVs) or fat-soluble vitamins (FSVs); water-soluble vitamins include the B vitamins, vitamin C, and biotin, and fat-soluble vitamins include vitamins A, D, E, and K. FSV methods of analysis may also measure carotenoids, some of which are converted to vitamin A (retinol) in the body.

Reliable measurements of the composition of dietary supplements are essential for clinical studies, for specification of appropriate dosage levels, for quality control in manufacturing, and in verification of product labeling. The need for improved precision and accuracy in the determination of micronutrients in food was recently evaluated by Phillips et al. from the results of the USDA's National Food and Nutrient Analysis Program (NFNAP).[2] Certified reference materials (CRMs) were distributed to contract laboratories for characterization of vitamin and element compositions. Performance among these laboratories varied, depending on the analyte; however, no laboratory exhibited acceptable proficiency for measurement of all vitamins and elements that were determined. In the NFNAP results, more than 20% of the measurements for certain vitamins were beyond two standard deviations from the assigned values.

* Author to whom correspondence should be addressed.
† Analytical Chemistry Division, National Institute of Standards and Technology (NIST).
‡ Biochemical Science Division, National Institute of Standards and Technology (NIST).
§ Statistical Engineering Division, National Institute of Standards and Technology (NIST).
|| U.S. Department of Agriculture (USDA).
⊥ Grocery Manufacturers Association (GMA).
▽ Office of Dietary Supplements, National Institutes of Health.

(1) Dietary Supplement Health and Education Act. Public Law 103-417 [S.784], Oct. 25, 1994.
(2) Phillips, K. M.; Wolf, W. R.; Patterson, K. Y.; Sharpless, K. E.; Holden, J. M. *Anal. Bioanal. Chem.* **2007**, *389*, 219–29.

10.1021/ac101953u Not subject to U.S. Copyright. Publ. 2011 Am. Chem. Soc.
Published on Web 12/03/2010

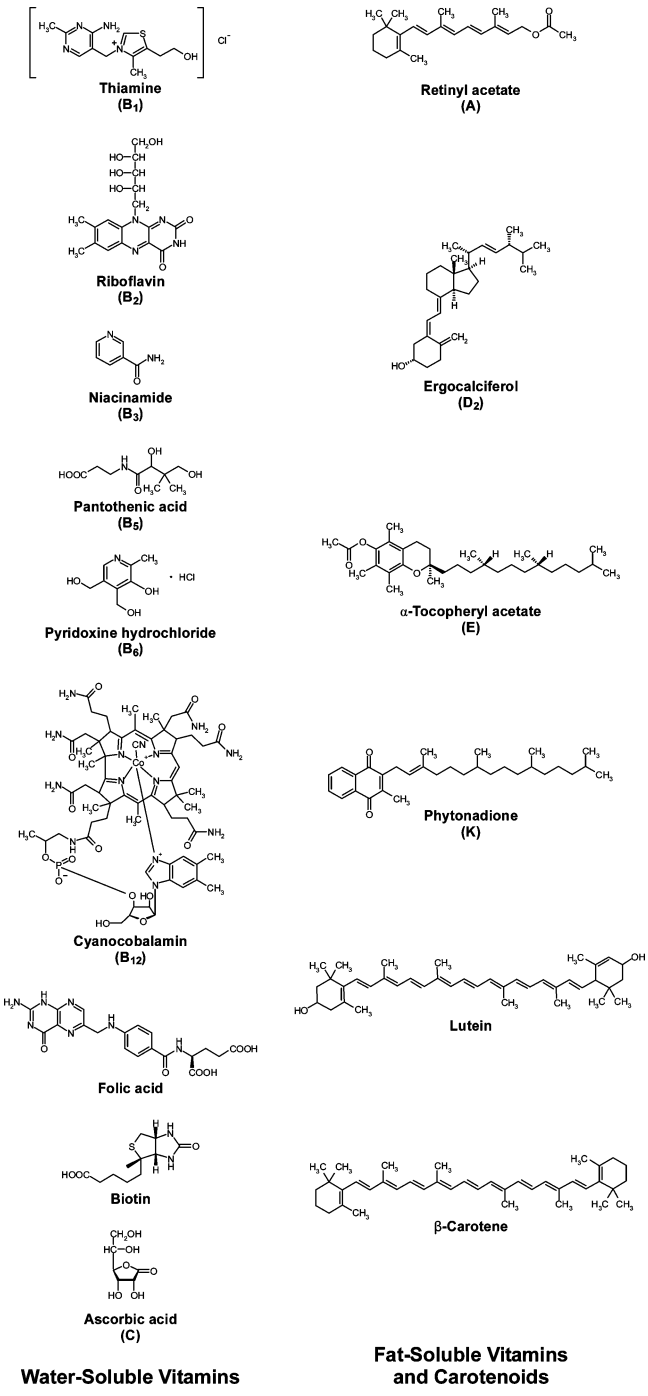

Figure 1. Structures of water-soluble vitamins (WSVs), fat-soluble vitamins (FSVs), and carotenoids contained in SRM 3280.

The determination of vitamins and nutrient elements in foods and dietary supplements presents a broad range of challenges to the analyst. These are dependent on the types of samples to be analyzed, the analytes to be determined, the range of constituent levels, and the precision and accuracy required for the results. Methods for the determination of vitamins include bioassays or microbiological assays, binding assays (e.g., immunoassays), and instrumental methods. In addition, a few instruments have been developed that utilize binding assays as part of their measurement technology. Bioassays and microbiological assays are dependent on an organism's biological response to micronutrient levels and may provide an indication of bioavailability, whereas instrumental methods and binding assays (with certain limitations) are indicative of molar response. Binding assays often provide high sensitivity and high throughput for vitamin determinations; however, variability of the assay kits among different manufacturers can lead to questionable accuracy and lack of traceability of measurements. Instrumental methods can also provide high sensitivity, although sample throughput may be reduced compared with binding assays. Chromatographic methods may allow discrimination of individual vitamers and vitamin metabolites.

CRMs are vital in chemical metrology of micronutrients: they are important in the development and validation of new methods, in the identification of biases among different methods, and ultimately, in the defense of claims of measurement traceability. Different types of CRMs are applicable to specific functions. For example, solution CRMs containing known quantities of organic or inorganic constituents are typically used for instrument calibration, whereas complex-matrix CRMs are more applicable for use as quality control materials or for method validation.

In 2001 NIST, the National Institutes of Health (NIH) Office of Dietary Supplements (ODS), and the Food and Drug Administration (FDA) began a collaboration to develop Standard Reference Materials (SRMs) to support the metrology of dietary supplement composition.[3,4] This program was planned to complement and extend NIST's ongoing efforts to develop food-matrix SRMs.[5] The majority of the dietary supplement SRMs have been of a botanical nature, and they have included natural, extracted, and more extensively processed materials. For example, a suite of SRMs has been issued that contains bitter orange as a dried and ground plant (SRM 3258 Bitter Orange Fruit), a spray-dried extract of immature bitter orange fruit (SRM 3259 Bitter Orange Extract), and a mixture of ground and sieved commercial formulations that contain bitter orange (SRM 3260 Bitter Orange-Containing Solid Oral Dosage Form).[6] Recently, efforts have been directed toward the characterization of nonbotanical, SRM 3280 Multivitamin/Multielement Tablets, which are intended for use primarily as a control material for analytical quality assurance, and for use in the development and validation of analytical methods for the determination of vitamins, carotenoids, and elements in dietary supplements. This report describes the value assignment of vitamin and carotenoid levels in SRM 3280. Independent extraction and sample processing steps, different approaches to quantitation, and independent analytical methods were utilized in the certification of analyte levels in this material. The certification of inorganic constituents will be reported elsewhere.

EXPERIMENTAL SECTION

Material Acquisition and Preparation. A manufacturer of multivitamin/multielement tablets prepared a noncommercial batch of tablets according to their normal procedure. SRM 3280

(3) Sharpless, K. E.; Sander, L. C.; Wise, S. A.; Nguyen Pho, A.; Lyon, R. C.; Ziobro, G. C.; Betz, J. M. *Herbal Gram* **2004**, *63*, 44–47.
(4) Wise, S. A.; Sharpless, K. E.; Sander, L. C.; May, W. E. *Accr. Qual. Assur.* **2004**, *9*, 543–50.
(5) Sharpless, K. E.; Brown Thomas, J. M.; Christopher, S. J.; Greenburg, R. R.; Sander, L. C.; Schantz, M.; Welch, M. J.; Wise, S. A. *Anal. Bioanal. Chem.* **2007**, *389*, 171–78.
(6) Sander, L. C.; Putzbach, K.; Nelson, B. C.; Rimmer, C. A.; Bedner, M.; Thomas, J. B.; Porter, B.; Wood, L. J.; Schantz, M. M.; Murphy, K. E.; Sharpless, K. E.; Wise, S. A.; Yen, J. H.; Siitonnen, P. H.; Nguyen Pho, A.; Roman, M. C.; Betz, J. M. *Anal. Bioanal. Chem.* **2008**, *391*, 2023–34.

is a direct-compression tablet formulation produced by blending a vitamin and a mineral premix with the remaining bulk of the formulation, compression, and tablet film coating. Fat-soluble vitamins and carotenoids (retinyl acetate, β-carotene, lutein, ergocalciferol, and D,L-α-tocopheryl acetate) were added as gelatin beadlets. The film coat consisted of triethyl citrate, polysorbate 80, Yellow #6 Aluminum Lake, hypromellose, and titanium dioxide. A complete list of vitamins, minerals, and excipients is provided in Table S1 in the Supporting Information.

Reagents (NIST). Sources of reference standards and stable isotope-labeled vitamins are listed in Table S2 in the Supporting Information. Reference standards were obtained commercially; certain stable isotope-labeled vitamins were obtained through custom synthesis (as indicated in Table S2). Corrections for the purity of the water-soluble vitamin reference standards (thiamine HCl, riboflavin, niacinamide, calcium pantothenate, pyridoxine HCl, cyanocobalamin, ascorbic acid, biotin, folic acid) and fat-soluble vitamins (retinyl acetate, tocopheryl acetate, phylloquinone, and ergocalciferol) were made based on the mean of purity measurements from multiple analytical methods. Moisture was determined in folic acid by Karl Fischer analysis, and purity corrections included this contribution. Levels of *trans*-β-carotene and lutein are based on spectrophotometric analysis of stock solutions with corrections made for chromatographic purity.[7] Collaborating laboratories used independently obtained reference standards and purity estimates.

Sample Preparation. Prior to removal of a test portion for analysis, 15−30 tablets were ground to obtain a homogeneous sample. A test portion was then removed from this powder. NIST analysts used either of two methods to grind pellets to a powder prior to analysis: (1) 30 tablets were ground in a Teflon disk mill, which involved shaking in an orbital pattern for 6 min, or (2) batches of 15, 20, or 30 tablets were ground for 10 min using an automated mortar and pestle.

Determination of Moisture. Moisture content of SRM 3280 was determined at NIST in ground tablets by (1) freeze-drying to constant mass over 8 days; (2) drying over magnesium perchlorate in a desiccator at room temperature for 5, 7, and 12 d; and (3) drying for 4 h in a forced-air oven at 80 °C. Unweighted results obtained using all three techniques were averaged to determine a conversion factor of 0.9863 ± 0.0051 gram dry mass per gram as-received mass, which was used to convert data from an as-received to a dry-mass basis; the uncertainty shown on this value is an expanded uncertainty. An uncertainty component for the conversion factor (0.26%) obtained from the moisture measurements is incorporated in the uncertainties of the certified and reference values, reported on a dry-mass basis.

Analytical Methods. A listing of the analytical methods is provided in Table 1, and the methods are summarized below. Additional details for some methods are provided in separate publications. Generally, both the levels of the internal standards and calibrants were adjusted to approximate levels of the analytes, for the purpose of calibration in the various NIST methods. However, efforts were not made to match the concentration of the calibrants and unknown samples exactly. Typically, 3−4 calibrants were prepared, based on independent weighings of the

(7) *Methods for Analysis of Cancer Chemopreventive Agents in Human Serum*, National Institute of Standards and Technology (NIST) Special Publication 874; U.S. Government Printing Office: Washington, DC, 1995.

Table 1. Summary of Methods Used in the Analysis of SRM 3280 Multivitamin/Multielement Tablets

number	method	laboratory	analytes	number of tablets ground	sample size (g)	column	configuration
1	LC/abs	NIST	A, E, β-carotene, lutein	30	2.0	Vydac 201TP C18[a]	250 × 4.6 mm, 5 μm
2	LC/abs	NIST	β-carotene, lutein	15	0.2−0.5	Bakerbond C18[b]	250 × 4.6 mm, 5 μm
3	LC/MS	NIST	A, D_2, E, K	15	0.6	ACE C18[c]	250 × 4.6 mm, 5 μm
4	LC/abs	NIST	β-carotene, lutein	15	0.6	YMC C30[d]	250 × 4.6 mm, 5 μm
5	LC/abs	NIST	B_1, B_3, B_6, C	30	2.0	YMC C18 Pro[d]	250 × 4.6 mm, 5 μm
6	LC/ID-MS	NIST	B_1, B_2, B_3, B_5, B_6		0.25	Cadenza CD-C18[e]	250 × 4.6 mm, 5 μm
7	LC/ELSD	NIST	biotin	15	1.5	Supelcosil CN[f]	250 × 4.6 mm, 5 μm
8	LC/ID-MS	NIST	biotin	15	1.5	Xterra RP C18[d]	150 × 4.6 mm, 5 μm
9	LC/ID-MS/MS pos ion	NIST	folic acid	15	0.3	Discovery HS-F5 pentafluoropropyl[f]	150 × 4.6 mm, 5 μm
10	LC/ID-MS/MS neg ion	NIST	folic acid	15	0.3	Discovery HS-F5 pentafluoropropyl[f]	150 × 4.6 mm, 5 μm
11	various	CEN[h]	A, B_1, B_2, B_3, B_5, B_6, B_{12}, C, D_2, E, K, folic acid, biotin, β-carotene, lutein				
12	various	GMA[i]	A, B_2, B_5, B_{12}, C, D_2, K, folic acid, biotin, lutein				
13	LC/ID-MS	USDA	B_1, B_3, B_5, B_6, folic acid, biotin	20	0.15	Phenomenex Hydro-RP C18[g]	250 × 2.0 mm, 4 μm
14	LC/abs or LC/FL	USDA	B_1, B_2, B_3, B_6, C	20	0.15	Phenomenex Hydro-RP C18[g]	250 × 2.0 mm, 4 μm

[a] The Separations Group, Hesperia, CA, USA. [b] J.T. Baker, Phillipsburg, NJ, USA. [c] Advanced Chromatography Technologies, Aberdeen, Scotland. [d] Waters Corporation, Milford, MA, USA. [e] Silvertone Sciences, Philadelphia, PA, USA. [f] Supelco, Bellefonte, PA, USA. [g] Phenomenex, Torrance, CA, USA. [h] European Committee for Standardization (CEN) Vitamin Working Group Laboratories. [i] Grocery Manufacturers Association (GMA) Food Industry Analytical Chemists Committee.

reference standards, to provide indications of potential mistakes in solution preparation. In the descriptions of individual methods that follow, all solvent percentages are volume fractions.

Method 1: Determination of Fat-Soluble Vitamins by Liquid Chromatography with Absorbance Detection (LC/Abs) (NIST).[8] Subsamples of ground tablets were combined with dilute hydrochloric acid and placed in an ultrasonic bath for ≈25 min, with intermittent manual shaking. An internal standard solution (containing δ-tocopherol) was added to each sample, and samples were returned to the ultrasonic bath for an additional 5 min. Analytes were extracted into hexane by shaking (inversion of sample tubes at ≈628 rad/s (100 rpm) for 8 h). At least three subsequent 1-h extractions into hexane were performed until the organic layer was colorless. Hexane layers were combined, and then 10 mL of extract was removed, evaporated to dryness under nitrogen, and reconstituted in ethanol that contained butylated hydroxytoluene (BHT). An isocratic LC method with a mobile phase (96% acetonitrile/4% methanol, containing 0.05% triethylamine) and a polymeric C_{18} column were used for LC/abs determination of retinyl acetate, α-tocopheryl acetate, and carotenoids, with absorbances measured at 325, 284, and 450 nm, respectively. The concentration of each carotenoid stock solution was determined by spectrophotometry using the following extinction coefficients in absolute ethanol: 2560 dL/(g·cm) for *trans*-β-carotene at 452 nm and 2765 dL/(g·cm) for lutein at 445 nm. The flow rate was 0.8 mL/min, and the column temperature was 25 °C.

Method 2: Determination of β-Carotene by LC/Abs (NIST). Subsamples of ground tablets were combined with an internal standard solution (containing *trans*-β-apo-8′-carotenal) and ethylenediamine tetraacetic acid (EDTA) solution and held at 45 °C to dissolve gel encapsulation. Analytes were then extracted into hexane (five successive 60-min extractions), and the extracts then were combined. A Bakerbond C_{18} column and gradient elution were used for LC/abs determination of β-carotene at 450 nm. Solvent A was acetonitrile, solvent B was methanol that contained 0.05 mol/L ammonium acetate, and solvent C was ethyl acetate. Each of the three solvents contained 0.05% triethylamine (TEA). The method consisted of two linear gradients and an isocratic component. The first gradient ran from 98% solvent A/2% solvent B to 75% solvent A/18% solvent B/7% solvent C in 10 min. A second linear gradient ran from this composition to 68% solvent A/25% solvent B/7% solvent C in 5 min. The flow rate was 1 mL/min, the column temperature was 29 °C, and absorbance detection was observed at 450 nm. The concentration of the *trans*-β-carotene stock solution was determined via spectrophotometry, using the extinction coefficient for *trans*-β-carotene in absolute ethanol at 452 nm (2560 dL/(g·cm)).

Method 3: Determination of Fat-Soluble Vitamins by LC with Mass Spectrometric Detection (LC/ID-MS) (NIST).[9] Subsamples of ground tablets were combined with EDTA solution and held at 45 °C to dissolve the gel encapsulation of some of the fat-soluble vitamins. Three internal standard solutions, containing retinyl acetate-d_6, vitamin K_1-d_4, and vitamin D_2-d_3, respectively, were added, and samples were placed in an ultrasonic bath for 10 min. Analytes were extracted into hexane by shaking overnight (inversion of sample tubes at ≈377 rad/s (60 rpm) for 8 h). At least five subsequent 1-h extractions into hexane were performed. An isocratic LC method with 40% methanol and 60% acetonitrile with 5 mmol/L ammonium acetate at a flow rate of 1 mL/min and a deactivated, monomeric C_{18} column were used for LC/MS determination of the fat-soluble vitamins. The mass spectrometry conditions were as follows: nebullizer pressure, 240 kPa (35 psi); fragmentor voltage, 100 V; drying gas temperature, 350 °C; drying gas flow rate, 6 L/min; corona current, 4 μA; capillary voltage, 3500 V; and vaporizer temperature, 350 °C. Retinyl acetate, $^2H_6$-retinyl acetate, and α-tocopheryl acetate were monitored together, with respective *m/z* ratios of 328, 334, and 473. Vitamin K_1 and $^2H_4$-K_1 were grouped together, with respective *m/z* ratios of 452 and 456. Finally, vitamin D_2 and $^2H_3$-D_2 were monitored together, with *m/z* ratios of 389 and 401. The separation was also monitored at 287 nm, and mass spectroscopy (MS) was used for quantitation.

Method 4: Determination of Lutein by LC/Abs (NIST). Samples were processed as in Method 2. A polymeric C_{30} column and gradient elution were used for determination of β-carotene and lutein. A linear 70-min gradient from 60% acetone/40% water (containing 2 mmol/L ammonium acetate) to 100% acetone was employed. The flow rate was 1 mL/min, the column temperature was 25 °C, and the absorbance was measured at 450 nm. The concentrations of the stock solutions was determined by spectrophotometry using the extinction coefficients for lutein in ethanol (2765 dL/(g·cm)).

Method 5: Determination of Vitamins B_1, B_6, and C, and Niacinamide, by LC/Abs (NIST). Subsamples of ground tablets were combined with hydrochloric acid, and an internal solution containing 4-pyridoxic acid was added. The mixture was sonicated and centrifuged, and a portion of the supernatant was removed and filtered prior to analysis by LC/abs. A gradient LC method with potassium phosphate dibasic buffer and acetonitrile and a C_{18} column were used for the LC/abs determination of vitamins B_1, B_6, and C, and niacinamide, with absorbance detection at 260 nm.

Method 6: Determination of Vitamins B_1, B_2, and B_6, Niacinamide, and Pantothenic Acid by LC/ID-MS (NIST).[9] Four internal standards were added to subsamples of ground tablets: $^{13}C_3$-thiamine chloride; $^2H_4$-niacinamide; $^{13}C_3$,^{15}N-calcium pantothenate; and $^{13}C_4$-pyridoxine hydrochloride. The analytes and internal standards were extracted into dilute acetic acid for analysis by positive-ion mode LC/MS. A gradient LC method with an ammonium formate buffer/methanol mobile phase and a C_{18} column were used for LC/MS determination of vitamins B_1, B_2, and B_6, niacinamide, and pantothenic acid. The mass

(8) Brown Thomas, J. M.; Sharpless, K. E.; Yen, J.; Rimmer, C. A. Determination of Fat-Soluble Vitamins and Carotenoids in Standard Reference Material 3280 Multivitamin/Multielement Tablets by Liquid Chromatography with Absorbance and Mass Spectrometric Detection. *J. AOAC Intl.*, **2010**, in press.

(9) Phinney, K. W.; Rimmer, C.; Brown Thomas, J. M.; Sander, L. C.; Sharpless, K. E.; Wise, S. A. Isotope Dilution Liquid Chromatography−Mass Spectrometry Methods for Fat- and Water-Soluble Vitamins in Nutritional Formulations. *Anal. Chem.*, **2010**, in press.

spectrometry conditions were as follows: nebullizer pressure, 345 kPa (50 psi); fragmentor voltage, 110 V; drying gas temperature, 350 °C; drying gas flow rate, 13.0 L/min; capillary voltage, 4000 V; and vaporizer temperature, 350 °C. Thiamine and $^{13}C_3$-thiamine were measured at m/z 265 and m/z 268, respectively. Niacinamide and $^{2}H_4$-niacinamide were measured at m/z 123 and m/z 127, respectively. Pantothenic acid and $^{13}C_3$,^{15}N-pantothenic acid were measured at m/z 220 and m/z 224, respectively. Pyridoxine and $^{13}C_4$-pyridoxine were measured at m/z 170 and m/z 174, respectively. Riboflavin was measured at m/z 377, with $^{13}C_4$-pyridoxine as the internal standard.

Method 7: Determination of Biotin by LC with Evaporative Light Scattering Detection (LC/ELSD) (NIST).[10,11] Desthiobiotin was added as an internal standard to subsamples of ground tablets, and the analytes were extracted into an aqueous formic acid solution. An isocratic LC method with a mobile phase composition of 95% water/5% methanol/0.1% formic acid and a cyanopropyl column were used for LC/ELSD determination of biotin. ELSD conditions were a temperature of 50 °C and a nitrogen gas pressure of 300 kPa.

Method 8: Determination of Biotin by LC/ID-MS (NIST).[10,11] $^{2}H_2$-biotin was added as an internal standard to subsamples of ground tablets, and the analytes were extracted into methanol. An isocratic LC method was used for the determination of biotin, with a C_{18} column and a mobile phase composition of 23% methanol/77% water/0.1% formic acid. Biotin and $^{2}H_2$-biotin were measured at m/z 245 and m/z 247, respectively. The MS instrument parameters were as follows: fragmentor voltage, 120 V, capillary volatage, 4500 V; drying gas temperature, 350 °C; drying gas flow, 13.0 L/min; nebullizer pressure, 276 kPa (40 psi).

Method 9: Determination of Folate by LC/ID-MS/MS [Positive-Ion] (NIST).[12] An internal standard, $^{13}C_5$-folic acid ([13C5]-FA), was added to ground subsamples of the tablets. The internal standard and folic acid (FA) were extracted into a 50% methanol/50% water mixture containing dithiothreitol for positive-ion-mode LC/MS/MS. A gradient LC method with a water/methanol/formic acid mobile phase and a pentafluorylphenyl column were used. The LC elution conditions were as follows (mobile phase A = 1% formic acid in water; mobile phase B = 1% formic acid in methanol): time program = 0 min, 60% A/40% B; 10.0 min, 60% A/40% B; 10.1 min, 0% A/100% B; 12.0 min, 0% A/100% B; 12.1 min, 60% A/40% B; and 15.0 min, 60% A/40% B. The flow rate was 0.5 mL/min. FA and [13C5]-FA were detected and quantified using multiple reaction monitoring (MRM) of the protonated analyte molecules $[M + H]^+$. The MS/MS operating parameters were as follows: FA, m/z 442 → m/z 295 and [13C5]-FA, m/z 447 → m/z 295; dwell time = 500 ms/ion; ion spray voltage = 5000 V; source temperature = 500 °C; curtain gas flow = 138 kPa (20 psi); gas 1 flow = 345 kPa (50 psi); gas 2 flow = 345 kPa (50 psi); collisionally activated dissociation gas (nitrogen) = 34 kPa (5 psi); declustering potential = 61 V; entrance potential = 10 V; collision energy = 23 V; and collision exit potential = 10 V.

Method 10: Determination of Folate by LC/ID-MS/MS [Negative-Ion] (NIST).[12] An internal standard, $^{13}C_5$-folic acid ([13C5]-FA), was added to subsamples of the ground tablets. The internal standard and folic acid were extracted into water that contained 0.1% dithiothreitol and 0.3% ammonium hydroxide solution (pH 11.1). A gradient LC method with a water/methanol/acetic acid mobile phase and a pentafluorylphenyl column were used. The LC elution conditions were as follows (all solvent percentages are volume fractions) (mobile phase A = 1% acetic acid in water; mobile phase B = 1% acetic acid in methanol): time program = 0 min, 55% A/45% B; 10.0 min, 55% A/45% B; 10.1 min, 0% A/100% B; 12.0 min, 0% A/100% B; 12.1 min, 55% A/45% B; and 15.0 min, 55% A/45% B. The flow rate was 0.5 mL/min. Folic acid and [13C5]-FA were detected and quantified using MRM of the deprotonated analyte molecules $[M - H]^-$. The MS/MS operating parameters were as follows: FA, m/z 440 → m/z 311 and [13C5]-FA, m/z 445 → m/z 311; dwell time = 500 ms/ion; ion spray voltage = −4000 V; source temperature = 700 °C; curtain gas flow = 69 kPa (10 psi); gas 1 flow = 138 kPa (20 psi); gas 2 flow = 276 kPa (40 psi); collisionally activated dissociation gas (nitrogen) = 68 kPa (10 psi); declustering potential = −95 V; entrance potential = −10 V; collision energy = −32 V; and collision exit potential = −23 V.

Method 11: CEN Laboratory Analyses. Five participants of the European Committee for Standardization (CEN) Vitamin Working Group reported measurements based on a variety of analytical methods. Outliers were excluded from calculation of the mean of the results. A listing of the individual methods used by the participating CEN laboratories is provided in Table S3 in the Supporting Information.

Method 12: GMA Laboratory Analyses. Participants in the Food Industry Analytical Chemists Committee (FIACC) of the Grocery Manufacturers Association (GMA) reported measurements based on analytical methods of their own choice. Outliers were excluded from calculation of the mean of the results. A listing of the individual methods used by the participating GMA laboratories is provided in Table S4 in the Supporting Information.

Method 13: Water-Soluble Vitamin Analysis by LC/ID-MS (USDA).[13] Details of the method used for the analysis of WSVs have been reported separately by Chen et al.[13] In summary, a gradient elution method was used to separate WSVs, via mass spectrometric detection. A C_{18} column designed for use with aqueous mobile phases was employed. The LC elution conditions were as follows (mobile phase A = 0.1% formic acid in water; mobile phase B = 0.1% formic acid in acetonitrile): time program: 0−5 min, isocratic at 100% A; 5−15 min, linear gradient from 0% B to 50% B; 15−17 min, linear gradient to 95% B; and 17.1−25 min, equilibration at 100% A. MRM was performed using electrospray ionization (ESI) in the positive-ion mode. Quantitation was based on the use of isotopically labeled vitamins as internal standards.

Method 14: Water-Soluble Vitamin Analysis by LC/Abs or LC with Fluorescence Detection (LC/FL) (USDA).[13] Details of the method used for the analysis of WSVs have been reported

(10) Nelson, B. C.; Sharpless, K. E.; Sander, L. C. *J. Agric. Food Chem.* **2006**, *54*, 8710–8716.
(11) Nelson, B. C. *Clin. Chem.* **2006**, *52*, A181.
(12) Nelson, B. C.; Sharpless, K. E.; Sander, L. C. *J. Chromatogr. A* **2006**, *1135*, 203–211.
(13) Chen, P.; Ozcan, M.; Wolf, W. R. *Anal. Bioanal. Chem.* **2007**, *389*, 343–347.

separately.[13] The LC conditions were similar to those described in Method 13; however, isotopically labeled internal standards were not utilized, and quantitation was based on the method of standard additions. Absorbance detection was performed at 260 nm (thiamin), 270 nm (riboflavin), 260 nm (nicotinamide), 292 nm (pyridoxine), and 280 nm (folic acid). Pyridoxine and riboflavin were also determined with fluorescence detection.

RESULTS AND DISCUSSION

The value assignment of vitamins and elements in SRM 3280 was approached through the use of multiple independent analytical methods, with measurements performed at NIST and by collaborating laboratories. Three types of data are reported for SRMs: certified concentration values, reference concentration values, and information concentration values. *Certified concentration values* are values for which NIST has the highest confidence in the accuracy of the data, and all known or suspected sources of bias have been investigated or taken into account. *Reference concentration values* are noncertified values that are the best estimate of the true values based on available data; however, estimates of measurement uncertainty may not include all sources of uncertainty or may reflect a lack of statistical agreement among methods. *Information concentration values* are usually reported without an estimate of uncertainty, and these are not recommended for quantitative comparisons. Details of the various approaches that NIST uses to assign values to SRMs have been published.[14] The use of independent methods of analysis is one of the classic NIST modes of certification. The principle is simple: agreement of the results from independent methods typically can occur only in the absence of measurement biases. Disagreement indicates that a bias exists in one or more of the methods. As an example, two orthogonal methods might use LC- and GC-based separations. It is unlikely that an interfering constituent that coeluted with the analyte by LC would coelute to the same extent with GC. Any indication of coelution with either approach would provide cause for additional investigation. Disagreement among methods (if small) may be incorporated into the expanded uncertainty or (if large and unexplained) may provide cause to exclude the analyte from certification.

As many as five datasets were combined to calculate an assigned value and associated expanded uncertainty for the vitamins and carotenoids in SRM 3280. The data used in the value assignment of the WSVs and FSVs is summarized in Table 2 for each method. Representative plots of these data are also provided in Figure 2 for α-tocopherol and riboflavin (see Figure S1 in the Supporting Information for plots of data for other analytes). Certified or reference concentration values are plotted in Figure 2 as solid horizontal lines. The expanded uncertainty is indicated with broken horizontal lines. Datasets are designated with the method number, as indicated in the Experimental Section. An example of the data treatment used to obtain certified values and expanded uncertainties is also provided in the Supporting Information and in the work of Levenson et al.[15]

Water-Soluble Vitamins. The mass fractions of WSVs were determined using two independent methods at NIST and up to three methods from collaborating laboratories. Blake recently reviewed methods for determining the presence of WSVs.[16] Although early approaches for the measurement of B vitamins relied heavily on microbiological assays, more recently instrumental LC-based methods with various modes of detection have been published.[17−22] Methods for the determination of WSVs in unfortified foods must carefully address extraction issues, because these nutrients are often bound to proteins within the matrix. Although multivitamin/multielement dietary supplements are formulated with encapsulated vitamin and mineral premixes in which single forms of the various vitamins are typically used, analytical methods must still utilize extraction approaches that achieve quantitative recovery of the analytes. In general, better sample recovery is achieved with multiple extraction cycles and with extended extraction intervals, with mechanical agitation. For greater method independence, extraction approaches were varied among the different methods used to characterize SRM 3280.

An LC-based method was developed with absorbance detection at 260 nm for thiamine, niacinamide, pyridoxine, and ascorbic acid (NIST LC/abs Method 5; see ref 9 for a description of the method). Although riboflavin (vitamin B_2) was readily separated by this method, the response for this analyte was insufficient for reliable quantitation. Levels of the B vitamins were also determined using liquid chromatography with isotope dilution mass spectrometry (LC/ID-MS; NIST Method 6).[9] The selectivity of the column used in this method differed from the column used in Method 5, and a different elution order for the B vitamins was observed. Two sets of data were contributed by USDA using LC/ID-MS (Method 13) and LC/abs or LC/fluorescence (Method 14). Data reported by CEN and GMA laboratories (Methods 11 and 12, respectively) included both microbiological and chemical assays for the B vitamins.

The best agreement among the methods for WSVs was obtained for niacinamide (vitamin B_3). Because the ultraviolet (UV) and MS responses for this vitamin are large, relative to those for the other B vitamins in SRM 3280, the challenge associated with the measurement of niacinamide is expected to be modest. Good agreement was achieved between instrumental methods and microbiological assay data reported by CEN and GMA laboratories. The expanded uncertainty represents an interval of ≈2% about the certified mass fraction value. For most of the other B vitamins, agreement among instrumental methods is very good, and the resulting expanded uncertainties range from 9% to 13%, relative to the assigned value. No trends are immediately apparent in comparisons of the accuracy or precision of the CEN and GMA microbiological

(14) May, W.; Parris, R.; Beck, C.; Fassett, J.; Greenberg, R.; Guenther, F.; Kramer, G.; Wise, S.; Gills, T.; Colbert, J.; Gettings, R.; MacDonald, B. Definitions of terms and modes used at NIST for value-assignment of reference materials for chemical measurements. National Institute of Standards and Technology. *NIST Special Publication 260-136*; U.S. Government Printing Office: Washington, DC, 2000.

(15) Levenson, M. S.; Banks, D. L.; Eberhardt, K. R.; Gill, L. M.; Guthrie, W. F.; Liu, H. K.; Vangel, M. G.; Yen, J. H.; Zhang, N. F. *J. Res. Natl. Inst. Stand. Technol.* **2000**, *105*, 571–579.

(16) Blake, C. J. *Anal. Bioanal. Chem.* **2007**, *389*, 63–76.

(17) Chen, P.; Wolf, W. R. *Anal. Bioanal. Chem.* **2007**, *387*, 2441–2448.

(18) Moreno, P.; Salvado, V. *J. Chromatogr. A* **2000**, *870*, 207–215.

(19) Li, H. B.; Chen, F.; Jiang, Y. *J. Chromatogr. A* **2000**, *891*, 243–247.

(20) Li, K. *Biomed. Chromatogr.* **2002**, *16*, 504–507.

(21) Dong, M. W.; Pace, J. L. *LC GC* **1996**, *14*, 794–803.

(22) Mittermayr, R.; Kalman, A.; Trisconi, M.; Heudi, O. *J. Chromatogr.* **2004**, *1032*, 1–6.

Table 2. Data Summary for Individual Methods Used in Value Assignment of Vitamins and Carotenoids (mg/g) in SRM 3280[a]

analyte	vitamin	certified and reference values[b]	k	5. NIST LC/abs WSV			6. NIST LC/MS WSV			11. CEN laboratories			12. GMA laboratories			13. USDA ID-LCMS			14. USDA LC/abs or FL		
				mean	s_{mean}	N	mean	s_{mean}	N	mean	s_{mean}	N	mean	s_{mean}	N	mean	s_{mean}	N	mean	s_{mean}	N
ascorbic acid	C	42.2 ± 3.7	3.15	40.9	0.3	6				41.3	1.8	4	45.8	0.5	4				41.0	0.4	5
thiamine HCl	B_1	1.06 ± 0.12	2.77	1.06	0.01	6	1.17	0.01	12	0.898	0.093	5				1.07	0.01	6	1.07	0.02	5
riboflavin	B_2	1.32 ± 0.17	3.17				1.47	0.03	12	1.30	0.07	5	1.30	0.05	3				1.21	0.03	5
niacinamide	B_3	14.1 ± 0.23	2.49	14.3	0.1	6	13.9	0.1	12	14.3	0.5	4				14.1	0.1	6	13.9	0.1	5
pantothenic acid	B_5	7.3 ± 0.96	3.17				8.13	0.04	12	6.83	0.09	3	6.87	0.11	2	7.36	0.07	6			
pyridoxine HCl	B_6	1.81 ± 0.17	2.76	1.75	0.01	6	1.86	0.01	12	1.79	0.02	4				1.64	0.02	6	2.01	0.04	5
cyanocobalamin	B_{12}	0.0049 ± 0.0019[c]	2.00							0.0055	0.0001	2	0.0043	0.0010	3						

		k	7. LC/ELSD Biotin			8. LC/ID-MS Biotin			9. LC/ID-MSMS Folate [1]			10. LC/ID-MSMS Folate [2]			13. USDA ID-LCMS			11. CEN laboratories	12. GMA laboratories
			mean	s_{mean}	N	mean	s_{mean}	N	mean	s_{mean}	N	mean	s_{mean}	N	mean	s_{mean}	N	mean	mean
folic acid	0.394 ± 0.022	2.74							0.373	0.001	12	0.385	0.001	12	0.390	0.003	12	0.420	0.400
biotin	0.0234 ± 0.0032	2.77	0.0251	0.0005	12	0.0268	0.0001	12							0.0224	0.0002	12	0.0225	0.0202

			k	1. NIST LC/abs FSV			2. NIST LC/abs Carotenoids [1]			3. NIST LC/MS FSV			4. NIST LC/abs Carotenoids [2]			11. CEN laboratories			12. GMA laboratories		
				mean	s_{mean}	N	mean	s_{mean}	N	mean	s_{mean}	N	mean	s_{mean}	N	mean	s_{mean}	N	mean	s_{mean}	N
retinol	A	0.78 ± 0.19[c]	2.36	0.803	0.004	12				0.928	0.025	6				0.659	0.021	4	0.733	0.065	4
ergocalciferol	D_2	0.00913 ± 0.00071	2.57							0.00878	0.00011	7				0.00956	0.00047	4	0.00906	0.00006	2
α-tocopherol	E	21.4 ± 3.5	2.78	21.9	0.1	12				18.7	0.9	6				22.7	1.7	4	22.1	0.9	4
phylloquinone	K	0.0228 ± 0.0022	2.36							0.0241	0.0004	7				0.0223	0.0011	3	0.0221	0.0034	2
total β-carotene[d]		0.514 ± 0.087	2.00	0.587	0.003	12	0.438	0.012	8							0.519	0.003	2			
trans-β-carotene[d]		0.42 ± 0.1	2.45	0.509	0.003	12	0.371	0.011	8							0.394	0.041	2			
cis-β-carotene[d]		0.072 ± 0.007[c]	2.06	0.0778	0.0007	12	0.0662	0.0016	8												
lutein		0.205 ± 0.05[c]	2.16	0.242	0.001	12							0.162	0.003	8	0.228	0.015	2	0.187	0.001	2

[a] Values are reported on a dry-mass basis. The standard deviation of the mean is represented by s_{mean}, whereas N is the effective number of measurements. [b] Each certified concentration value, expressed as a mass fraction on a dry-mass basis, is an equally weighted mean of the results from 2−5 analytical methods carried out at NIST and at collaborating laboratories. The uncertainty in the certified value is expressed as an expanded uncertainty (U) about the mean (x), following the ISO/NIST Guide to the Expression of Uncertainty in Measurement.[15,36,37] The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory, within-laboratory, and drying components of uncertainty. The coverage factor (k) is determined from the Student's t-distribution, corresponding to the appropriate associated degrees of freedom and ≈95% confidence for each analyte. [c] Reference value. Each reference concentration value, expressed as a mass fraction, is an equally weighted mean of the individual sets of results provided by the individual NIST methods, the mean of the CEN laboratories' data, and the mean of the GMA data, where available. The uncertainty in the reference value, calculated according to the method described in the ISO Guide,[15,36,37] is expressed as an expanded uncertainty (U). The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of within-laboratory and drying components of uncertainty. The coverage factor (k) is determined from the Student's t-distribution, corresponding to the appropriate associated degrees of freedom and ≈95% confidence for each analyte. [d] Total β-carotene includes *cis*- and *trans*-β-carotene isomers.

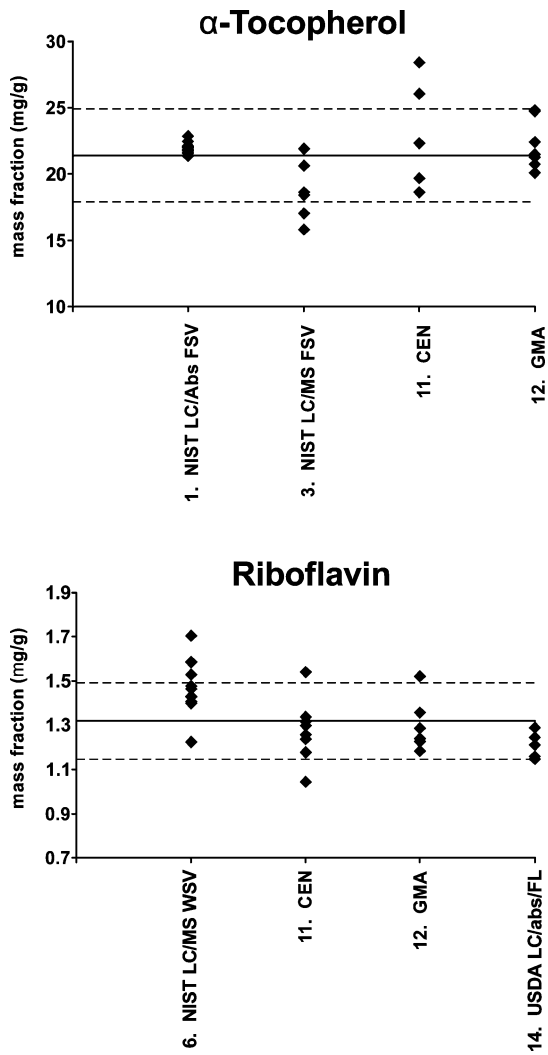

Figure 2. Individual measurements for representative fat- and water-soluble vitamins, plotted as a function of method number. The assigned value is shown as a solid line; broken lines indicate the expanded uncertainty.

data with data from instrumental methods. The relative standard deviations (RSDs) of data from a single method were quite consistent among the methods, with RSDs typically less than ≈5%. Not surprisingly, the data exhibiting the broadest range of values were the composite data from multiple laboratories.

The presence of ascorbic acid was determined with measurements by NIST and collaborating laboratories (four datasets). The methods that were used exhibit considerable independence: LC/abs with UV absorbance detection at 260 nm, LC with electrochemical detection, LC with fluorescence detection, and electrochemical titration. In principle, an LC/ID-MS method could be developed; however, high levels of ascorbic acid might make the use of a labeled internal standard at matching levels impractical. Ascorbic acid is easily oxidized, and care must be taken to maintain sample integrity during processing. Despite these inherent difficulties and potential sources of variability, excellent agreement was achieved among the methods (relative expanded uncertainty of ≈9%).

Folic acid measurements were made using ID-MS measurements at NIST and at USDA, microbiological assays at CEN and GMA laboratories, and additional LC measurements at CEN laboratories. Isotope dilution methods for the determination of folic acid have been developed by Rychlik,[23−25] Nelson et al.,[26] and Chen et al.[13] These methods are based on LC/MS or LC/MS/MS approaches, with the use of a stable isotope-labeled internal standard for quantitation. Different folate vitamers occur in unfortified samples (e.g., foods and biological fluids), and, for the best accuracy, ID-MS methods should utilize stable isotope-labeled internal standards for each of the analyte forms.[25] Because SRM 3280 is fortified with a single folate vitamer (folic acid), isotopically labeled folic acid (folic acid-[$^{13}C_5$]) was used as an internal standard in NIST and USDA measurements. Agreement among the methods was excellent; however, the levels of folic acid determined by NIST were slightly lower than those reported by USDA, CEN, and GMA laboratories. This may be due to a correction factor for moisture that was applied to the folic acid reference standard used by NIST. NIST measurements were corrected for a moisture content of ≈8% (mass fraction) water. The assigned value utilized all data provided, and the relative expanded uncertainty (≈6%) is sufficient to encompass most of the measurements.

The measurement of biotin is challenging, because of the lack of a chromophore for absorbance detection, and the low concentration level that is typical of supplement samples. Two independent methods have been developed at NIST that are applicable to the determination of biotin in dietary supplements.[10,11] An LC method with evaporative light scattering detection (Method 7) was used with an analog of biotin (desthiobiotin) as an internal standard. Sufficient selectivity and sensitivity were achieved with this method to provide good precision (RSD 6.7%). An LC/ID-MS/MS method (Method 8) provided improved sensitivity and better precision; the RSD for these measurements was 1.1%. The relative expanded uncertainty among all methods was ≈14%.

The development of instrumental methods for cyanocobalamin remains challenging, because of the low levels and difficulty in selecting an appropriate internal standard.[18,19,27−32] Efforts are in progress at NIST to develop a robust instrumental method for cyanocobalamin that will permit future certification of this constituent in SRM 3280 and other reference materials. Cyanocobalamin (B_{12}) is present in SRM 3280 at a concentration of ≈4.9 ± 1.9 μg/g. This value was determined by microbiological assays performed by 10 CEN and GMA laboratories. No measurements were made using instrumental methods, nor were any measurements carried out at NIST at the time of the

(23) Rychlik, M.; Freisleben, A. *J. Food Compos. Anal.* **2002**, *15*, 399–409.
(24) Freisleben, A.; Schieberle, P.; Rychlik, M. *Anal. Bioanal. Chem.* **2003**, *376*, 149–156.
(25) Freisleben, A.; Schieberle, P.; Rychlik, M. *Anal. Biochem.* **2003**, *315*, 247–255.
(26) Nelson, B. C.; Satterfield, M. B.; Sniegoski, L. T.; Welch, M. J. *Anal. Chem.* **2005**, *77*, 3586–3593.
(27) Chassaigne, H.; Lobinski, R. *Analyst* **1998**, *123*, 131–137.
(28) Lambert, D.; Adjalla, C.; Felden, F.; Benhayoun, S.; Nicolas, J. P.; Gueant, J. L. *J. Chromatogr.* **1992**, *608*, 311–315.
(29) Marszall, M. L.; Lebiedzinska, A.; Czarnowski, W.; Szefer, P. *J. Chromatogr. A* **2005**, *1094*, 91–98.
(30) Papadoyannis, I. N.; Tsioni, G. K.; Samanidou, V. F. *J. Liq. Chromatogr. Relat. Technol.* **1997**, *20*, 3203–3231.
(31) Vinas, P.; Campillo, N.; Lopez Garcia, I.; Hernandez Cordoba, M. *Chromatographia* **1996**, *42*, 566–570.
(32) Wongyai, S. *J. Chromatogr. A* **2000**, *870*, 217–220.

original certification of this material, and the mass fraction of cyanocobalamin in SRM 3280 is reported as a reference value.

Fat-Soluble Vitamins. Four FSVs and two carotenoids were determined in SRM 3280 (see Figure 1). As with WSV analyses, multiple independent methods were used at NIST and at collaborating laboratories. All of these methods represent instrumental approaches, and include LC/abs, LC/FL, and LC/MS. Collaborating laboratories were asked to use their usual methods of analysis, which, in many instances, included saponification as a step in the sample preparation. Saponification can release bound species to provide measures of total analyte levels; it also converts retinyl acetate to retinol, and α-tocopheryl acetate to α-tocopherol. Results reported as retinyl acetate and α-tocopheryl acetate were converted to retinol and α-tocopherol equivalents, respectively, for combination with data provided for saponified samples.

Retinyl acetate, α-tocopheryl acetate, lutein, and *β*-carotene were determined at NIST using a wavelength-programmed LC/abs method (Method 1) with *δ*-tocopherol as an internal standard.[8] Precision of the method was excellent, with relative standard deviations (RSDs) ranging from ≈2% to ≈3%. *Trans-β*-carotene was resolved from *cis-β*-carotene isomers. These species were quantified separately and then combined to calculate a concentration level representative of total *β*-carotene.

A second FSV method was developed at NIST to include measurement of retinyl acetate, ergocalciferol, α-tocopheryl acetate, and phylloquinone (Method 3).[9] This LC/MS method utilized stable isotope-labeled internal standards for the quantification of retinyl acetate, ergocalciferol, and phylloquinone (see Table S1 in the Supporting Information). Measurement precision was slightly poorer for Method 3 than for Method 1; however, in principle, the use of labeled internal standards provides added confidence in the accuracy of the method. It is interesting to compare the measurement precision obtained for α-tocopheryl acetate, which was quantified using retinyl acetate-[$^2H_6$] as an internal standard, with the other analytes, which were each quantified using corresponding labeled internal standards. The RSDs for retinyl acetate, ergocalciferol, and phylloquinone ranged from ≈3% to 6%, whereas the measurement precision for α-tocopheryol acetate was represented by an RSD of 12%. These data are consistent with other measurements reported for LC/MS methods with which labeled internal standards were not used. For example, in the certification of ephedra alkaloids in SRM 3240 *Ephedra sinica* Stapf Aerial Parts, ephedrine-[$^2H_3$] was used as an internal standard for the LC/MS determination of six analytes. The best measurement precision was obtained for ephedrine (RSD ≈ 6%), whereas the measurement precision for the other alkaloids was represented by RSD values in the range from ≈9% to ≈21%.[33]

Measurements provided by CEN and GMA laboratories for ergocalciferol and phylloquinone agreed very well with NIST data, and the the relative expanded uncertainties ranged from 8% to 10%. Somewhat-larger expanded uncertainties resulted for measurements of α-tocopheryl acetate (≈16%) and retinyl acetate (≈24%). Because of the larger-than-expected dispersion in the data for retinyl acetate, the mass fraction is reported as a reference value.

Carotenoids were measured at NIST by independent LC/abs methods. Method 1 utilized a wide-pore polymeric C_{18} column, Method 2 utilized a C_{18} column with alternate selectivity toward carotenoids, and Method 4 utilized a C_{30} column with enhanced carotenoid selectivity. Additional data were provided by CEN and GMA laboratories. The relatively large dispersion in the data for *β*-carotene and lutein is not unexpected; similar performance has been observed in intercomparison exercises of the Micronutrient Measurement Quality Assurance Program that was organized by NIST.[34]

Homogeneity and Stability. SRM 3280 is provided in sealed bottles that contain 30 tablets each. In the design of SRM 3280, whole tablets were formulated with premixes of coated or encapsulated vitamins to enhance stability and to provide a format comparable to commercial dietary supplements. Initial characterization of the material indicated sample heterogeneity between individual tablets. For the certified and reference concentration values to be valid, at least 15 tablets must be finely ground and subsamples of the ground powder analyzed. The use of smaller tablet portions for analysis may result in decreased homogeneity that is not taken into account in the uncertainty estimates.

The specified storage conditions of the SRM (i.e., unopened bottles at 20−25 °C) are based on the recommendations of the original manufacturer of the vitamin tablets. After the sealed bottles have been opened, the contents are estimated to be stable for at least 4 days, if recapped and stored properly. Upon grinding, the protective coatings are fractured, and the sample must be utilized immediately. Generally, accelerated stability studies are not carried out as part of the certification protocol at NIST. The long-term stability of SRMs are periodically assessed at NIST though stability studies and through use of the SRMs as control materials. The frequency of stability assessment is based on reported or suspected analyte instability. In cases for which constituent levels have changed, values are recertified (or removed from certification), and users are notified.

Traceability. Certain FSVs (retinyl acetate, D,L-α-tocopheryl acetate, phylloquinone) are susceptible to oxidation and/or isomerization, and care must be taken to evaluate the purity of these compounds to maintain traceability to the amount-of-substance units of the International System of Units (SI). Purity was assessed via LC and confirmed with spectrophotometry. For carotenoids (*β*-carotene and lutein), quantitation based on the masses of reference standards of known purity was not deemed to be technically feasible. Efforts to prepare, purify, characterize, and maintain these reference standards have not been successful, and a different approach is utilized by the measurement community. Calibrant concentrations are assigned based on spectrophotometry of the solutions, with corrections made for purity. Purity determinations are performed via LC with detection at the maximum absorbance wavelength. Measurement traceability for such analytes is ultimately based on the absorption coefficient used in the assignment of calibrant concentrations rather than the amount-of-substance units of the SI. High-purity reference standards were available for WSVs, and mass fractions of calibrants were corrected after assessment of the purity. Measurement of these compounds is traceable to the amount-of-substance

(33) Sander, L. C.; Sharpless, K. E.; Satterfield, M. B.; Ihara, T.; Phinney, K. W.; Yen, J. H.; Wise, S. A.; Gay, M. L.; Lam, J. W.; McCooeye, M.; Gardner, G.; Fraser, C.; Sturgeon, R.; Roman, M. *Anal. Chem.* **2005**, *77*, 3101–3112.

(34) Duewer, D. L.; Kline, M.; Sharpless, K. E.; Brown Thomas, J. M.; Stacewicz-Sapuntzakis, M.; Sowell, A. L. *Anal. Chem.* **2000**, *72*, 3611–3619.

units of the SI based on the masses of measurands, purity assessments, and appropriate uncertainties.

CONCLUSIONS

Standard Reference Material SRM 3280 is provided by NIST as part of a continuing effort to support the chemical metrology of foods and dietary supplements. This Standard Reference Material is intended primarily for use as a control material and in the development of new analytical methods. This material may be useful to analysts and manufacturers of dietary supplements to improve measurement precision and accuracy, assist in the establishment of traceability, and improve the quality of information provided on product labels.[35]

(35) Sharpless, K. E.; Duewer, D. L. *J. AOAC Int.* **2008**, *91*, 1298–1302.

(36) *Guide to the Expression of Uncertainty in Measurement*, 1st Edition; International Organization for Standardization (ISO): Geneva, Switzerland, 1993. (ISBN No. 92-67-10188-9.).

(37) Taylor, B. N.; Kuyatt, C. E. *Guidelines for Evaluating and Expressing Uncertainty of National Institute of Standards and Technology Measurements Results*. National Institute of Standards and Technology (NIST) Technical Note 1297; U.S. Government Printing Office: Washington, DC, 1994.

SUPPORTING INFORMATION AVAILABLE

Individual measurements for vitamins and carotenoids (Figure S1), and tables describing the ingredients, standards, and analytical methods used by CEN and GMA laboratories with SRM 3280 (Tables S1−S4). This material is available free of charge via the Internet at http://pubs.acs.org.

ACKNOWLEDGMENT

Partial funding for this work was provided by the National Institutes of Health, Office of Dietary Supplements. Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology or other governmental agency, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Received for review July 23, 2010. Accepted October 29, 2010.

AC101953U