National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 3280

Multivitamin/Multielement Tablets

This Standard Reference Material (SRM) is intended primarily for use in validating analytical methods for the determination of vitamins, carotenoids, and elements in dietary supplement tablets and similar matrices. This SRM can also be used for quality assurance when assigning values to in-house control materials. A unit of SRM 3280 consists of five bottles, each containing 30 tablets. The SRM is provided as whole tablets because some of the vitamins are coated or encapsulated to provide stability and grinding would compromise this coating. Each tablet weighs approximately 1.5 g.

The development of SRM 3280 was a collaboration between the National Institute of Standards and Technology (NIST) and the National Institutes of Health (NIH), Office of Dietary Supplements (ODS).

Values were derived from the combination of results provided by NIST and collaborating laboratories. The certified and reference values in this material are the equally weighted means of the individual sets of NIST results and the means of the individual sets of measurements made by collaborating laboratories, as available. The associated uncertainties are expanded uncertainties at the 95 % level of confidence, as described below [1–4]. Values are reported on a dry-mass basis in mass fraction units [5].

Certified Mass Fraction Values: The certified mass fraction values of selected vitamins, carotenoids, and elements are provided in Tables 1 and 2. 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 have been investigated or taken into account [6].

Reference Mass Fraction Values: Reference mass fraction values for additional vitamins, carotenoids, and elements are provided in Tables 3 and 4. Reference values are noncertified values that are the best estimate of the true values based on available data; however, the values do not meet the NIST criteria for certification [6] and are provided with associated uncertainties 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.

Expiration of Certification: The certification of **SRM 3280** is valid, within the measurement uncertainty specified, until **31 October 2021**, provided the SRM is handled and stored in accordance with instructions given in this certificate (see "Warning and Instructions for Storage and Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet or register online) will facilitate notification.

Support for the development of SRM 3280 was provided in part by the NIH-ODS. Technical consultation was provided by J.M. Betz of NIH-ODS.

Coordination of the technical measurements leading to the certification of this SRM was performed by L.C. Sander and S.A. Wise of the NIST Chemical Sciences Division and K.E. Sharpless of the Special Programs Office. Acquisition of the material was coordinated by K.E. Sharpless.

Statistical analysis was provided by J.H. Yen of the NIST Statistical Engineering Division.

Carlos A. Gonzalez, Chief
Chemical Sciences Division

Steven J. Choquette, Director
Office of Reference Materials

Gaithersburg, MD 20899

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Analytical measurements from the NIST Chemical Sciences Division were performed by C.Q. Burdette, S.J. Christopher, D. Cleveland, R.D. Day, C.G. Jongsma, S.E. Long, E.A. Mackey, A.F. Marlow, B.C. Nelson, R.L. Paul, K.W. Phinney, B.J. Porter, C.A. Rimmer, J.R. Sieber, R.O. Spatz, J.B. Thomas, R.Q. Thompson, L.J. Wood, L.L. Yu, and R. Zeisler.

Analytical measurements from the U.S. Department of Agriculture (USDA, Beltsville, MD) were performed by R. Atkinson, P. Chen, and R. Goldschmidt under the direction of W.R. Wolf and E. Greene under the direction of J. Harnly.

Laboratories participating in a European Committee for Standardization (CEN) interlaboratory comparison exercise that provided results are: Danish Institute for Food and Veterinary Research, Søborg, Denmark; DSM Nutritional Products, Research and Development Analytical Research Center, Kaiseraugst, Switzerland; Food and Consumer Product Safety Authority (VWA), Eindhoven, The Netherlands; Nestlé Research Center, Quality and Safety Assurance Department, Lausanne, Switzerland; Swedish National Food Administration, Research and Development Department, Uppsala, Sweden.

Laboratories participating in an interlaboratory comparison exercise organized by the Grocery Manufacturers Association (GMA) Food Industry Analytical Chemists Committee (FIACC) that provided results are: Campbell Soup Company, Camden, NJ; Covance, Madison, WI; General Mills, Inc., James Ford Bell Technical Center, Golden Valley, MN; Krueger Food Laboratories, Inc., Billerica, MA; Novartis Nutrition Corporation, St. Louis Park, MN. The GMA FIACC interlaboratory comparison exercise was coordinated by I-P. Ho of GMA (Washington, DC).

Support aspects involved with the issuance of this SRM were coordinated through the NIST Office of Reference Materials.

WARNING AND INSTRUCTIONS FOR STORAGE AND USE

Warning: For research use. Not for human consumption. Individual tablets should not be analyzed because of tablet-to-tablet variability. The variation of measured element mass fractions from tablet to tablet ranges from approximately 15 % to 25 %, therefore instructions for use (below) must be followed.

Storage: The material should be stored at controlled room temperature (20 °C to 25 °C), in an unopened bottle, until required for use. Freshly ground powder was observed to gain an average of 0.075 % of its original mass in 3 h after grinding. After 24 h, the average gain was 0.11 % at 44 % relative humidity and a temperature of 21 °C. Vitamins are stable for at least 4 d following opening of the bottle; some vitamins have been observed to be unstable in ground material but this instability has not been fully investigated. Use of a freshly ground portion for vitamin analyses is recommended.

Use: At least 15 tablets must be ground to obtain a homogeneous sample prior to removal of a test portion for analysis. NIST analysts used two methods to grind tablets to a powder prior to analysis: (1) thirty tablets were ground in a disk mill, which involved shaking in an orbital pattern for 6 min, (2) batches of 15, 20, or 30 tablets were ground for 10 min using an automated mortar and pestle. (Note that 6 min of shaking in a disk mill did not grind the tablets, particularly the coating material, as finely as did the other technique.) For certified values to be valid, test portions of the powder equal to or greater than 0.6 g to 2 g for carotenoids and fat-soluble vitamins, 0.3 g to 2 g for water-soluble vitamins, and 0.25 g to 4.5 g for elements should be used. Test portions should be analyzed as received and results converted to a dry-mass basis by determining moisture content (described below) on a separate test portion.

PREPARATION AND ANALYSIS[1]

Material Acquisition and Preparation: A manufacturer of multivitamin/multielement tablets prepared a non-commercial batch of tablets according to their normal procedure. SRM 3280 is a direct-compression tablet formulation produced by blending a vitamin and a mineral pre-mix with the remaining bulk of the formulation, compression, and tablet film coating. Fat-soluble vitamins and carotenoids (retinyl acetate, β-carotene, lutein, ergocalciferol, and dl-α-tocopheryl acetate) were added as gelatin beadlets. The film coating consisted of triethyl citrate, polysorbate 80, yellow #6 aluminum-lake, hypromellose, and titanium dioxide.

Analytical Approach for Determination of Vitamins and Carotenoids: Value assignment of the mass fractions of the vitamins and carotenoids in SRM 3280 was based on the combination of measurements from several different analytical methods at NIST and USDA and in two interlaboratory comparison exercises involving the GMA FIACC

(1)Certain commercial equipment, instruments, or materials are identified in this certificate 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.

and CEN laboratories [7]. NIST provided measurements by using a combination of liquid chromatography (LC) methods with different detection methods, i.e., evaporative light scattering detection (ELSD) and mass spectrometry (MS), positive-ion and negative-ion MS/MS, and absorbance detection (abs), as described below. USDA used LC/abs, LC/fluorescence, and LC/MS for measurement of water-soluble vitamins. GMA and CEN laboratories used their usual methods. Methods used for measurement of vitamins and carotenoids are listed in Table A1.

NIST Analyses for Carotenoids and Fat-Soluble Vitamins: Vitamin E (as α-tocopheryl acetate) and vitamin K (phylloquinone or phytonadione) were measured by using a combination of LC/abs and LC/MS methods, and carotenoids (lutein, *cis*-β-carotene, and *trans*-β-carotene) were measured by using two LC/abs methods [8,9]; vitamin A (measured as retinyl acetate and reported as retinol equivalents) and vitamin D_2 (ergocalciferol) were measured at NIST using LC/MS [9]. Calibrants were prepared gravimetrically at levels intended to approximate the levels of the analytes in the SRM. Internal standards were employed; a single solution was used for the calibrants and samples.

LC/MS Detection for Measurement of Retinyl Acetate, α-Tocopheryl Acetate, Ergocalciferol, and Phylloquinone (Phytonadione). Tablets were ground as described above in "Warning and Instructions for Storage and Use". Single 0.6 g portions of the powder from each of six bottles were combined with ethylenediaminetetracetic acid (EDTA) solution and held at 45 °C to dissolve the gel encapsulation of some of the fat-soluble vitamins. An internal standard solution containing retinyl acetate-d_6, vitamin K_1-d_4, or vitamin D_2-d_3, was added and samples were placed in an ultrasonicating bath for 10 min. Analytes were extracted into hexane by shaking overnight. The extraction was repeated five times. An isocratic LC method with a methanol/acetonitrile (ACN)/ammonium acetate mobile phase and a polymeric C_{18} column were used for LC/MS determination of the fat-soluble vitamins. The separation was monitored at 287 nm, and MS was used for quantitation. A typical separation is provided in Appendix B [9].

LC/abs for Measurement of Carotenoids, Retinyl Acetate, and α-Tocopheryl Acetate – Method 1. Tablets were ground as described above. Two 2 g portions of the powder from each of six bottles were combined with dilute hydrochloric acid and were placed in a 37 °C ultrasonicating bath for about 25 min with intermittent manual shaking. An internal standard solution (δ-tocopherol) was added to each sample, and samples were returned to the ultrasonicating bath for an additional 5 min. Analytes were extracted into hexane by shaking overnight. At least three subsequent extractions into hexane were performed (1 h on the shaker) until the organic layer was colorless. Hexane layers were combined, and 10 mL extract was removed, evaporated to dryness under nitrogen, and reconstituted in ethanol containing butylated hydroxytoluene (BHT). An isocratic LC method with a methanol/triethylamine (TEA)/ACN mobile phase and a polymeric C_{18} column held at 25 °C were used for the determination of carotenoids, retinyl acetate, and α-tocopheryl acetate, with absorbance measured at 450 nm, 325 nm, and 284 nm, respectively. The absorbance of the internal standard, δ-tocopherol, was measured at 284 nm. A typical separation is provided in Appendix B [8]. (Note that this method was used for determination of the mass fraction of retinyl acetate and its homogeneity on the original certificate dated 14 January 2009. Results obtained by using this method were not used when the reference value for retinol was updated in 2011.)

LC/abs Detection for Measurement of Carotenoids – Methods 2a and 2b. Tablets were ground as described above. Approximately 0.6 g portions of the powder from each of eight bottles were combined with an EDTA solution and an internal standard solution, containing either *trans*-β-apo-8'-carotenal or *trans*-β-apo-10'-carotenal oxime, and held at 45 °C to dissolve gel encapsulation. Analytes were then extracted into hexane for 60 min five times, and the extracts were combined. For Method 2a, a C_{30} carotenoid column and water/ammonium acetate/acetone gradient were used for the determination of lutein with absorbance measured at 450 nm. For Method 2b, a C_{18} column and an ACN/methanol (containing ammonium acetate)/ethyl acetate gradient were used for the determination of *cis*- and *trans*-β-carotene at 450 nm. Typical separations are provided in Appendix B [8].

NIST Analyses for Water-Soluble Vitamins: Water-soluble vitamins, including vitamin B_1 (thiamine HCl), vitamin B_2 (riboflavin), vitamin B_3 (niacinamide), vitamin B_5 (pantothenic acid), vitamin B_6 (pyridoxine HCl), folic acid, biotin, cyanocobalamin, and vitamin C (ascorbic acid), were measured by using combinations of two LC methods with absorbance detection, ELSD, MS, MS/MS, or inductively coupled plasma mass spectrometry (ICP-MS). Calibrants were prepared gravimetrically at levels intended to approximate the levels of the vitamins in the SRM. In cases where an internal standard was employed, a single solution was used for the calibrants and samples.

LC/abs for Analysis of Vitamins B_1, B_2, B_6, C, and Niacinamide. Tablets were ground as described above in "Warning and Instructions for Storage and Use". Vitamins B_1, B_2, B_6, C, and niacinamide were measured by LC/abs in single 2 g test portions taken from each of six bottles. Test portions were combined with HCl, 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. A gradient LC method with potassium phosphate dibasic buffer and ACN and a C_{18} column were used with absorbance detection at 260 nm for vitamins B_1, B_6, C, and niacinamide, and at 266 nm for vitamin B_2. A typical separation is provided in Appendix C.

LC/MS for Analysis of Vitamins B_1, B_2, B_6, Niacinamide, and Pantothenic Acid. Vitamins B_1, B_2, B_6, niacinamide, and pantothenic acid were measured by LC/MS in two 0.25 g test portions taken from each of six bottles. Four internal standards were added: $^{13}C_3$-thiamine chloride, $^{2}H_4$-niacinamide, $^{13}C_3$,^{15}N-calcium pantothenate, and $^{13}C_4$-pyridoxine HCl. 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 determination of B_1, B_2, B_6, niacinamide, and pantothenic acid. Transitions were measured at: m/z 265 for thiamine, m/z 268 for $^{13}C_3$-thiamine, m/z 123 for niacinamide, m/z 127 for $^{2}H_4$-niacinamide, m/z 220 for pantothenic acid, m/z 224 for $^{13}C_3$,^{15}N-pantothenic acid, m/z 170 for pyridoxine and m/z 174 for $^{13}C_4$-pyridoxine. The transition for riboflavin was measured at m/z 377, with $^{13}C_4$-pyridoxine as the internal standard. A typical separation is provided in Appendix C [9].

Negative-Ion Mode LC/MS/MS for Analysis of Folic Acid. Tablets were ground as described above. Folic acid measurements were made on two 0.3 g test portions taken from each of six bottles. An internal standard, $^{13}C_5$-folic acid, was added. The internal standard and folic acid were extracted into water containing dithiothreitol and ammonium hydroxide for negative-ion mode LC/MS/MS. A gradient LC method with a water/methanol/acetic acid mobile phase and a pentafluorylphenyl column were used. The transitions at m/z 440 → m/z 311 (folic acid) and m/z 445 → m/z 311 ($^{13}C_5$-folic acid) were monitored [10].

Positive-Ion Mode LC/MS/MS for Analysis of Folic Acid. Tablets were ground as described above. Folic acid measurements were made on two 0.3 g test portions taken from each of six bottles. An internal standard, $^{13}C_5$-folic acid, was added. The internal standard and folic acid were extracted into a methanol/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 transitions at m/z 442 → m/z 295 (folic acid) and m/z 447 → m/z 295 ($^{13}C_5$-folic acid) were monitored [10].

LC/MS for Analysis of Biotin. Tablets were ground as described above. Biotin was measured in two 1.5 g test portions taken from each of six bottles. $^{2}H_2$-biotin was added as an internal standard, and the analytes were extracted into methanol [11]. An isocratic LC method with a water/methanol/formic acid mobile phase and a C_{18} column were used. Transitions for biotin and $^{2}H_2$-biotin were measured at m/z 245 and m/z 247, respectively.

LC/ELSD for Analysis of Biotin. Tablets were ground as described above. Biotin was measured in two 1.5 g test portions taken from each of six bottles. Desthiobiotin was added as an internal standard, and the analytes were extracted into an aqueous formic acid solution. An isocratic LC method with a water/methanol/formic acid mobile phase and a cyanopropyl column were used for LC/ELSD determination of biotin.

LC/ICP-MS for Analysis of Cyanocobalamin. Tablets were ground as described above. Cyanocobalamin was measured in 4.5 g test portions taken from each of ten bottles. Water-soluble vitamins were extracted into water, and gallium was added as an internal standard. An isocratic LC method with a methanol/water mobile phase and a C_{18} column were used for separation of cyanocobalamin from cobalt and other extracted constituents. Cyanocobalamin was measured as cobalt using LC/ICP-MS.

Analytical Approach for Determination of Elements: Value assignment of the mass fractions of the elements in SRM 3280 was based on the combination of measurements from several different analytical methods at NIST and in an interlaboratory comparison exercise involving GMA FIACC laboratories. NIST provided measurements by using prompt gamma activation analysis (PGAA), instrumental neutron activation analysis (INAA), radiochemical neutron activation analysis (RNAA), X-ray fluorescence spectrometry (XRF), and inductively coupled plasma spectrometry with optical emission (ICP-OES) or ICP-MS detection with isotope dilution (ID) in some cases. GMA laboratories used their usual methods. Table A2 lists the methods used for measurement of the elements.

NIST Analyses for As, B, Cd, Cu, Hg, I, K, Mo, Ni, P, Pb, Se, Sn, V, and Zn by Using ICP-OES and ICP-MS: Tablets were ground as described above in “Warning and Instructions for Storage and Use”. Copper, molybdenum, nickel, phosphorus, potassium, vanadium, and zinc were measured by ICP-OES in duplicate test portions (0.35 g to 0.4 g) taken from each of six bottles of SRM 3280. Samples for ICP-OES analysis were digested in Teflon beakers in nitric, perchloric, and hydrofluoric acids. Arsenic, boron, iodine, nickel, selenium, and tin were measured by ICP-MS in single test portions (0.25 g to 0.45 g) taken from each of six or ten bottles. Except for samples in which iodine was measured, samples for ICP-MS and ID-ICP-MS analyses were digested in microwave systems; samples in which arsenic, boron, nickel, selenium, and tin were measured were digested in nitric and hydrofluoric acids, samples in which cadmium was measured were digested in nitric and hydrofluoric acids, and samples in which lead was measured were digested in nitric acid. Samples in which iodine was measured were digested in an alkaline solution of sodium hydroxide and sodium sulfite and were measured by ICP-MS with standard additions. Cadmium was isolated from interferences (Mo and Sn, in particular) by solid-phase extraction and measured by ID-ICP-MS in collision-cell mode in single test portions (0.25 g) taken from each of ten bottles. Lead was measured by ID-ICP-MS in single test portions (0.5 g) taken from each of six bottles. Mercury was measured by using ID cold vapor ICP-MS (ID-CV-ICP-MS) in two 0.5 g test portions taken from a single bottle. Samples for mercury analysis were digested in a mixture of nitric acid and hydrogen peroxide. The mass fraction of mercury was too low for quantitation. Quantitation for ICP-OES and non-ID-ICP-MS analyses was based on the method of standard additions.

NIST Analyses for Ca, Co, Cr, Cu, Fe, I, La, Mg, Mn, Mo, Na, Sb, Se, V, and Zn by Using INAA: Tablets were ground as described above, and antimony, calcium, cobalt, chromium, copper, iodine, iron, lanthanum, magnesium, manganese, molybdenum, selenium, sodium, vanadium, and zinc were measured using INAA. Individual disks were prepared from 0.2 g test portions taken from each of eight bottles; a duplicate was prepared from one of the bottles. Disks were formed using a stainless steel die and hydraulic press. Standards were prepared by transferring a weighed portion of a solution containing a known amount of each element onto filter papers or from pure elements or compounds of known purity. For analysis of short-lived nuclides (calcium, copper, iodine, magnesium, manganese, sodium, and vanadium), samples, standards, and controls were packaged individually in clean polyethylene bags and irradiated individually at 20 MW. For analysis of long-lived nuclides (antimony, cobalt, chromium, iron, lanthanum, molybdenum, selenium, and zinc), samples, standards, and controls were irradiated for 3 h; irradiation capsules were then inverted 180º, and materials were irradiated another 3 h. Short-lived nuclides were counted for 5 min after a 2 min decay and again for 20 min following a 15 min decay. For the long-lived irradiations, a 4 h count followed a 5 d decay and an 8 h count followed a 25 d decay.

NIST Analyses for B, Cl, Cu, Fe, K, and Ti by Using PGAA: Tablets were ground as described above, and boron, chlorine, copper, iron, potassium, and titanium were measured by using PGAA. Individual disks were formed from 0.75 g test portions taken from each of six bottles and duplicate test portions taken from each of two bottles of the SRM. Disks were formed using a stainless steel die and hydraulic press. Standards were prepared by transferring a weighed portion of a solution containing a known amount of each element onto filter papers. Disks were formed from the dried filter papers. Samples, standards, and controls were packaged individually in clean polyethylene bags and irradiated individually at 20 MW, which provided a neutron fluence rate of 3.0×10^{8} $cm^{-2}s^{-1}$. The following γ-ray lines were used for quantitation: 477 keV line from ^{10}B (corrected for ^{10}B in the background and for ^{23}N at 472 keV from the sample), 770 keV line from ^{39}K, 6111 keV line from ^{35}Cl, 341 keV and 1381 keV lines from ^{48}Ti, 278 keV line from ^{63}Cu, and 352 keV line from ^{56}Fe.

NIST Analyses for As by Using RNAA: Tablets were ground as described above, and arsenic was measured by using RNAA. Individual disks were formed from 0.2 g test portions taken from each of five bottles of the SRM. Disks were formed using a stainless steel die and hydraulic press. Standards were prepared by transferring a weighed portion of a solution containing a known amount of arsenic onto filter papers. Disks were formed from the dried filter papers. Samples, standards, and controls were packaged individually in clean polyethylene bags and irradiated in one polyethylene irradiation vessel for 2 h at 20 MW, which provided a neutron fluence rate of 1.0×10^{14} $cm^{-2}s^{-1}$. Samples and controls were combined with ^{77}As tracer and digested in nitric and perchloric acids. Arsenic was sequestered on hydrated manganese dioxide resins, which were then counted. The 559 keV line from decay of ^{76}As was used for quantitation. The 239 keV line from decay of ^{77}As was evaluated for yield determination.

NIST Analyses for Ca, Cr, Fe, K, Mg, Mn, Mo, P, Si, and Sr by Using XRF: Tablets were ground as described above, and calcium, chromium, iron, magnesium, manganese, molybdenum, phosphorus, potassium, silicon, and strontium were measured by using XRF in duplicate or triplicate test portions of 4.5 g taken from each of six bottles. Samples were prepared by borate fusion, and samples were cast as 40 mm diameter beads. (Average loss on fusion at 975 °C was 53.2 % of the average (as-received) mass.) The K-$L_{2,3}$ characteristic X-ray lines of calcium, chromium, iron, magnesium, manganese, molybdenum, phosphorus, potassium, silicon, and strontium were used for quantitation. Sample beads were bracketed with at least four synthetic standards for calibration [12,13].

Determination of Moisture: Moisture content of SRM 3280 was determined at NIST in ground tablets (see "Warning and Instructions for Storage and Use") by (1) freeze-drying to constant mass over 8 d; (2) drying over magnesium perchlorate in a desiccator at room temperature for 5 d, 7 d, 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, that are provided in this certificate.

Homogeneity Assessment: The homogeneity of carotenoids, retinol, and α-tocopheryl acetate was assessed at NIST by using the LC/abs method described above. The homogeneity of vitamins B_1, B_2, B_6, niacinamide, and pantothenic acid was assessed at NIST by using the LC/MS method described above. The homogeneity of folic acid and biotin was assessed at NIST using both value-assignment methods described above. The homogeneity of various elements was assessed at NIST by using ICP-OES, INAA, PGAA, and XRF. Analysis of variance did not show inhomogeneity for the test portions analyzed. All measurands were treated as though they were homogeneously distributed, although homogeneity of all measurands was not assessed.

Certified Mass Fraction Values for Vitamins, Carotenoids, and Elements: Each certified mass fraction value, expressed on a dry-mass basis, is an equally weighted mean of the individual sets of results provided by the individual NIST methods, individual means of two USDA methods, the mean of the CEN laboratories' data, and the mean of the GMA data, where available. The uncertainty in the certified value, calculated according to the method described in the ISO/JCGM Guide and its Supplement 1 [1–4], 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 between-laboratory, within-laboratory, and drying components of uncertainty. The coverage factor k corresponds to approximately 95 % confidence for each analyte. The measurand is the total mass fractions of each vitamin, carotenoid, and element in Tables 1 and 2. Metrological traceability to the SI derived unit for mass fraction (expressed as milligrams per gram, micrograms per gram, or nanograms per gram as noted in the tables).

Table 1. Certified Mass Fraction Values for Vitamins and Selected Carotenoids in SRM 3280

	Mass Fraction (mg/g)	Coverage Factor, k
α-Tocopherol[a,b,c,d,e]	21.4 ± 3.5	2.78
Ascorbic acid[b,d,e,g]	42.2 ± 3.7	3.15
Thiamine hydrochloride[b,c,d,f,g]	1.06 ± 0.12	2.77
Riboflavin[b,c,e,h]	1.32 ± 0.17	3.17
Niacinamide[b,c,d,f,g]	14.10 ± 0.23	2.49
Pantothenic acid[c,d,e,g]	7.30 ± 0.96	3.17
Pyridoxine hydrochloride[b,c,d,g,h]	1.81 ± 0.17	2.76
	Mass Fraction (µg/g)	**Coverage Factor, k**
Folic acid[c,d,e,g]	394 ± 22	2.74
Cyanocobalamin[d,e,i]	4.8 ± 1.0	2.00
Biotin[c,d,e,g,j]	23.4 ± 3.2	2.77
Phylloquinone[c,d,e]	22.8 ± 2.2	2.36
Trans-β-carotene[b,d]	420 ± 100	2.45
Total β-carotene[b,d]	514 ± 87	2.00

[a] α-Tocopherol was added to SRM 3280 as α-tocopheryl acetate. The certified value is expressed as α-tocopherol equivalents.
[b] LC/abs (NIST)
[c] LC/MS (NIST)
[d] CEN
[e] GMA
[f] LC/abs (USDA)
[g] LC/MS (USDA)
[h] LC/fluorescence (USDA)
[i] LC/ICP-MS (NIST)
[j] LC/ELSD (NIST)

Table 2. Certified Mass Fraction Values for Selected Elements in SRM 3280

	Mass Fraction (mg/g)			Coverage Factor, *k*
Boron (B)[a,b]	0.141	±	0.007	2.36
Calcium (Ca)[c,d,e]	110.7	±	5.3	2.45
Chloride (Cl)[b,e]	53.0	±	2.3	2.45
Copper (Cu)[b,c,e,f]	1.40	±	0.17	2.12
Iodine (I)[a,c]	0.1327	±	0.0066	2.23
Iron (Fe)[b,c,d,e]	12.35	±	0.91	3.18
Magnesium (Mg)[c,d,e]	67.8	±	4.0	2.31
Manganese (Mn)[c,d,e]	1.44	±	0.11	2.57
Phosphorus (P)[d,e,f]	75.7	±	3.2	2.16
Potassium (K)[b,d,e,f]	53.1	±	7.0	2.02
Zinc (Zn)[c,e,f]	10.15	±	0.81	2.00

	Mass Fraction (µg/g)			Coverage Factor, *k*
Arsenic (As)[a,g]	0.132	±	0.044	2.00
Chromium (Cr)[c,d]	93.7	±	2.7	2.06
Lead (Pb)[h]	0.2727	±	0.0024	2.14
Molybdenum (Mo)[c,d,f]	70.7	±	4.5	2.57
Nickel (Ni)[a,e]	8.43	±	0.30	2.00
Selenium (Se)[a,c]	17.42	±	0.45	2.00

	Mass Fraction (ng/g)			Coverage Factor, *k*
Cadmium (Cd)[h]	80.15	±	0.86	2.03

[a] ICP-MS (NIST)
[b] PGAA (NIST)
[c] INAA (NIST)
[d] XRF (NIST)
[e] GMA
[f] ICP-OES (NIST)
[g] RNAA (NIST)
[h] ID ICP-MS (NIST)

Reference Mass Fraction Values for Vitamins A and D, Carotenoids and Elements: Reference mass fraction values for carotenoids and elements, expressed on a dry-mass basis, are equally weighted means 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 values, calculated according to the method described in the ISO/JCGM Guide [1–3], 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 approximately 95 % confidence for each analyte. The measurand is the mass fraction of each vitamin, carotenoid, and element listed in Tables 3 and 4, as determined by the methods indicated. Metrological traceability to the SI derived unit for mass fraction (expressed as micrograms per gram).

The reference mass fraction value for vitamin A is expressed on a dry-mass basis as retinol equivalents and is the mean of NIST results provided using LC/MS. The uncertainty in the reference value, calculated according to the method described in the ISO/JCGM Guide [1], is expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U = ku_c$, where u_c incorporates within-method uncertainty and components for the use of two different retinyl acetate absorptivities (1535 dL/g cm and 1560 dL/g cm) and for spectrophotometric measurement of the calibrants, and k is a coverage factor corresponding to approximately 95 % level of confidence.

The reference mass fraction value for vitamin D, expressed on a dry-mass basis, is the mean of NIST results provided using LC/MS. The uncertainty in the reference value is expressed as an expanded uncertainty, U calculated as $U = ku_c$, where u_c incorporates within-method uncertainty and a component for inhomogeneity, consistent with the ISO/JCGM Guide and its Supplement 1 [1,3,4], and k is a coverage factor corresponding to approximately 95 % level of confidence. The value for vitamin D was redetermined in 2013 following questions about its stability. The variability in measurements had increased since the original characterization, and a component for heterogeneity is now included in the assigned value.

Table 3. Reference Mass Fraction Values for Vitamins A and D and Selected Carotenoids in SRM 3280

	Mass Fraction (µg/g)	Coverage Factor, k
Retinol[(a,b)]	444 ± 46	2.00
Lutein[(c,d,e)]	205 ± 50	2.16
Cis-β-carotene[(c)]	72 ± 7	2.06
Ergocalciferol[(b)]	8.6 ± 2.6	2.00

[(a)] Retinol was added to SRM 3280 as retinyl acetate. The reference value is expressed as retinol equivalents.
[(b)] LC/MS (NIST)
[(c)] LC/abs (NIST
[(d)] CEN
[(e)] GMA

Table 4. Reference Mass Fraction Values for Selected Elements in SRM 3280

	Mass Fraction (µg/g)	Coverage Factor, k
Antimony (Sb) [(a)]	0.159 ± 0.008	2.30
Cobalt (Co)[(a)]	0.81 ± 0.01	2.22
Lanthanum (La)[(a)]	0.70 ± 0.01	2.23
Silicon (Si)[(b)]	2010 ± 10	2.00
Sodium (Na)[(a)]	330 ± 20	2.36
Strontium (Sr)[(b)]	29.8 ± 0.2	2.00
Tin (Sn)[(c)]	11.1 ± 0.9	2.56
Titanium (Ti)[(d)]	5400 ± 300	2.25
Vanadium (V)[(a,e)]	8 ± 2	2.00

[(a)] INAA (NIST)
[(b)] XRF (NIST)
[(c)] ICP-MS (NIST)
[(d)] PGAA (NIST)
[(e)] ICP-OES (NIST)

REFERENCES

[1] 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/utils/common/documents/jcgm/JCGM_100_2008_E.pdf (accessed Aug 2019); 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 Aug 2019).

[2] 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.; *An Approach to Combining Results From Multiple Methods Motivated by the ISO GUM;* J. Res. Natl. Inst. Stand. Technol., Vol. 105, pp. 571–579 (2000).

[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/utils/common/documents/jcgm/JCGM_101_2008_E.pdf (accessed Aug 2019).

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

[5] Thompson, A.; Taylor, B.N.; Guide for the Use of the International System of Units (SI); NIST Special Publication 811; U.S. Government Printing Office: Washington, DC (2008); available at https://www.nist.gov/pml/pubs/sp811/index.cfm (accessed Aug 2019).

[6] May, W.; Parris, R.; Beck II, C.; Fassett, J.; Greenberg, R.; Guenther, F.; Kramer, G.; Wise, S.; Gills, T.; Colbert, J.; Gettings, R.; MacDonald, B.; Definition of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements; NIST Special Publication 260-136 U.S. Government Printing Office: Washington, DC (2000); available at https://www.nist.gov/srm/upload/SP260-136.PDF (accessed Aug 2019).

[7] Sander, L.C.; Sharpless, K.E.; Wise, S.A.; Long, S.E.; Mackey, E.A.; Malow, A.F.; Nelson, B.C.; Phinney, K.W.; Porter, B.J.; Rimmer, C.A.; Sieber, J.R.; Spatz, R.O.; Thomas, J.B.; Turk, G.C.; Wood, L.J.; Yu, L.L.; Zeisler, R.; Yen, J.H.; Duewer, D.L.; Atkinson, R.; Chen, P.; Goldschmidt, R.; Greene, E.; Harnly, J.; Wolf, W.R.; Ho, I-P.; Betz, J.M.; *Certification of SRM 3280 Multivitamin/Multielement Tablets*; Anal. Chem., Vol. 83, pp. 99–108 (2011).

[8] Phinney, K.W.; Rimmer, C.A.; Thomas, J.B.; 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., Vol. 83, pp. 92–98 (2011).

[9] Thomas, J.B.; Sharpless, K.E.; Yen, J.H.; Rimmer, C.A.; *Determination of Fat-Soluble Vitamins and Carotenoids in Standard Reference Material 3280 Multivitamin/Multi-element Tablets by Liquid Chromatography with Absorbance Detection*, J. AOAC Intl., Vol. 94, pp. 815–822 (2011).

[10] Nelson, B.C.; Sharpless, K.E.; Sander, L.C.; *Quantitative Determination of Folic Acid in Multivitamin/ Multielement Tablets Using Liquid Chromatography/Tandem Mass Spectrometry;* J. Chromatogr. A., Vol. 1135, pp. 203 (2006).

[11] Nelson, B.C.; Sharpless, K.E.; Sander, L.C.; *Improved Liquid Chromatography Methods for the Separation and Quantification of Biotin in Multivitamin/Multielement Tablets*; J. Agric. Food Chem., Vol. 54, pp. 8710–8715 (2006).

[12] Sieber, J.R.; *Matrix-Independent XRF Methods for Certification of Standard Reference Materials*; Adv. X-Ray Anal., Vol. 45, pp. 493–504 (2002).

[13] Sieber, J.; Broton, D.; Fales, C.; Leigh, S.; MacDonald, B.; Marlow, A.; Nettles, S.; Yen, J.; *Standard Reference Materials for Cements*; Cem. Concr. Res., Vol. 32, pp. 1899–1906 (2002).

Certificate Revision History: 01 August 2019 (Change of expiration date; editorial changes); (**11 May 2016** (Change of expiration date; editorial changes); **05 August 2014** (Extension of certification period; editorial changes); **31 July 2013** (Changed Vitamin D_2 value from certified to reference; editorial changes); **11 February 2013** (Corrected Table 1 footnotes for ascorbic acid; editorial changes); **09 May 2012** (Corrected Table 1 footnotes for ascorbic acid and riboflavin, Table 2 footnotes for iron and molybdenum, Table 3 footnote for *Cis*-β-carotene, Table 4 footnote for vanadium; editorial changes); **31 October 2011** (Addition to the description of preparation and analysis of ICP-MS I samples); **12 September 2011** (Correction to the units for Cd in Table 2 and units in Table 4); **17 June 2011** (Addition of certified values for As, Cd, and Pb; change of reference values to certified values for Ni, Se, and Vitamin B_{12}; change in the reference value for retinol; editorial changes); **14 January 2009** (Original certificate date).

Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 948-3730; e-mail srminfo@nist.gov; or via the Internet at https://www.nist.gov/srm.

APPENDIX A

Table A1. Summary of CEN and GMA FIACC Analytical Methods Used for Measurement of Vitamins and Carotenoids.

	Methods
Retinol	Saponification - RPLC/abs (3), Saponification - NPLC/abs (5), Extraction - RPLC/abs (1),Extraction - NPLC/abs (1)
Ergocalciferol (Vitamin D_2)	Saponification - RPLC/abs (4), Saponification - NPLC/abs (1), Extraction - NPLC/abs (1), Saponification - RPLC/NPLC/abs (1)
α-Tocopherol (Vitamin E)	Saponification - NPLC/fluorescence (3), Saponification - NPLC/abs (1), Extraction - RPLC/abs (1)
Phylloquinone (Phytonadione, Vitamin K)	RPLC/reduction - fluorescence (4), Enzymatic reduction - fluorescence (1)
β-carotene	Extraction - RPLC/abs (3)
Lutein	Saponification - RPLC/abs (1), Extraction - RPLC/abs (1), Extraction - NPLC/abs (1)
Ascorbic acid (Vitamin C)	Abs (1), Fluorescence (4), RPLC/electrochemical (1), Electrochemical titration (1)
Thiamine HCl (Vitamin B_1 HCl)	Digestion - fluorescence (1), Extraction - RPLC/abs (1), Extraction - RPLC/fluorescence (2)
Riboflavin (Vitamin B_2)	Digestion - fluorescence (3), Extraction - RPLC/abs (1) Extraction - RPLC/fluorescence (3)
Niacin (Niacinamide)	Microbiological (2), Extraction - RPLC/abs (1)
Pantothenic Acid (Vitamin B_5)	Microbiological (3), RPLC/abs (1), RPLC/MS (1)
Pyridoxine HCl (Vitamin B_6 HCl)	Microbiological (1), Extraction - RPLC/fluorescence (2)
Biotin (Vitamin B_7)	Microbiological (3), RPLC/abs (1), RPLC/MS (1)
Folic acid (Vitamin B_9)	Microbiological (4), RPLC/abs (2)
Cyanocobalamin (Vitamin B_{12})	Microbiological (4)

NOTE: RPLC: Reversed-phase LC, NPLC: Normal-phase LC. Number in () corresponds to number of laboratories using the method.

Table A2. Summary of GMA FIACC Analytical Methods Used for Measurement of Elements.

Element	Methods
Calcium	ICP-OES (4), FAAS (1)
Chloride	Potentiometric titration (2)
Copper	ICP-OES (3), FAAS (1)
Iron	ICP-OES (4), FAAS (1)
Magnesium	ICP-OES (4), FAAS (1)
Manganese	ICP-OES (3), FAAS (1)
Phosphorus	ICP-OES (3), Colorimetry (1)
Potassium	ICP-OES (4), FAAS (1)
Zinc	ICP-OES (4), FAAS (1)

NOTE: Number in () corresponds to number of laboratories using the method.

APPENDIX B

Chromatograms of fat-soluble vitamins and carotenoids by LC/MS (Figure B1) and LC/abs Method 1 (Figure B2) and Method 2 (Figure B3).

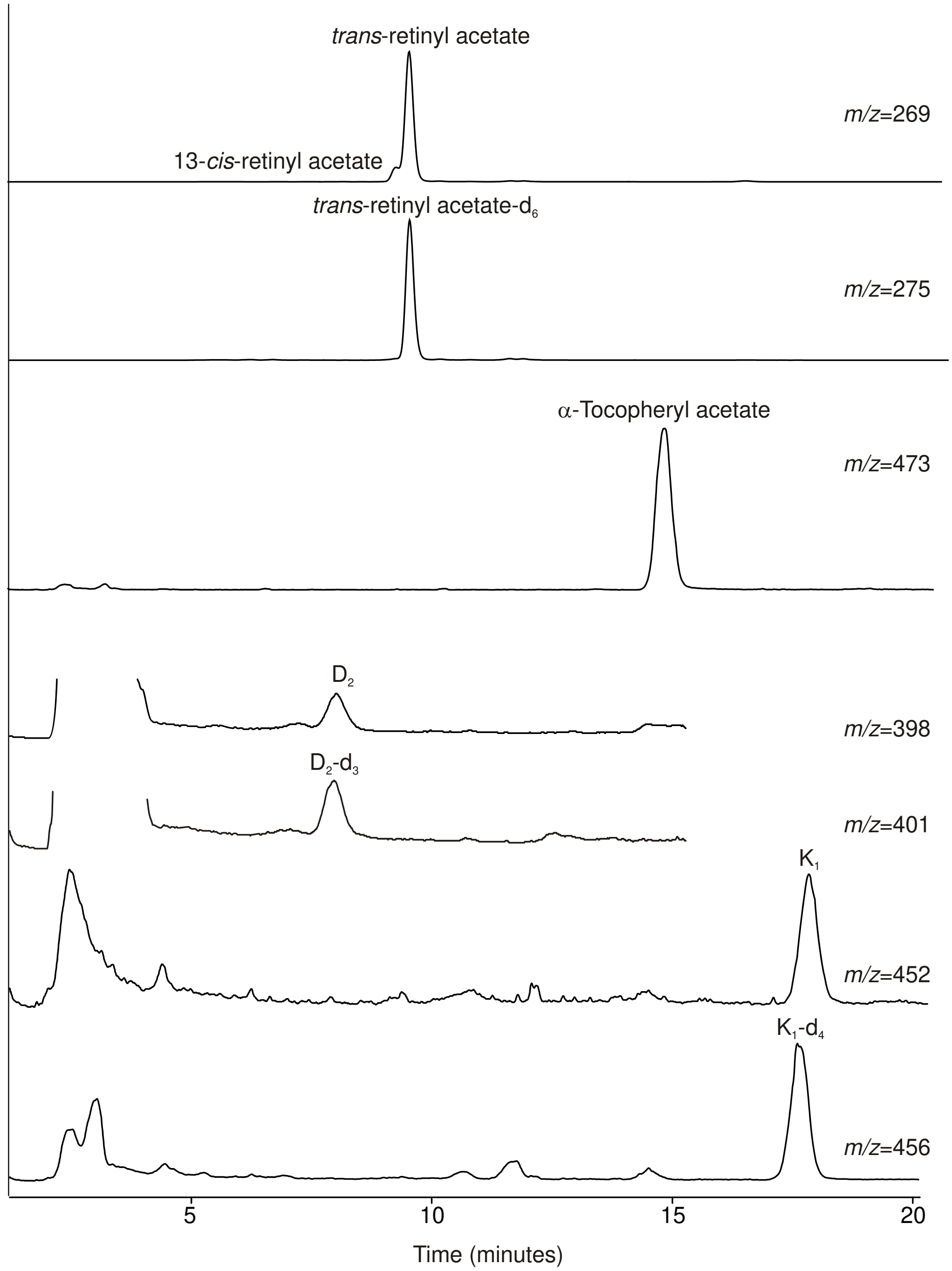

Figure B1. Chromatographic conditions for LC/MS were as follows: A 15 cm ACE C_{18} column (Advanced Chromatography Technologies, Aberdeen, Scotland) was held at 25 °C. The isocratic mobile phase consisted of methanol: ACN (40 %:60 % volume fractions) containing 5 mmol/L ammonium acetate at a flow rate of 1 mL/min. MS detection conditions were as follows: nebulizer pressure 240 kPa (35 psi), fragmentor 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.

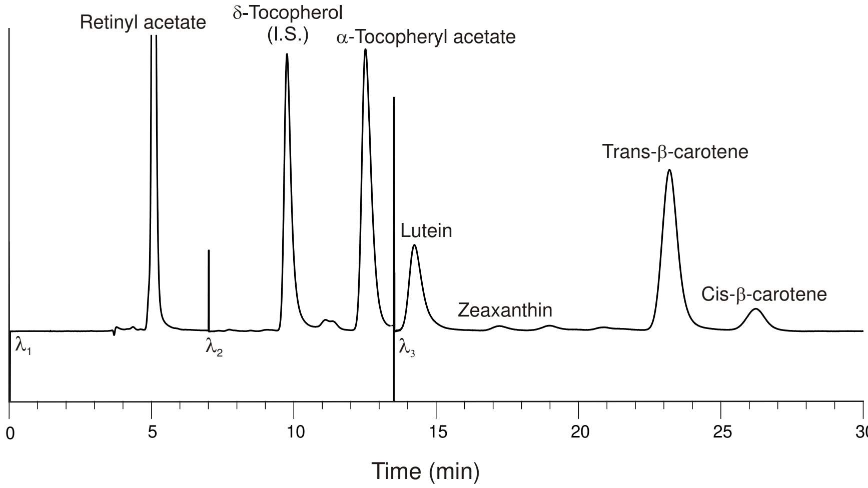

Figure B2. Chromatographic conditions for LC/abs Method 1. Polymeric C_{18} column (Vydac 201TP; The Separations Group, Hesperia, CA) was held at 25 ºC and a mobile phase consisting of 96 % ACN/4 % methanol containing 0.05 % TEA at a flow rate of 0.8 mL/min. Absorbance was measured at 325 nm (retinyl acetate), 284 nm (tocopherols), and 450 nm (carotenoids).

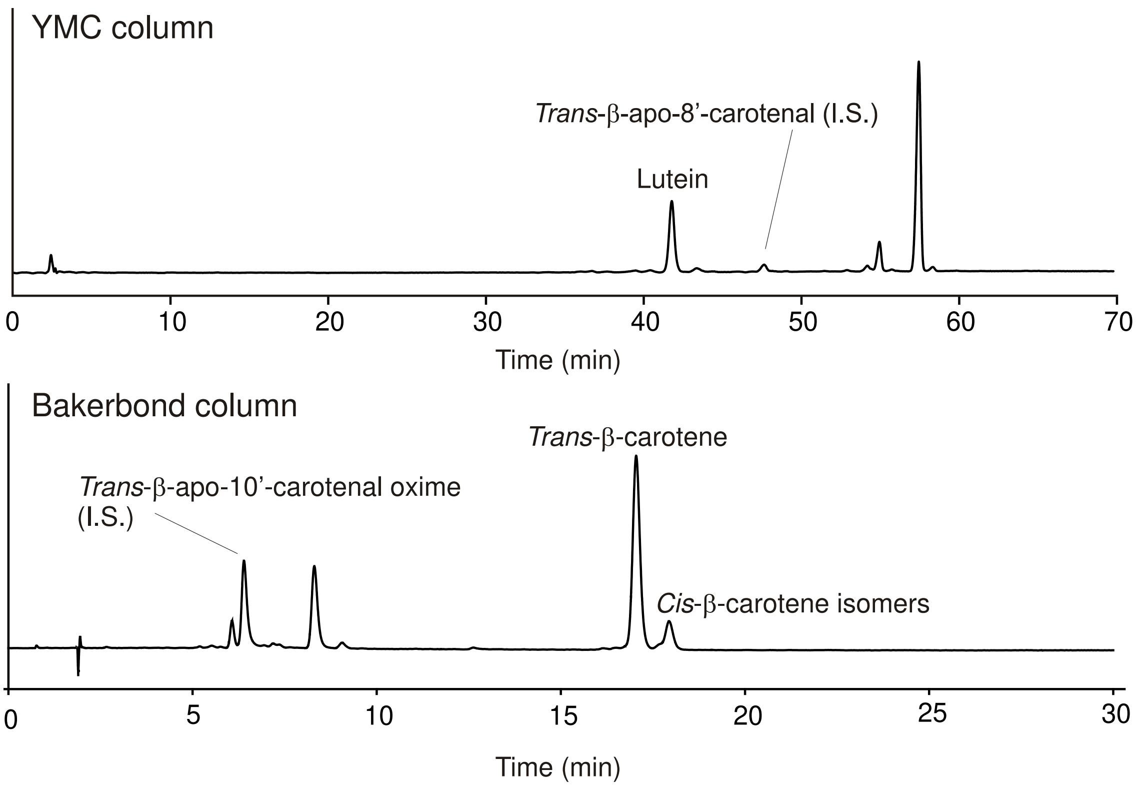

Figure B3. Chromatographic conditions for LC/abs Methods 2a and 2b: For Method 2a, on the YMC C_{30} carotenoid column (Waters Corporation, Milford, MA), the separation was performed using a gradient of 40 % solvent A (2 mmol/L ammonium acetate in water) and 60 % solvent B (acetone) to 100 % solvent B (volume fractions) at a flow rate of 1 mL/min. Column temperature was 25 °C, and absorbance detection was at 450 nm. For Method 2b, on the Bakerbond C_{18} column (J.T. Baker, Phillipsburg, NJ), a ternary solvent method was used. Solvent A was ACN, solvent B was methanol containing 0.05 mol/L ammonium acetate, and solvent C was ethyl acetate. Each of the three solvents contained a volume fraction of 0.05 % 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, column temperature was 29 °C, and absorbance detection was at 450 nm.

APPENDIX C

Chromatograms of water-soluble vitamins by LC/MS (Figure C1) and LC/abs Method 1 (Figure C2). Peak identities: B_1 = thiamine hydrochloride, B_2 = riboflavin, B_3 = niacinamide, B_5 = pantothenic acid, B_6 = pyridoxine hydrochloride, C = ascorbic acid, and 4-PA = 4-pyridoxic acid (internal standard).

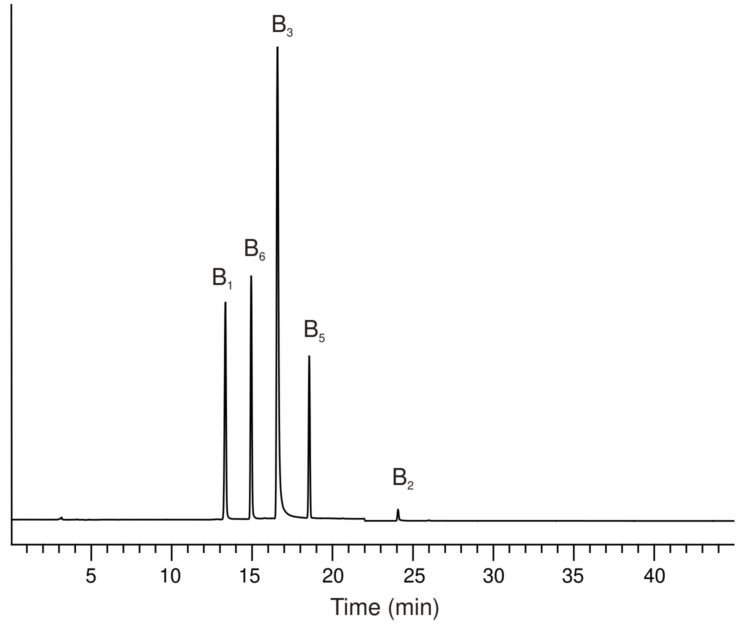

Figure C1. Chromatograms of water-soluble vitamins by LC/MS: A 25 cm Cadenza CD-C_{18} column (Silvertone Sciences, Philadelphia, PA) was held at 22 °C. The gradient mobile phase consisted of methanol and an aqueous solution of 20 mmol/L ammonium formate (pH 4.0) at a flow rate of 0.8 mL/min. MS detection conditions were as follows: nebulizer pressure, 350 kPa (50 psig); fragmentor, 110 V; drying gas temperature, 350 °C; drying gas flow rate, 13 L/min; and capillary voltage, 4000 V.

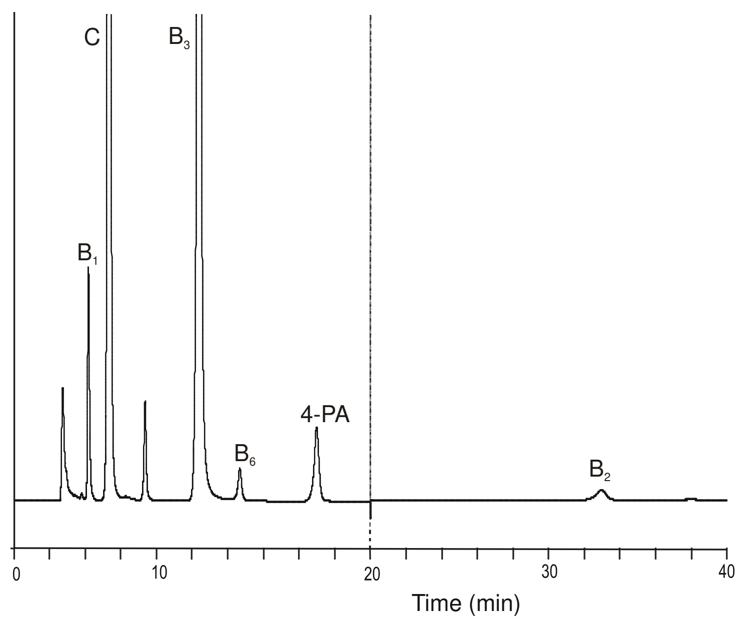

Figure C2. Chromatographic conditions for LC/abs: Pro C_{18} column (YMC, Waters, Milford, MA) held at 26 ºC and a gradient mobile of 0.02 mol/L potassium phosphate dibasic (pH 3.1) as solvent A and ACN as solvent B) at a flow rate of 1 mL/min. Solvent A was held constant at 100 % for 20 min, then conditions were changed to 75 % A and 25 % B over 5 min. These conditions were held constant for an additional 15 min. Absorbance was measured at 260 nm for vitamins B_1, B_3, B_6, and C and at 266 nm for vitamin B_2.