DIETARY SUPPLEMENTS

Preparation and Characterization of a Suite of Ephedra-Containing Standard Reference Materials

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The National Institute of Standards and Technology, the U.S. Food and Drug Administration, Center for Drug Evaluation and Research and Center for Food Safety and Applied Nutrition, and the National Institutes of Health, Office of Dietary Supplements, are collaborating to produce a series of Standard Reference Materials (SRMs) for dietary supplements. A suite of ephedra materials is the first in the series, and this paper describes the acquisition, preparation, and value assignment of these materials: SRMs 3240 *Ephedra sinica* Stapf Aerial Parts, 3241 *E. sinica* Stapf Native Extract, 3242 *E. sinica* Stapf Commercial Extract, 3243 Ephedra-Containing Solid Oral Dosage Form, and 3244 Ephedra-Containing Protein Powder. Values are assigned for ephedrine alkaloids and toxic elements in all 5 materials. Values are assigned for other analytes (e.g., caffeine, nutrient elements, proximates, etc.) in some of the materials, as appropriate. Materials in this suite of SRMs are intended for use as primary control materials when values are assigned to in-house (secondary) control materials and for validation of analytical methods for the measurement of alkaloids, toxic elements, and, in the case of SRM 3244, nutrients in similar materials.

Ephedra-containing products once represented a large share of the U.S. market for dietary supplements (1) until concerns about their safety were raised, and they were ruled as adulterated by the U.S. Food and Drug Administration (FDA) in February 2004 (2). Before the ruling in 2004, FDA had concerns about the safety of ephedra-containing products and had proposed a regulation in 1997 to set dosage limits (3, 4). In late 2001, FDA began working with the National Institute of Standards and Technology (NIST) and the National Institutes of Health's Office of Dietary Supplements (NIH/ODS) to produce a suite of 5 ephedra-containing Standard Reference Materials (SRMs) against which analytical methods could be validated and the accuracy of analytical results could be judged (5). This paper describes the acquisition of materials for preparation and value assignment of the SRMs in the suite: SRMs 3240 *Ephedra sinica* Stapf Aerial Parts, 3241 *E. sinica* Stapf Native Extract, 3242 *E. sinica* Stapf Commercial Extract, 3243 Ephedra-Containing Solid Oral Dosage Form, and 3244 Ephedra-Containing Protein Powder. Materials in this suite of SRMs are intended for use as primary control materials when values are assigned to in-house (secondary) control materials and for validation of analytical methods for the measurement of alkaloids, toxic elements, and, in the case of SRM 3244, nutrients in similar materials. Analytical methods used by NIST and by collaborating laboratories participating in the value-assignment process for alkaloids in this suite of materials have been described elsewhere (6–10). Complete details of the value assignment process for the ephedrine alkaloids are provided by Sander et al. (8). Assigned values for toxic and some other elements (Cr in SRM 3243, nutrients in SRM 3244) were determined by using results obtained by NIST and by collaborating laboratories. Assigned values for additional elements, proximates, fatty acids, and amino acids in SRM 3244 were obtained by using data provided solely by the collaborating laboratories. Collaborating laboratories included the National Research Council Canada (NRCC; Ottawa, Canada), FDA's Center for Food Safety and Applied Nutrition (CFSAN; College Park, MD), ChromaDex, Inc. (Clearwater, FL), and the Food Products Association (FPA; formerly the National Food Processors Association, Washington, DC) Food Industry Analytical Chemists Subcommittee (FIACS).

Values for this suite of SRMs were assigned through measurements by NIST and collaborating laboratories and were designated as certified, reference, or information. (The value assignment scheme is depicted in Figure 1.) A NIST-certified value is a value for which NIST has the highest confidence of its accuracy in that all known or suspected sources of bias have been fully investigated or taken into account (11); certified values were provided for analytes that were measured by both NIST and the collaborating laboratories. NIST reference values represent a best estimate of the true value for which all known or suspected sources of bias have not been fully investigated; reference values have associated uncertainties that may not include all sources of uncertainty and may represent only a measure of the precision of the measurement method(s) (11). Reference values may be assigned if no NIST data are available, or if sources of bias in NIST measurements have not been fully resolved (11); reference values for these materials were provided for analytes measured only by the collaborating laboratories or for which there is less assurance as to analyte integrity or

Received April 12, 2006. Accepted by AP May 12, 2006.
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Certain commercial products are identified to specify adequately the experimental procedure. Such identification does not imply endorsement or recommendation by the National Institute of Standards and Technology, nor does it imply that the materials identified are necessarily the best available for the purpose.

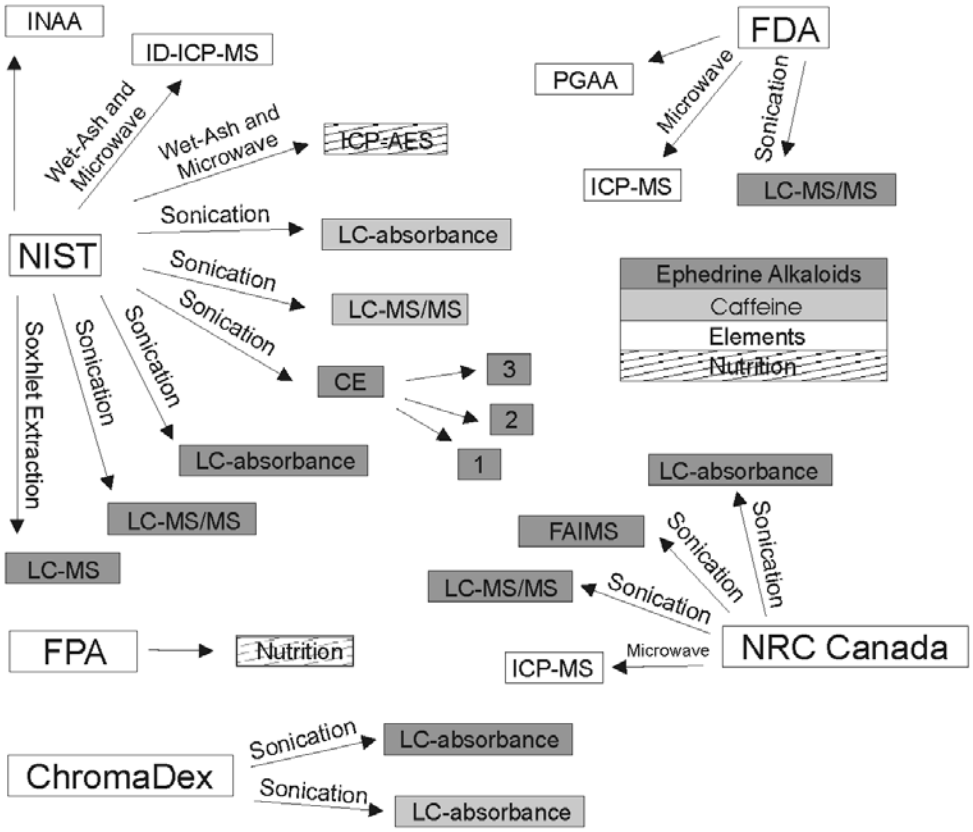

Figure 1. Schematic diagram of the value assignment process for the ephedra-containing SRMs.

greater variability in results. NIST information values may be provided for analytes that may be of interest to the SRM user, but for which insufficient information is available to assign the uncertainty associated with the value (and therefore, typically, no uncertainty is reported; 11); information values were provided for 3 low-level ephedrine alkaloids in SRM 3244.

Source Materials and Processing

Sufficient material to prepare nominally 12 500 bottles of each of the 5 SRMs was acquired as follows:

A single year's harvest of *E. sinica* from a single field in China was acquired in 2002 from Jinke Group USA, Inc. (Diamond Bar, CA) through Modern Nutrition and Biotech (Appleton, WI). The crop was examined by a Chinese taxonomist (Xian-Chun Zhang, Institute of Botany, Chinese Academy of Sciences, Beijing, People's Republic of China) who verified its identity; voucher specimens were collected at time of flower and shipped with the dried botanical following harvest in the same year. The herbarium sheets were deposited at FDA's herbarium (CFSAN; FDA Accession No. 1221) and the Missouri Botanical Garden (St. Louis, MO; Herbarium sheet No. 5827116; http://www.mobot.org/MOBOT/research/diversity/herbarium/compendium_model.aspx?id=3; click on *Ephedra sinica* Specimen 1). While still in China, the plant material (aerial parts) was dried, powdered, sieved to 177 μm (80 mesh), and sterilized by using a 6 kGy dose of ^{60}Co. Approximately 100 kg dried powdered plant material was shipped to NIST in 5 drums and processed as the powdered botanical raw material (SRM 3240). A prebottling analysis of the 5 drums of material indicated that the material was not uniformly blended. The material was blended by combining it in 2 drums—one containing the contents of 3 of the original drums and the other containing the contents of 2. These drums were rolled; then the material was transferred from drum 1 to drum 2 and rolled again; material was transferred from drum 2 to drum 1 and rolled, etc. Following this process, analysis for ephedrine showed that the material was sufficiently blended at the 1.5 g level.

While still in China, a portion of the plant material was extracted with hot water under pressure, and the resulting extract was used in the production of the "native extract" and the "commercial extract". The water extract was filtered, concentrated, and spray-dried to produce the native extract (SRM 3241), which is a 14-fold concentrate of the plant material. A second portion of the water extract was filtered, concentrated, and then fortified with ephedrine to yield nominally 8% total ephedrine alkaloids before spray drying to produce the commercial extract (SRM 3242). Approximately 15 kg of each of the extracts was shipped to NIST. Prebottling analyses for ephedrine indicated that the contents of the 2 individual drums

containing each of the 2 extracts were uniform at the 280 mg level, and no additional blending was performed.

Both SRM 3243 Ephedra-Containing Solid Oral Dosage Form and SRM 3244 Ephedra-Containing Protein Powder were prepared from several brands of commercially available products that were purchased in the U.S. marketplace. To obtain material from several production lots of each brand, materials were purchased from more than one vendor. Materials for SRM 3243 were ground and sieved at NIST. A 36.0 cm diameter Teflon disk mill set was operated at room temperature. Ephedra-containing tablets were arranged around the inside of the mill, which contained a concentric Teflon ring and a Teflon puck (12). The mill was placed on a shaker for 6 min, and the tablets were ground to a fine powder. Individual brands of materials were ground separately and were sieved by using a 177 μm (80 mesh) stainless steel sieve on an automatic shaker. Material from each brand that did not pass through the 177 μm sieve was reground by following the same protocol as described above. Some component materials of SRM 3243 were purchased in capsule form; the capsules were emptied, and the capsule contents were sieved as described above. Materials that did not pass through the sieve were ground and sieved again. Materials were ground/reground a total of 3 times. Component materials for SRMs 3243 and 3244 were separately blended for 20 min by using a V-blender; prebottling analyses for ephedrine indicated that the materials were uniformly blended at the 1.5 and 10 g levels, respectively.

Bottling

The suite of SRMs was bottled by using a Micro 109 bottling apparatus (Actionpac Scales, Ventura, CA), which used a vibrating hopper to fill the bottles. Bottles were flushed with nitrogen before introduction of the SRMs. SRM 3240 (5.1 ± 0.1 g) was packaged in 30 mL amber high-density polyethylene bottles with polypropylene screw caps. SRMs 3241 and 3242 (1.3 ± 0.1 g) were packaged in 7.5 mL bottles of the same type; SRM 3243 (2.6 ± 0.1 g) was packaged in bottles of the same size. SRM 3244 (12.6 ± 0.1 g) was packaged in a 30 mL bottle of the same type. The bottling operation—filling and capping the bottles—was a manual operation.

Irradiation

As noted above, SRM 3240 plant material was irradiated while still in China by using a 6 kGy dose of ^{60}Co. After bottling, all 5 materials were irradiated. The absorbed dose for SRMs 3240, 3241, and 3242 was 12.8–15.4 kGy. The absorbed dose for SRMs 3243 and 3244 was 12.5–15.7 kGy.

Moisture Assessment

The moisture content of each of the 5 materials was determined in order to report assigned concentration values on a dry-mass basis. Moisture was determined by drying in a desiccator over magnesium perchlorate for 5 d (SRMs 3240 and 3244), 17 d (SRMs 3241 and 3242), and 35 d (SRM 3243); drying in a forced-air oven for 4 h at 85°C (all 5 materials); and freeze-drying for 7 d (SRMs 3240, 3243, and 3244) or 11 d (SRMs 3241 and 3242) until samples reached constant mass. Unweighted results obtained by using all 3 techniques were averaged to convert NIST and FPA FIACS data from an as-received to a dry-mass basis. Other collaborating laboratories converted their data to a dry-mass basis by using their own moisture determinations.

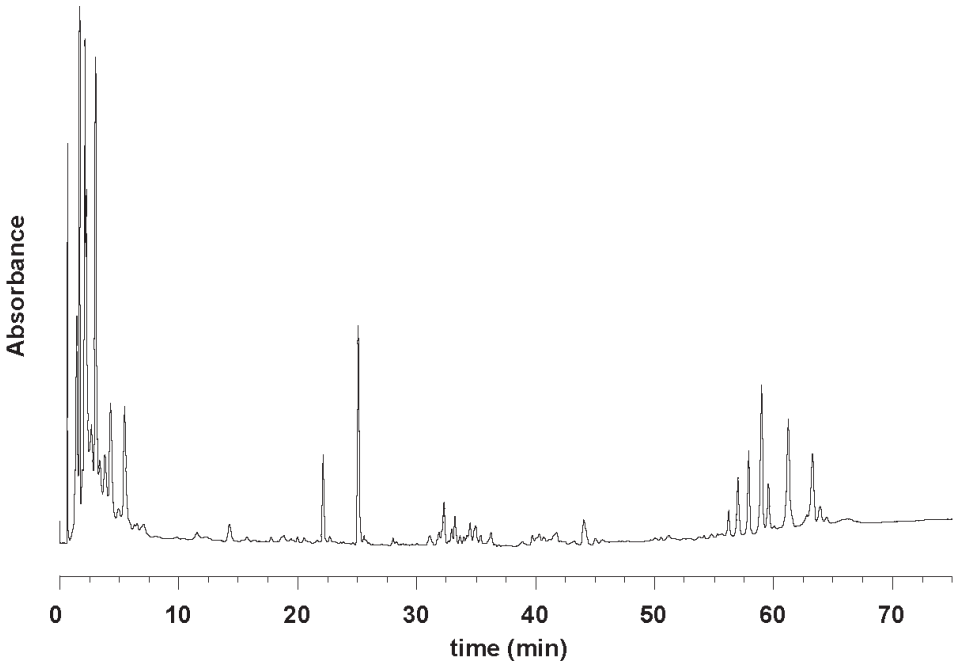

Figure 2. "Fingerprint" chromatogram from the analysis of SRM 3240 *E. sinica* Stapf Aerial Parts.

Table 1. Certified[a] and reference[b] concentration values for alkaloids in the suite of ephedra SRMs

	Mass fraction, mg/g				
Alkaloid	SRM 3240	SRM 3241	SRM 3242	SRM 3243	SRM 3244
Ephedrine	11.31 ± 0.76[a]	28.86 ± 1.17[a]	78.1 ± 2.3[a]	11.21 ± 0.42[a]	0.242 ± 0.038[a]
Methylephedrine	1.18 ± 0.14[a]	2.61 ± 0.51[a]	2.77 ± 0.57[a]	0.323 ± 0.031[a]	0.0075 ± 0.0024[a]
Pseudoephedrine	3.53 ± 0.26[a]	10.74 ± 1.11[a]	9.27 ± 0.94[a]	2.81 ± 0.11[a]	0.0361 ± 0.0086[a]
Methylpseudoephedrine	0.046 ± 0.015[b]	0.11 ± 0.09[b]	0.124 ± 0.044[b]	0.020 ± 0.011[b]	0.00028 ± 0.00011[a]
Norephedrine	0.44 ± 0.09[b]	0.48 ± 0.20[b]	0.57 ± 0.18[b]	0.160 ± 0.026[b]	0.0030[b,c]
Norpseudoephedrine	0.65 ± 0.14[b]	0.44 ±0.17[b]	0.40 ± 0.16[b]	0.186 ± 0.029[b]	0.0034[b,c]
Total ephedrine alkaloids	17.0 ± 1.2[a]	43.3 ± 2.7[a]	91.2 ± 2.0[a]	14.78 ± 0.54[a]	0.296 ± 0.067[a]
Synephrine				0.54 ± 0.19[a,d]	
Caffeine				76.5 ± 4.1[a,e]	2.99 ± 0.54[a,d]
Theobromine					0.762 ± 0.026[b,f]
Theophylline					0.080 ± 0.003[b,f]

[a,b] Each certified (a) and reference (b) concentration value, expressed as a mass fraction on a dry-mass basis, is an equally weighted mean of results from 8 or 9 analytical methods performed at NIST and at collaborating laboratories; *see* footnotes d and e and ref. 8 for details of the methods. The uncertainty in the assigned values, calculated according to the method described in the ISO Guide (18–20), is expressed as an expanded uncertainty.

[c] Information value only.

[d] Certified concentration values for synephrine and caffeine were based on results from 4 and 3 analytical methods, respectively.

[e] Expanded uncertainty includes a contribution of 1.6% due to inhomogeneity.

[f] Reference concentration values for theobromine and theophylline were based on results from a single analytical method.

Value Assignment

Analytical Approach for Determination of Ephedrine Alkaloids

(*Note*: The methods listed do not replace current official methods used for enforcement purposes.)

Value assignment of concentrations of ephedrine alkaloids in the 5 materials was based on the combination of measurements from different analytical methods at NIST and at 3 collaborating laboratories (NRCC, FDA, and ChromaDex) and is fully described elsewhere (8). A total of 9 sets of measurements was used for value assignment of the concentrations of ephedrine alkaloids. NIST provided measurements by using a combination of 2 sample extraction procedures and 3 liquid chromatography (LC) methods with different detection techniques, i.e., ultraviolet (UV) absorbance spectrometry, mass spectrometry (MS), tandem mass spectrometry (MS/MS), and capillary electrophoresis (CE). NRCC provided results from 3 analytical methods: LC/UV, LC/MS/MS, and high-field asymmetric waveform ion mobility spectrometry (FAIMS). (FAIMS is a new MS technique that provides results without using a chromatographic separation; 7.) FDA results were based on LC/MS/MS (6), and ChromaDex results were based on LC/UV (9). Two collaborating laboratories analyzed a minimum of 6 subsamples, one from each of 6 bottles or 2 from each of 3 bottles; one laboratory analyzed one subsample from 3 bottles of each of the 5 materials.

Analytical Approach for Determination of Caffeine, Synephrine, Theobromine, and Theophylline

Value assignment of concentrations of synephrine in SRM 3243 was based on the combination of measurements from 2 different analytical methods at NIST and at 2 collaborating laboratories. Synephrine was determined at NIST by using LC/MS/MS and LC/MS, at FDA by using LC/MS/MS, and at ChromaDex by using LC/UV.

Value assignment of concentrations of caffeine in SRMs 3243 and 3244 was based on the combination of measurements from 2 analytical methods at NIST and the method of one collaborating laboratory. Caffeine was determined at NIST by using LC/UV (13) and LC/MS/MS and at ChromaDex by using LC/UV. Theobromine and theophylline were determined at NIST in the same LC/UV analyses in which caffeine was determined (13).

Analytical Approach for Determination of Trace Elements

Elements of primary interest for the 5 materials were the potentially toxic contaminants arsenic, cadmium, lead, and mercury. Value assignment of the concentrations of toxic trace elements in the materials was based on the combination of measurements at NIST obtained by using a single analytical method and results from one or 2 collaborating laboratories (NRCC and FDA). At NIST, instrumental neutron activation analysis (INAA) was used for the determination of arsenic; isotope dilution (ID) inductively coupled plasma mass

Table 2. Comparison of certified[a] and reference[b] values with values permitted by NSF/ANSI Standard 173 (22), in units of mg/kg[c]

SRM	As	Cd	Hg	Pb[d]
3240	0.265 ± 0.016[a,e,f]	0.0906 ± 0.0039[a,g–i]	0.0167 ± 0.0005[a,h,j]	
3241	1.285 ± 0.081[a,e,f]	0.0587 ± 0.0036[a,g–i]	0.00383 ± 0.00029[a,h,j]	0.241 ± 0.012[a,g–i]
3242	1.030 ± 0.033[a,e,f]	0.0538 ± 0.0032[a,g–i]	0.00418 ± 0.00042[a,h,j]	0.362 ± 0.014[a,g–i]
Raw material NTE	5[b]	0.3[b]	0.2[b]	10[b]
3243	0.554 ± 0.018[a,e,f]	0.1218 ± 0.0033[a,g–i]	0.00900 ± 0.00044[a,j]	0.692 ± 0.056[a,g–i]
Finished product NTE	1[b]	0.6[b]	2[b]	2[b]
3244	0.0196 ± 0.0027[b,e]	0.01266 ± 0.00069[a,i,k]	0.000253 ± 0.000033[a,j]	0.0270 ± 0.0027[a,g–i]
Finished product NTE	0.05[b]	0.03[b]	0.1[b]	0.1[b]

[a] Each certified concentration value, expressed as a mass fraction on a dry-mass basis, is an equally weighted mean of the results from NIST and collaborating laboratories. The uncertainty in the certified value, calculated according to the method described in the ISO Guide (18–20), is expressed as an expanded uncertainty.

[b] Each reference concentration value, expressed as a mass fraction on a dry-mass basis, is the equally weighted mean of results provided by a collaborating laboratory. The uncertainty in the reference values, calculated according to the method described in the ISO Guide (18–20), is expressed as an expanded uncertainty.

[c] For the raw ingredients (plant and extract), mass fractions not to exceed (NTE) are as specified by the Standard. Mass fractions NTE for finished products were calculated from the maximum daily intake specified by the Standard and the maximum number of servings and serving sizes specified on the Supplement Facts panels of the SRM component products where applicable. Analytical methods used for value assignment are provided in the footnotes associated with each value.

[d] A concentration value is not assigned for lead in SRM 3240. Analyses by collaborating laboratories showed that lead was not homogeneous in this material, with concentrations ranging from 1.3 to 16 mg/kg.

[e] NRCC hydride generation graphite furnace atomic absorption spectrometry.

[f] NIST INAA.

[g] NRCC ID ICP-MS.

[h] FDA ICP-MS.

[i] NIST ID ICP-MS.

[j] NIST ID cold vapor ICP-MS.

[k] The reference concentration value, expressed as a mass fraction on a dry-mass basis, is the equally weighted mean of results from one analytical method (ID ICP-MS) at NIST. The uncertainty in the reference value, calculated according to the method described in the ISO Guide (18–20) is expressed as an expanded uncertainty.

spectrometry (ICP-MS) was used for the determination of cadmium and lead (14, 15); and ID cold vapor ICP-MS was used for the determination of mercury (16). NRCC used ID ICP-MS for the determination of cadmium and lead and hydride generation graphite furnace atomic absorption spectrometry for the determination of arsenic. FDA provided results for cadmium, lead, and mercury by using ICP-MS. All collaborating laboratories analyzed a minimum of 6 subsamples (one from each of 6 bottles or 2 from each of 3 bottles) of the 5 materials.

Analytical Approach for Determination of Nutrients

Up to 3 sources of data were used to determine nutrients in SRM 3244. Proximates (protein, carbohydrate, etc.), individual fatty acids, amino acids, water-soluble vitamins, and elements of nutritional interest were measured by the following laboratories participating in an interlaboratory comparison exercise organized by the FPA FIACS: Campbell Soup Co., Camden, NJ; Covance Laboratories, Madison, WI; Eurofins Scientific, Inc., Memphis, TN; General Mills, Inc., Golden Valley, MN; Hormel Foods Corp., Austin, MN; Kraft East, East Hanover, NJ; Kraft Foods, Glenview, IL (analyses performed by Silliker Laboratories, Homewood, IL); Nestlé Foods Corp., Dublin, OH; Nestlé-Purina Pet Care, St. Louis, MO; Novartis Nutrition Corp., St. Louis Park, MN; and U.S. Department of Agriculture (USDA), Nutrient Composition Laboratory, Beltsville, MD. Two B vitamins were determined by NIST, using LC with absorbance detection. Nutritive elements were determined by NIST, using inductively coupled plasma atomic emission spectrometry (ICP-AES). Nutritive and other elements, including boron, calcium, carbon, chlorine, gadolinium, hydrogen, iron, magnesium, nitrogen, phosphorus, potassium, samarium, silicon, sodium, sulfur, and zinc, were also determined by FDA, using prompt gamma activation analysis (PGAA; 17).

Calculation of Assigned Values

The equally weighted means of results submitted by NIST, FDA, NRCC, and ChromaDex were used for value assignment of alkaloids and toxic elements in the 5 materials, as appropriate. If a laboratory's results for a particular analyte disagreed with the other laboratories' results and were beyond 3 standard deviations from the mean of the other laboratories' combined data, then that laboratory's results for that analyte

Table 3. Reference[a] and certified[b] concentration values for selected elements in the suite of ephedra SRMs[c]

Element	Mass fraction, %				
	SRM 3240	SRM 3241	SRM 3242	SRM 3243	SRM 3244
C	45.0 ± 1.1[a]	41.3 ± 1.5[a]	41.5 ± 1.1[a]	38.5 ± 1.1[a]	44.7 ± 1.5[a]
Ca	2.69 ± 0.08[a]	0.845 ± 0.050[a]	0.742 ± 0.087[a]	1.03 ± 0.05[a]	1.328 ± 0.090[b]
Cl	0.460 ± 0.012[a]	1.83 ± 0.05[a]	2.75 ± 0.06[a]	1.07 ± 0.03[a]	0.0800 ± 0.0048[a]
H	5.51 ± 0.13[a]	5.59 ± 0.19[a]	6.06 ± 0.14[a]	5.32 ± 0.10[a]	6.13 ± 0.06[a]
K	0.547 ± 0.012[a]	3.08 ± 0.09[a]	2.46 ± 0.05[a]	1.39 ± 0.03[a]	1.60 ± 0.18[b]
Mg	0.338 ± 0.041[a]	0.719 ± 0.060[a]		4.80 ± 0.14[a]	0.310 ± 0.012[b]
N	1.58 ± 0.08[a]	3.20 ± 0.18[a]	2.88 ± 0.07[a]	4.45 ± 0.21[a]	
Na		0.248 ± 0.028[a]	0.244 ± 0.028[a]	0.196 ± 0.014[a]	0.091 ± 0.010[b]
P				0.68 ± 0.10[a]	1.220 ± 0.088[b]
S	0.177 ± 0.005[a]	0.385 ± 0.017[a]	0.325 ± 0.011[a]	0.263 ± 0.010[a]	0.650 ± 0.010[a]
Si	0.360 ± 0.023[a]	0.248 ± 0.030[a]	0.278 ± 0.037[a]	1.62 ± 0.03[a]	0.499 ± 0.022[a]
Zn				0.325 ± 0.031[a]	0.01264 ± 0.00077[b]
	Mass fraction, mg/kg				
B	13.0 ± 0.4[a]	62.2 ± 1.8[a]	52.5 ± 1.1[a]	70.6 ± 1.4[a]	3.56 ± 0.13[a]
Cr				63.4 ± 1.3[a]	
Cu					10.2 ± 1.0[b]
Gd	0.085 ± 0.016[a]			0.133 ± 0.007[a]	
Fe	457 ± 67[a]	900 ± 100[a]	870 ± 230[a]	760 ± 160[a]	107 ± 15[a,d]
Mn					30.0 ± 1.4[b]
Sm	0.097 ± 0.015[a]			0.132 ± 0.009[a]	

[a] Each reference concentration value, expressed as a mass fraction on a dry-mass basis, is the equally weighted mean of results provided by one collaborating laboratory using ICP-AES (for Cr) or PGAA (all other reference values except for iron in SRM 3244; *see* footnote d). The uncertainty in the reference value, calculated according to the method described in the ISO Guide (18–20), is expressed as an expanded uncertainty.

[b] Each certified concentration value, expressed as a mass fraction on a dry-mass basis, is the equally weighted mean of results from NIST and collaborating laboratories. The uncertainty in the certified values, calculated according to the method described in the ISO Guide (18–20), is expressed as an expanded uncertainty. Analytical methods used for value assignment are provided in footnote c.

[c] Analytical methods used to provide assigned values in SRM 3244: (No. of collaborating laboratories in parentheses): calcium: direct current plasma AES (1), PGAA (1), ICP-AES (7 + NIST); copper: direct current plasma AES (1), ICP-AES (6 + NIST); iron: ICP-AES (7 + NIST); magnesium: direct current plasma AES (1), ICP-AES (7 + NIST); manganese: ICP-AES (6 + NIST); phosphorus: absorption spectrophotometry (3), ICP-AES (9 + NIST), PGAA (1); potassium: direct current plasma AES (1), ICP-AES (6 + NIST); sodium: direct current plasma AES (1), ICP-AES (6 + NIST); zinc: direct current plasma AES (1), ICP-AES (7 + NIST).

[d] The reference concentration value for iron, expressed as a mass fraction on a dry-mass basis, is the weighted mean of results provided by NIST and the FPA FIACS interlaboratory exercise. The uncertainty in the reference value, calculated according to the method described in the ISO Guide (18–20), is expressed as an expanded uncertainty.

were not used. The NIST and FPA means were used to assign values for nutrient elements and 2 B vitamins in SRM 3244. The FPA mean was used to assign reference values for additional vitamins, proximates, individual fatty acids, and amino acids in SRM 3244; outliers were identified as described above. Reference values for additional elements were assigned by using FDA's PGAA data.

Uncertainties in the assigned values were calculated according to the method described in the Guide of the International Organization for Standardization (ISO; 18–20). The uncertainty for each value is expressed as an expanded uncertainty, U, calculated as $U = ku_c$, where u_c is intended to represent, at the level of 1 standard deviation, the combined effect of between-laboratory, within-laboratory, and drying components of uncertainty. (Deviations, e.g., incorporation of an inhomogeneity component in the uncertainty for caffeine in SRM 3243, are noted in the tables that follow.) 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.

Homogeneity Assessment

The homogeneity of ephedrine in SRMs 3240, 3241, 3242, and 3243 was assessed at NIST by using the LC/UV method

Table 4. Reference values[a] for proximates, selected fatty acids (as triglycerides), and caloric content in SRM 3244[b]

Nutrient	Mass fraction, %
Solids	96.4 ± 1.2
Ash	9.11 ± 0.36
Protein	66.1 ± 1.3
Fat[c]	1.41 ± 0.18
Carbohydrate (by difference)	20.0 ± 4.9
Dodecanoic acid (C12:0) (lauric acid)	0.021 ± 0.005
Tetradecanoic acid (C14:0) (myristic acid)	0.075 ± 0.008
Hexadecanoic acid (C16:0) (palmitic acid)	0.375 ± 0.040
Octadecanoic acid (C18:0) (stearic acid)	0.253 ± 0.025
(*Z*)-9-Octadecenoic acid (C18:1 n-9) (oleic acid)	0.342 ± 0.042
(*Z,Z*)-9,12-Octadecadienoic acid (C18:2 n-6) (linoleic acid)	0.192 ± 0.009
(*Z,Z,Z*)-9,12,15-Octadecatrienoic acid (C18:3 n-3) (linolenic acid)	0.024 ± 0.002
Calories, kcal/100 g[d]	366.5 ± 9.6

[a] Each reference concentration value, expressed as a mass fraction on an as-received basis, is the mean of results provided by the collaborating laboratories. The uncertainty in the reference values, calculated according to the method described in the ISO Guide (18–20), is expressed as an expanded uncertainty.

[b] Analytical methods used for value assignment (No. of collaborating laboratories in parentheses): solids: moisture determined by mass loss after oven-drying using forced-air oven (2 + NIST) or vacuum oven (7), freeze-dryer (NIST), desiccator with $Mg(ClO_4)_2$ (NIST); ash: mass loss after ignition in muffle furnace (9); fatty acids: hydrolysis followed by gas chromatography (GC; 9); nitrogen: Kjeldahl (4), thermal conductivity (2), pyrolysis, GC (2), PGAA (1); protein: calculated (a factor of 6.38 was used to calculate protein from nitrogen results); carbohydrate: calculated (solids – [protein + fat as the sum of fatty acids + ash]), amino acids: hydrolysis, derivatization, LC (5), amino acid analyzer (1); calories: calculated (9 [fat] + 4 [protein] + 4 [carbohydrate]).

[c] Based on fat as the sum of the fatty acids.

[d] The value for caloric content is the mean of individual caloric calculations from the laboratories participating in the FPA FIACS interlaboratory excercise. The equivalent energy in units of kilojoules is 1530 kJ/100 g. If the proximate values above are used for calculation, with caloric equivalents of 9, 4, and 4 for fat (as the sum of the fatty acids), protein, and carbohydrate, respectively, the mean caloric content is 357 kcal/100 g (equivalent to 1490 kJ/100 g).

Table 5. Reference concentration[a] values for amino acids in SRM 3244[b]

Amino acid	Mass fraction, %
Alanine	2.12 ± 0.96
Arginine	2.26 ± 0.52
Aspartic acid	5.29 ± 0.28
Cystine	0.48 ± 0.14
Glutamic acid	14.3 ± 2.1
Glycine	1.23 ± 0.13
Histidine	1.73 ± 0.17
Isoleucine	3.00 ± 0.61
Leucine	6.16 ± 0.88
Lysine	4.78 ± 0.77
Methionine	1.71 ± 0.28
Phenylalanine	3.48 ± 0.50
Proline	6.64 ± 0.73
Serine	3.80 ± 0.35
Threonine	2.76 ± 0.54
Tryptophan	0.84 ± 0.29
Tyrosine	3.16 ± 0.71
Valine	3.67 ± 0.98

[a] Each reference concentration value, expressed as a mass fraction on an as-received basis, is the mean of results provided by the laboratories participating in the FPA FIACS interlaboratory exercise. The uncertainty in the reference values, calculated according to the method described in the ISO Guide (18–20), is expressed as an expanded uncertainty.

[b] Amino acids were measured by 5 laboratories using a hydrolysis followed by derivatization and LC, and one laboratory using an amino acid analyzer.

results used for value assignment (8). There was no trend in ephedrine data across bottles for 1 g test portions of SRM 3240. An analysis of variance (ANOVA) using measurements for ephedrine did not show inhomogeneity for a 0.3 g test portion of SRM 3241 and a 0.15 g test portion of SRM 3242. Other analytes in these 3 materials were treated as though they were homogeneously distributed, although homogeneity was not assessed.

An ANOVA using LC/UV measurements for ephedrine showed homogeneity for a 1 g test portion of SRM 3243. The homogeneity of SRM 3243 for caffeine was assessed at NIST by using the LC/UV method results that were used for value assignment. An ANOVA of results for caffeine did show a mean bottle difference of 1.6% for 150 mg test portions, and an inhomogeneity component has been included in the expanded uncertainty for the caffeine. Other analytes in SRM 3243 were treated as though they were homogeneously distributed, although homogeneity was not assessed.

The homogeneity of SRM 3244 for selected elements was assessed at NIST by using the ICP-AES results used for value assignment. An ANOVA using NIST's measurements of Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn did not show inhomogeneity for 0.5 g test portions. An ANOVA using NIST's LC/UV measurements of caffeine (600–900 mg test portions) and vitamins B_2 and B_6 (2 g test portions) also did not show inhomogeneity. Other analytes in SRM 3244 were treated as though they were homogeneously distributed, although homogeneity was not assessed.

Table 6. Certified[a] and reference[b] concentration values for selected water-soluble vitamins in SRM 3244[c]

Vitamins	Mass fraction, mg/kg
Vitamin C	890 ± 100[b]
Vitamin B_1[d]	20.5 ± 3.6[b]
Vitamin B_2	29.9 ± 2.3[b]
Vitamin B_6	34.1 ± 2.2[a]
Niacin	304 ± 10[a]
Vitamin B_{12}	0.107 ± 0.017[b]
Pantothenic acid	172 ± 33[b]
Biotin	4.36 ± 0.38[b]
Folic acid	5.4 ± 1.2[b]
Choline ion	1500 ± 600[b]
Inositol	1550 ± 450[b]

[a] Each certified concentration value, expressed as a mass fraction on a dry-mass basis, is an equally weighted mean of the results from NIST and collaborating laboratories. The uncertainty in the certified value, calculated according to the method described in the ISO Guide (18–20), is expressed as an expanded uncertainty.

[b] Each reference concentration value, expressed as a mass fraction on an as-received basis, is the mean of results provided by the laboratories participating in the FPA FIACS interlaboratory exercise. The uncertainty in the reference values, calculated according to the method described in the ISO Guide (18–20), is expressed as an expanded uncertainty.

[c] Analytical methods used for value assignment (No. of laboratories in parentheses): vitamin C: colorimetric titration (1), LC–fluorescence detection (1), LC–absorbance detection (1), LC (1), fluorescence (3); total vitamin B_1: digestion–fluorescence detection (3), extraction–reversed-phase liquid chromatography (RPLC)–fluorescence detection (1), microbiological (1); total vitamin B_2: digestion–fluorescence detection (2), extraction–RPLC–fluorescence detection (3), microbiological (1); total vitamin B_6: LC-fluorescence detection (2), microbiological methods (4), RPLC–absorbance detection (NIST); niacin: microbiological (6), RPLC–absorbance detection (NIST); total vitamin B_{12}: microbiological (6); folic acid: microbiological (6); biotin: microbiological (6); pantothenic acid: microbiological (6); choline (ion): digestion–absorption spectrometry (2), microbiological (1), extraction, Reinckate method (1); inositol: digestion–GC with flame-ionization detection (1), size-exclusion chromatography–refractive index detection (1), microbiological (2).

[d] Thiamine, not thiamine hydrochloride.

Supplemental Information

Users of these materials may want assurance that the materials are taxonomically authentic and may want to compare a plant material's anatomy with those of other materials purported to be the same species. The voucher specimen from which SRM 3240 *E. sinica* Stapf was prepared has been archived. Microscopic studies of this material as well as SRM 3240 itself, and micrographs, photographs, and specimen data are available on the Missouri Botanical Garden's Website at http://www.mobot.org/MOBOT/research/diversity/herbarium/compendium_model.aspx?id=3.

In addition to the analyses of the material described above, further characterization of SRM 3240 was provided by using LC with absorbance detection and thin-layer chromatography (TLC). A "fingerprint" chromatogram (Figure 2) from the analysis of SRM 3240 *E. sinica* Stapf Aerial Parts was created by using the method reported by Schaneberg et al. (21). A 0.58 g test portion of the SRM was extracted with 6 mL acetone by sonication for 15 min. The slurry was centrifuged for 5 min and decanted. This procedure was repeated twice, and the 3 extracts were combined and evaporated to dryness. The extract was reconstituted in ethanol, and analyzed by gradient elution LC using a 4.6 × 150 mm, 5 μm particle size, C_{18} column (XTerra RP_{18}; Waters, Inc., Milford, MA) operated at 40°C, with UV absorbance detection at 320 nm (9). Mobile phase conditions consisted of an initial 10 min isocratic separation with water–acetonitrile (75 + 25, v/v), followed by a linear gradient to 100% acetonitrile over 45 min. End conditions were held for 20 min, for a total run time of 75 min. The flow rate was 1 mL/min, and the injection volume was 10 μL.

Results and Discussion

All 5 materials in the suite of ephedra SRMs have values assigned for ephedrine and other alkaloids (Table 1) as well as toxic elements (Table 2). To make these materials more broadly useful, values have been assigned for additional alkaloids (caffeine, theobromine, theophylline, and synephrine), nutrients, and other elements (Table 3), and other analytes of nutritional interest in SRM 3244, e.g., proximates, fatty acids, vitamins, amino acids (Tables 4–6), as appropriate. Thus, for example, SRM 3240 *E. sinica* Stapf could be used as a control material in the measurement of elements in some other type of plant material, and SRM 3243 Ephedra-Containing Solid Oral Dosage Form could be used as a control material in the measurement of caffeine in "diet pills".

Maximum recommended daily intakes of toxic elements from various sources are set by a number of parties, including the U.S. Environmental Protection Agency, the FDA, the World Health Organization, and the British Herbal Pharmacopoeia. NSF International has derived acceptable limits in dietary supplements from these sources and has specified them in NSF/ANSI Standard 173, Dietary Supplements, Product Formulation and Raw Materials (22). A comparison of the certified and reference values for As, Cd, Hg, and Pb in the 5 ephedra-containing SRMs with these limits is provided in Table 2. Daily intake limits were converted to a mass-fraction basis (mg/kg) by using the maximum daily intake specified by NSF Standard 173 and the maximum number of servings and serving sizes specified on the Supplement Facts panels of the SRM component products. Because the 2 extract SRMs (3241 and 3242) were prepared from the plant material that is SRM 3240, it is interesting to compare the change in levels resulting from the extraction process; note that arsenic is concentrated by this process, whereas the mass fractions of cadmium and mercury are lower than those in the starting material. Lead was not homogeneously distributed in SRM 3240; therefore, a value was not assigned. The concentrations of toxic elements in

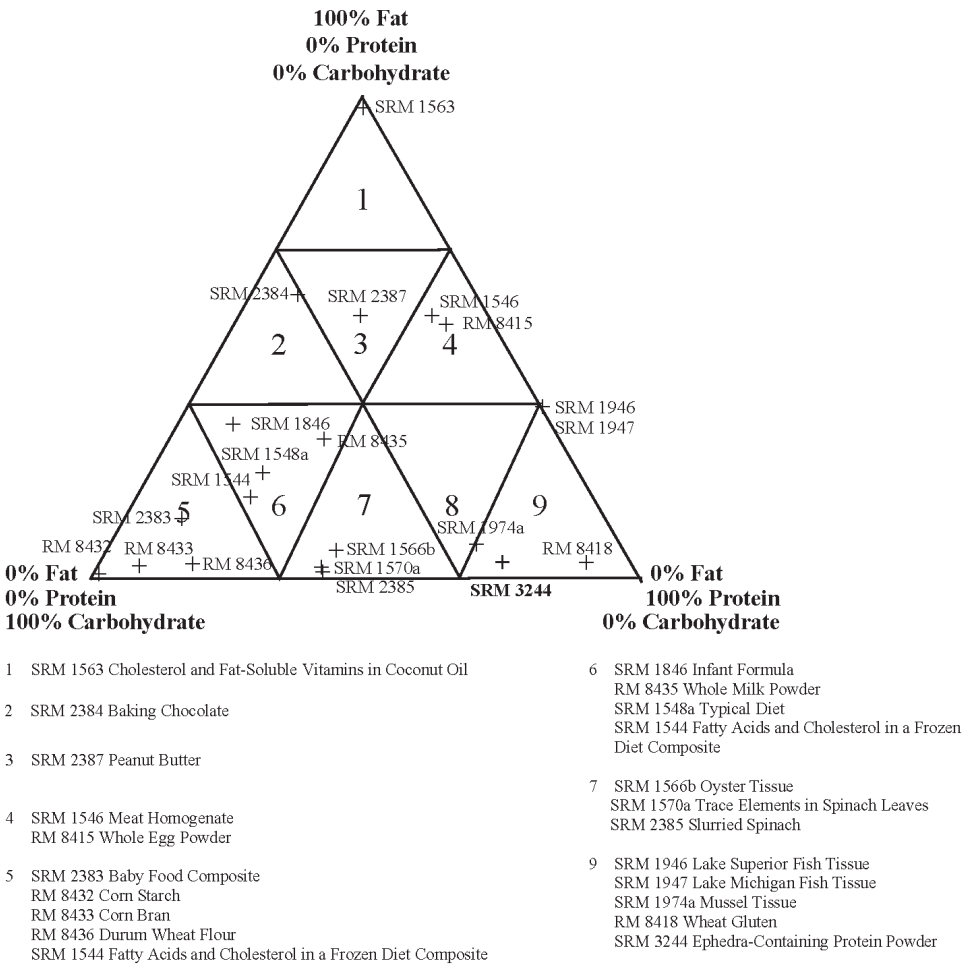

Figure 3. Location of SRM 3244 Ephedra-Containing Protein Powder in the fat–protein–carbohydrate triangle developed by AOAC INTERNATIONAL for categorization of food matrixes. Other food-matrix SRMs and RMs available from NIST with values assigned for proximates are shown.

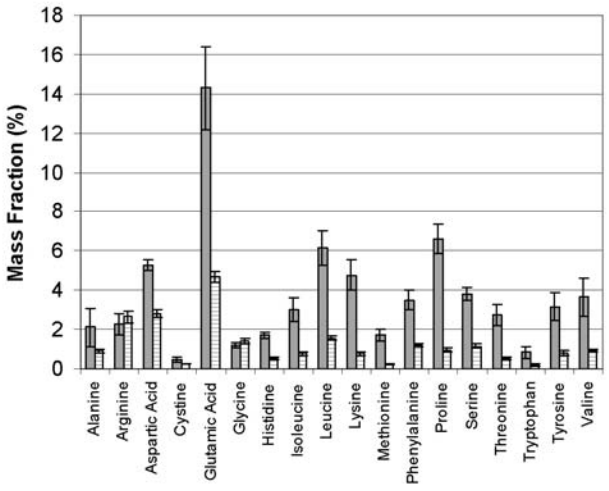

Figure 4. Comparison of reference values for amino acids in SRM 3244 Ephedra-Containing Protein Powder (solid bars) and SRM 2387 Peanut Butter (striped bars). Error bars represent the 95% confidence interval.

these 3 materials are 2–30% of the allowed maximum, with mercury and lead generally occurring at lower percentages than arsenic and cadmium. In the finished products, mercury was 0.3% of the maximum permitted level, whereas the other elements were found at 20–50% of the allowed maximum.

SRMs 3241 and 3242 were originally prepared as free-flowing powders. It became clear soon after the material was bottled that these materials did not maintain their powdery consistency in all cases. When the bottles are shaken, some material sounds granular rather than powdery, and some bottles contain solidified pellets of material. A number of causes for the conversion have been proposed: heat, moisture, and irradiation. However, the conversion has not been definitively attributed to any of these factors. As the bottles were filled, they were packaged in boxes of 100; a given bottle in the box may contain powder, although its neighbors are granular or pellets, implying that variability in atmospheric moisture during packaging cannot be the culprit. Material that was bottled but not irradiated has formed pellets, implying that irradiation did not cause the conversion. Ampoules of powder have been heated,

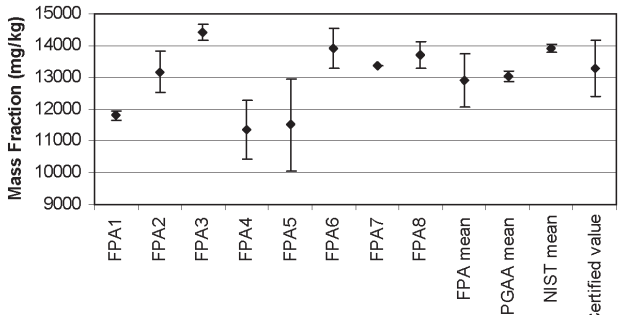

Figure 5. Comparison of data used to assign the value for calcium in SRM 3244 Ephedra-Containing Protein Powder. Error bars for the FPA, PGAA, and NIST data represent 2 standard deviations of each mean. The error bar for the certified value represents the expanded uncertainty.

and some have formed a pellet while others remain powder, implying that heating did not cause the conversion to a solid pellet. Fortunately—although perhaps not what one would intuitively expect—analyses of powder, lumps, and pellets for the ephedrine alkaloids yield equivalent results. Thus, although the problem has not been explained and results in an SRM with variable consistency in form, the conversion does not appear to cause analytical problems (aside from the difficulty of removing sample from the bottle!).

NIST has a number of food-matrix reference materials with values assigned for constituents of nutritional interest. This effort was driven largely by the requirements of the Nutrition Labeling and Education Act of 1990 (23). As shown in Figure 3, reference materials have been developed for a wide range of food compositions (24). Foods are positioned in this AOAC-developed triangle on the basis of their fat, protein, and carbohydrate content. One or 2 foods within each sector are expected to be representative of—and useful as control materials for analysis of—other foods within that sector (25, 26). With materials available within, or on borders between, all sectors of this triangle, this effort has reached a natural conclusion, and there are no immediate plans to introduce new food-matrix SRMs directed at nutrition labeling. However, food-matrix materials that are developed for other purposes will also be characterized for nutrients. For example, the ephedra-containing protein powder, SRM 3244, which was mainly produced as a reference material for the ephedrine alkaloids, also has values assigned for nutrients. Thus, materials that might otherwise be important to a small sector of the analytical community can be made more broadly useful.

Table 7. Data for calcium (mass fraction, in mg/kg, on a dry-mass basis) in SRM 3244 Ephedra-Containing Protein Powder, and the calculation of its assigned value and associated uncertainty[a]

Data source	Values	Mean	SD of mean
FPA lab 1	11724, 11874	11799	76
FPA lab 2	13492, 12838	13165	327
FPA lab 3	14269, 14523	14396	127
FPA lab 4	10889, 11822	11355	467
FPA lab 5	12237, 10785	11511	726
FPA lab 6	13585, 14207	13896	311
FPA lab 7	13378, 13378	13378	0
FPA lab 8	13896, 13481	13689	207
FPA mean		12899	416
PGAA	13152, 13327, 12862, 13069, 12786, 13013	13035	80
NIST	13721, 13729, 13926, 14135, 13990, 13968	13911	66
Mean of laboratory means		13282	
Between-method uncertainty		292	
Within-method uncertainty		143	
Effective total degrees of freedom		4	
Coverage factor (k)		2.8	
Standard uncertainty		326	
Expanded uncertainty		900	
Final assigned value		13280 ± 900	

[a] The same raw data—the value for the mean and standard deviation (SD) of the mean for calcium reported by the individual laboratories, as well as the means for the 3 sources of data—are plotted in Figure 5 for graphical comparison. Each of the 6 NIST values listed represents the mean of 2 measurements. Data were averaged as (NIST + PGAA + FPA means)/3 for calculation of the certified value.

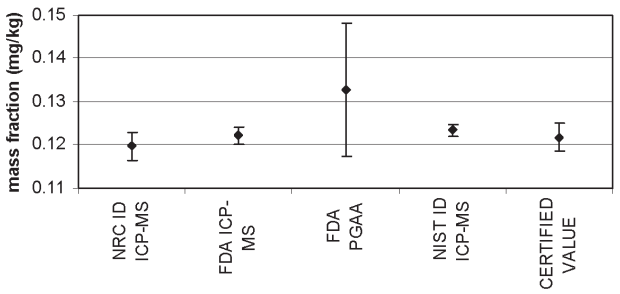

Figure 6. Comparison of data used to assign the value for cadmium in SRM 3243 Ephedra-Containing Solid Oral Dosage Form. Error bars for the FPA, PGAA, and NIST data represent 2 standard deviations of each mean. The error bar for the certified value represents the expanded uncertainty. These PGAA data were not used for value assignment because cadmium was below the limit of quantitation, causing the variability in the data to be large.

The USDA has been including amino acid values in its nutrient databases for several years (27), and until the introduction of SRM 2387 Peanut Butter in 2003, NIST had no food-matrix SRMs available with values assigned for amino acids to provide quality assurance for these measurements. SRM 3244 is the second food-matrix SRM with values assigned for amino acids; the amino acid profiles of SRMs 3244 and 2387 are compared in Figure 4. Amino acid values may be added to existing food-matrix SRMs over time so that materials for amino acid analyses are available in all relevant sectors of the triangle.

A comparison of the values used to calculate the certified value for calcium in SRM 3244 is provided in Figure 5, and the values themselves are shown in Table 7, for comparison. A similar plot for cadmium in SRM 3243 is provided in Figure 6; the raw data are not provided, but values were calculated similarly. (Cadmium and calcium were selected as examples because data were provided by NIST and all collaborative sources in each case.) In both cases, the means of the individual data sources were combined to provide the final assigned value. Because cadmium was below the limit of quantitation by PGAA, and the variability in the data was large, the PGAA data for cadmium were not used for value assignment.

Values are assigned in the ephedra materials for a number of elements that, although not classified as nutritive elements, are obviously essential components in biological systems, i.e., carbon, chlorine, hydrogen, and sulfur (Table 3). Although animal data also suggest nutritional requirements for arsenic, boron, and silicon, as well, no biological function in humans has been identified (28). Elemental profiles including these and other elements can also be used to identify the geographical origin of a material (29, 30).

This suite of ephedra SRMs is the first in a series of dietary supplement reference materials being produced as part of an interagency agreement among NIST, NIH, and FDA. These SRMs and others in the series will be used to validate analytical methods and to judge the accuracy of analytical results. Other botanical-containing SRMs being produced include suites containing *Ginkgo biloba*, saw palmetto, bitter orange, green tea, and St. John's wort. In addition, a multivitamin/multielement tablet and oils containing β-carotene, tocopherols, and Ω-3 fatty acids are being characterized.

Acknowledgments

We thank the members of the FPA FIACS and the other collaborating laboratories for analyzing this suite of SRMs; we recognize and appreciate the amount of time and effort the collaborating laboratories contributed in assisting in the value assignment of these materials.

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