National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 3244

Ephedra-Containing Protein Powder

Standard Reference Material (SRM) 3244 is intended primarily for use in validating analytical methods for the determination of ephedrine alkaloids, caffeine, nutrients, and toxic elements in protein powder and similar matrices. This SRM can also be used for quality assurance when assigning values to in-house control materials. SRM 3244 is part of a suite of ephedra dietary supplement SRMs that have been developed to cover a range of natural matrices and ephedrine alkaloid levels. A unit of SRM 3244 consists of ten bottles of protein powder, each containing approximately 12 g of material.

Certified Concentration Values: 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 accounted for [1]. The certified concentration values of selected ephedrine alkaloids and caffeine, water-soluble vitamins, and elements are provided in Tables 1 through 3. Certified values were derived from the combination of results provided by NIST and collaborating laboratories. The certified values for the alkaloids 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. The certified values for the water-soluble vitamins and elements in this material are the equally weighted means of the results provided by NIST, the mean of results provided by the Food Products Association (FPA, formerly the National Food Processors Association) Food Industry Analytical Chemists Subcommittee (FIACS), and results provided by the Food and Drug Administration (FDA) and National Research Council Canada (NRCC) where available. The associated uncertainties are expanded uncertainties at the 95 % level of confidence [2,3]. Values are reported on a dry-mass basis in mass fraction units [4].

Reference Concentration Values: A NIST reference value is a noncertified value that is the best estimate of the true values based on available data; however, the value does not meet the NIST criteria for certification [1] and is 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. Reference concentration values for theobromine, theophylline, methylpseudoephedrine, water-soluble vitamins, and additional elements, proximates (protein, carbohydrates, etc.), fatty acids, and amino acids are provided in Tables 4 through 8.

Information Concentration Values: Information values are considered to be values that will be of interest to the SRM user; however, either insufficient information is available to assess the uncertainty or the uncertainty is relatively large. Information values are generally reported with no associated uncertainty. Information concentration values for additional ephedrine alkaloids are provided in Table 9.

Expiration of Value Assignment: The value assignment of this SRM is valid until **31 March 2014**, within the measurement uncertainties specified, provided the SRM is handled and stored in accordance with the instructions given in this certificate. Value assignment is nullified if the SRM is damaged, contaminated, or modified.

Maintenance of SRM Value Assignment: NIST will monitor this SRM over the period of its value assignment. If substantive technical changes occur that affect the value assignment before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet) will facilitate notification.

The coordination of the technical measurements leading to the certification of this SRM was performed by L.C. Sander, K.E. Sharpless, and S.A. Wise of the NIST Analytical Chemistry Division.

Stephen A. Wise, Chief
Analytical Chemistry Division

Gaithersburg, MD 20899
Certificate Issue Date: 10 August 2007

Robert L. Watters, Jr., Chief
Measurement Services Division

See Certificate Revision History on Page 13

The development of SRM 3244 was a collaboration among the National Institute of Standards and Technology (NIST); the National Institutes of Health (NIH), Office of Dietary Supplements (ODS); and the Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition (CFSAN) and Center for Drug Evaluation and Research (CDER).

Acquisition and preparation of the material was coordinated by K.E. Sharpless of the NIST Analytical Chemistry Division.

Analytical measurements at NIST were performed by J.M. Brown Thomas, T.A. Butler, T. Ihara, S.E. Long, E.A. Mackey, K.E. Murphy, K.W. Phinney, B.J. Porter, L.C. Sander, M.B. Satterfield, and L.J. Wood of the NIST Analytical Chemistry Division. Analyses for value assignment were also performed by C. Fraser, G. Gardner, J.W. Lam, M. McCooeye, C. Scriver, and L. Yang of the National Research Council Canada (Ottawa, ON); D.L. Anderson, J. Cheng, M.L. Gay, and W. Mindak at the FDA's CFSAN (College Park, MD); S. Mitvalsky and M. Roman at ChromaDex, Inc. (Clearwater, FL); and laboratories participating in an interlaboratory comparison exercise organized by the FPA FIACS. The FPA FIACS interlaboratory comparison exercise was coordinated by K.E. Sharpless of the NIST Analytical Chemistry Division and I-P. Ho of the FPA (Washington, DC). FPA FIACS laboratories that contributed to value assignment of SRM 3244 are listed in Appendix A.

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

Support for the development of SRM 3244 was provided in part by the NIH Office of Dietary Supplements and the FDA CFSAN and CDER. Technical consultation from these agencies was provided by J. Betz (NIH-ODS), A. NguyenPho (FDA CDER), and G. Ziobro (FDA CFSAN).

The support aspects involved in the issuance of this SRM were coordinated through the NIST Measurement Services Division.

NOTICE AND WARNING TO USERS

Note: This material is exempt from requirements of Title 21 Code of Federal Regulations Section 1310 (21 CFR 1310). Concentrations of ephedrine alkaloids in this material are less than those specified in section 1310.12 (c) of the regulation, thereby exempting this material.

Storage: The material should be stored at controlled room temperature (20 °C to 25 °C) in its unopened bottle until required for use.

WARNING: FOR LABORATORY USE ONLY. NOT FOR HUMAN CONSUMPTION.

INSTRUCTIONS FOR USE

Prior to removal of a test portion for analysis, the contents of a bottle of material should be mixed thoroughly. Test portions used for NIST analyses (see "Preparation and Analysis" section) were 2.5 g and 5 g for ephedrine alkaloids, 0.6 g to 1 g for caffeine, 0.2 g for arsenic, 1.0 g for cadmium and lead, 0.3 g for mercury, and 0.5 g and 1.0 g for elements of nutritional interest.

PREPARATION AND ANALYSIS[1]

Material Acquisition and Preparation

SRM 3244 Ephedra-Containing Protein Powder was prepared from several brands of commercially available products that were purchased in the marketplace. The products were intentionally purchased from multiple vendors to obtain material from different production lots. These materials were primarily milk-based products, although some egg protein was present. Individual amino acids, flavorings, botanicals (including *E. sinica*), vitamins, and elements were among the other ingredients in the products that were combined.

[1]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, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Materials for blending as SRM 3244 were shipped to Sun-Ten (Irvine, CA) in their original containers. Sun-Ten blended the materials for 20 min, to uniformity, using a V-blender. Following blending, SRM 3244 was placed in four polyethylene-lined drums. The material was then transferred to ChromaDex, Inc. (Santa Ana, CA) where it was bottled under nitrogen in amber high-density polyethylene bottles with polypropylene screw caps. After bottling, the materials were irradiated by ^{60}Co to an absorbed dose of 12.5 kGy to 15.7 kGy.

Analytical Approach for Determination of Ephedrine Alkaloids, Caffeine, Theobromine, and Theophylline

Value assignment of the concentrations of the ephedrine alkaloids in SRM 3244 was based on the combination of measurements from different analytical methods at NIST and at three collaborating laboratories. As many as eight sets of measurements were used for the value assignment of the concentrations of ephedrine alkaloids. NIST provided measurements by using a combination of two sample extraction procedures and three liquid chromatography (LC) methods with different detection, i.e., ultraviolet absorbance spectrometry (UV), mass spectrometry (MS), tandem mass spectrometry (MS/MS), and a capillary electrophoresis (CE) method as described below. Results for ephedrine alkaloids were provided by three collaborating laboratories: National Research Council Canada (NRCC), FDA, and ChromaDex. NRCC provided results from two analytical methods: LC/UV and LC/MS/MS. FDA results were based on LC/MS/MS [5], and ChromaDex results were based on LC/UV [6]. Two collaborating laboratories analyzed six subsamples (one from each of six bottles or two from each of three bottles); one laboratory analyzed one subsample from three bottles of SRM 3244.

Value assignment of the concentration of caffeine in SRM 3244 was based on the combination of measurements from different analytical methods at NIST and at one collaborating laboratory. Caffeine was determined at NIST by using LC/UV and LC/MS/MS and at ChromaDex by using LC/UV. Value assignment of concentrations of theobromine and theophylline in SRM 3244 was based on measurements at NIST using LC/UV.

NIST Analyses for Ephedrine Alkaloids

Ephedrine alkaloids were measured by using combinations of two sample preparation methods, three LC methods, and one CE method as described below and in reference 7. Four independently prepared calibrants were used for each of the methods. Calibrants were prepared gravimetrically, at levels intended to approximate the levels of the alkaloids in the extracts of the SRM. A single internal standard solution was used for the calibrants and samples. Calculations are based on average response factors for the calibrants (typically duplicate analyses of four calibrant solutions, n = 8).

Sonication Extraction: Six 5 g portions of the SRM were placed in 50 mL polyethylene centrifuge tubes or glass pressurized-fluid extraction tubes, followed by the addition of a measured mass of internal standard solution. Approximately 30 g of methanol was added to the tubes, and the tubes were capped. The solid matter was suspended by shaking, and the tubes were placed in an ultrasonic bath for 90 min. At the completion of the sonication extraction, the samples were centrifuged or allowed to settle, and an aliquot of the supernatant solution was filtered through a 0.45 μm × 2.5 cm syringe filter. Samples prepared by this approach were analyzed by LC/UV or LC/MS/MS.

Soxhlet Extraction: Ten 5 g portions of the SRM were weighed into glass-fritted Soxhlet thimbles, each containing an approximate 1 cm layer of diatomaceous earth (Hydromatrix, Isco, Lincoln, NE). After stirring the sample, additional diatomaceous earth was added (approximately 1 cm). A measured mass of internal standard solution (ephedrine-d_3) was transferred to the Soxhlet thimble. The samples were extracted with approximately 200 mL methanol for at least 18 h. Extracts were concentrated to approximately 1 mL under nitrogen, and the sides of the tube were rinsed with methanol for a final volume of approximately 10 mL. This extract was filtered through a 0.45 μm × 2.5 cm syringe filter and analyzed. Samples prepared by this approach were analyzed by LC/MS.

LC with UV Absorbance Detection (LC/UV): An isocratic LC method with a methanol/phosphate buffer mobile phase was utilized for LC/UV determination of the alkaloids, similar to the method of Roman [6]. A 250 mm × 4.6 mm alkylphenyl bonded-phase column (Synergy Polar RP, Phenomenex, Torrance, CA) was used with a precolumn and an in-line filter. Column temperature was controlled at 29.0 ºC ± 0.5 ºC with a circulating-fluid column jacket and water bath. The flow rate of the mobile phase was set at 1.5 mL/min, and detection was at 208 nm. Terbutaline was used as the internal standard for LC/UV measurements. A typical separation is provided in Appendix B.

LC with Mass Spectrometric Detection (LC/MS): A 250 mm × 4.6 mm phenyl bonded-phase column (YMC Phenyl, Waters, Inc., Milford, MA) was used at ambient temperature (21 °C ± 1 °C) with an isocratic mobile phase (water/methanol/acetic acid/ammonium acetate) at 1.0 mL/min. The mass spectrometer was operated in positive ion, atmospheric pressure ionization, electrospray mode (API-ES). Quantification of the six alkaloids was based on monitoring ions (*m/z*) at 134 and 152 (norephedrine and norpseudoephedrine), 166 (ephedrine and pseudoephedrine), 180 (methylephedrine and methylpseudoephedrine), and 169 (ephedrine-d_3). Ephedrine-d_3 was used as the internal standard for LC/MS measurements. A typical separation is provided in Appendix B.

LC with Tandem Mass Spectrometric Detection (LC/MS/MS): Chromatographic conditions were similar to those used in the LC/MS method; however, the flow rate was reduced to 0.5 mL/min and column temperature was set at 30 °C ± 2 °C. A program was designed to measure each individual analyte using multiple reaction monitoring (MRM). The protonated precursor of each analyte was selected in the first quadrupole, these ions collisionally dissociated in the collision cell (the second quadrupole), and the predetermined fragment ions monitored in the third quadrupole. The following precursor and fragment ions (m/z) were monitored: 151.8, 134.0 (norephedrine and norpseudoephedrine), 165.9 and 148.0 (ephedrine and pseudoephedrine), 179.9 and 162.0 (methylephedrine and methylpseudoephedrine), and 168.9 and 151.0 (ephedrine-d_3). Ephedrine-d_3 was used as the internal standard for LC/MS/MS measurements. A typical separation is provided in Appendix B.

Capillary Electrophoresis (CE): Portions (2.5 g) of the SRM were weighed into polyethylene centrifuge tubes, followed by the addition of a measured mass of internal standard solution (β-phenylethylamine hydrochloride) and approximately 18 mL of methanol. The samples were placed in an ultrasonic bath for 30 min and the supernatant solution was filtered through 0.2 μm nylon syringe filters. The filtered extract was concentrated and then diluted to 1 mL with 10 % methanol in water. Electrophoretic measurements were performed on a CE system with a photodiode array detector (data collected at 210 nm) with a high-sensitivity UV detection cell. Three chiral CE methods (utilizing different cyclodextrin-based chiral selectors) were used to analyze the samples. The methods were sufficiently independent to provide slightly different selectivity, thereby reducing the likelihood of undetected peak overlap and providing additional confidence in the enantiomeric identity of the analytes. Separations were performed in unmodified fused silica capillaries maintained at 25 °C, and injections were performed by pressure. Applied voltages were in the range of 15 kV to 30 kV. A detailed discussion of the CE method is provided in reference 8. A typical separation is provided in Appendix B; note that only the (–)-ephedrine and (+)-pseudoephedrine enantiomers that are naturally occurring in *E. sinica* were found in this material, indicating that the material was not altered through the addition of synthetic alkaloids.

NIST Analyses for Caffeine, Theobromine, Theophylline, Niacin, and Vitamin B_6

Caffeine was determined using LC/UV and LC/MS/MS. For the LC/UV analyses, duplicate subsamples of approximately 600 mg to 900 mg were removed from each of six bottles of SRM 3244. Approximately 7 g to 10 g of a methanol solution of β-hydroxyethyltheophylline was added to the subsample for use as an internal standard; the methanol served as the extraction solvent. After sonication for 1 h, the samples were processed as described above and analyzed using reversed-phase LC/UV on a C_{18} column as described in reference 9. Theobromine and theophylline were determined in the same LC/UV analyses as the caffeine. The LC/MS/MS measurements of caffeine were obtained during the LC/MS/MS analyses for the ephedrine alkaloids described above. $^{13}C_3$-caffeine was added to the subsamples of SRM 3244 for use as an internal standard in the LC/MS/MS analyses. Niacin and pyridoxine hydrochloride (vitamin B_6) were determined using LC/UV. Subsamples of approximately 2 g from each of six bottles of SRM 3244 were analyzed over three days. The subsamples were dissolved in water, and 4-pyridoxic acid (the internal standard), metaphosphoric acid, and acetontrile were added, with mixing after each addition. The solution was centrifuged, and the bottom layer was analyzed by using reversed-phase LC/UV on a C_{18} column.

Analytical Approach for Determination of Nutrients

Up to three sources of data were used to assign values for nutrients in SRM 3244. Proximates (protein, carbohydrate, etc.), individual fatty acids, amino acids, water-soluble vitamins, and elements of nutritional interest were determined as part of an FPA FIACS interlaboratory comparison exercise. (Participating laboratories are listed in Appendix A). The FPA laboratories were asked to use AOAC methods or their equivalent to perform single measurements from each of two bottles of SRM 3244. Nutritive elements were determined by NIST using inductively coupled plasma optical emission spectrometry (ICP-OES). Nutritive and other elements were also determined by FDA using prompt gamma activation analysis (PGAA). A summary of the methodological information and the number of laboratories using a particular analytical technique is provided in Appendix C.

NIST Analyses for Nutritive Elements

Calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc were measured in six bottles of SRM 3244 using ICP-OES. Two 0.5 g portions were taken from each bottle and digested in Teflon beakers in a nitric, perchloric, and hydrofluoric acid mixture. Digests were transferred to plastic bottles and diluted with the appropriate volume of 1.5 % (volume fraction) nitric acid. To correct for matrix effects caused by differences between samples and calibrants, the method of standard additions was used; spikes were added to one aliquot prepared from each 0.5 g test portion. Four measurements using ICP-OES were made and averaged for each sample and each spiked solution. Results were corrected for spike recoveries.

A second set of samples was prepared for determination of copper, iron, manganese, phosphorus, potassium, and sodium. A single 1.0 g test portion was taken from each of five bottles, and two 1.0 g test portions were taken from one bottle of SRM 3244. Samples were digested in quartz microwave vessels with 12 mL nitric acid. Two digestions were required because of the large sample size. Digests were transferred to plastic bottles and diluted with the appropriate volume of 1.5 % (volume fraction) nitric acid. To correct for matrix effects caused by differences between samples and calibrants, the method of standard additions was used; spikes were added to one aliquot prepared from each test portion. Four measurements using ICP-OES were made and averaged for each sample and each spiked solution. Results were corrected for spike recoveries.

Analytical Approach for Determination of Toxic Elements

Potentially toxic elements (arsenic, cadmium, lead, and mercury) were also of interest in SRM 3244. Value assignment of the concentrations of toxic trace elements in SRM 3244 was based on the combination of measurements at NIST using a single analytical method and results from one or two collaborating laboratories (NRCC and FDA). At NIST, instrumental neutron activation analysis (INAA) was used for the determination of arsenic, isotope dilution inductively coupled plasma mass spectrometry (ID ICP-MS) was used for the determination of cadmium and lead, and cold vapor (CV) ID ICP-MS was used for determination of mercury. For all NIST measurements, botanical-matrix SRMs with certified values for the elements of interest were analyzed concurrently as control samples. NRCC used ID ICP-MS for the determination of lead and hydride generation graphite furnace atomic absorption spectrometry (HG GFAAS) for the determination of arsenic. FDA provided results for lead using ICP-MS. FDA also provided results using prompt gamma activation analysis (PGAA) for the concentrations of boron, calcium, carbon, chlorine, hydrogen, iron, magnesium, nitrogen, potassium, silicon, and sulfur. All collaborating laboratories analyzed a minimum of six subsamples (one from each of six bottles or two from each of three bottles) of SRM 3244.

NIST Analyses for Toxic Elements

Arsenic was measured using instrumental neutron activation analysis (INAA). Individual disks were formed from 200 mg test portions of the SRM using a stainless steel die and hydraulic press. Portions of three additional SRMs were prepared in the same manner and included for quality control. 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, placed together in a polyethylene irradiation container, and exposed to a neutron fluence rate of 1×10^{14} $cm^{-2} \cdot s^{-1}$ for a total of 2 h. Decay times were approximately 5 d to 7 d. Gamma rays were collected using an intrinsic germanium detector with a relative efficiency of 35 % and a resolution of 1.75 keV (full-width at half maximum peak height for the 1333-keV line from ^{60}Co). The arsenic concentration in SRM 3244 was below the 30 ng/g limit of detection.

Lead, cadmium, and mercury were quantified by ID ICP-MS. For cadmium and lead determinations, a single 1 g portion was taken from each of six bottles of the SRM and weighed by difference into Teflon vessels. Isotopically enriched ^{111}Cd and ^{206}Pb were added and the samples digested in a microwave oven with nitric and hydrofluoric acids. For lead, a small portion of the digested sample was diluted with nitric acid to contain a ^{206}Pb concentration of approximately 5 ng/g, and the $^{206}Pb/^{208}Pb$ ratio was measured by ICP-MS [10]. Preliminary investigation showed that matrix-related interferences were present at the cadmium masses. Thus the remaining sample digest was converted to the chloride and re-dissolved in a mixture of hydrochloric and hydrofluoric acids, and cadmium was separated from the matrix using anion exchange chromatography. After separation, sample components were reconverted to the nitrate, the samples were diluted with nitric acid to contain a ^{111}Cd mass fraction of approximately 2.5 ng/g, and the $^{111}Cd/^{114}Cd$ measured by ICP-MS [10]. For mercury determinations, a single 0.30 g portion was taken from each of six bottles of the SRM. Isotopically enriched ^{201}Hg was added to the samples prior to digestion in quartz vessels with nitric acid in a high-pressure microwave system. Following digestion, samples were diluted to contain an approximate ^{201}Hg concentration of 0.05 ng/g. Samples were allowed to degas overnight at 4 °C. Measurements were made by using cold-vapor mercury generation (using tin [II] chloride reductant) coupled with ID ICP-MS [11].

NIST Determination of Moisture

Moisture content of SRM 3244 was determined by (1) freeze-drying to constant mass over 7 days; (2) drying over magnesium perchlorate in a desiccator at room temperature for 5 days; and (3) drying in a forced-air oven at 85 °C for 4 h. Unweighted results obtained using all three techniques were averaged to determine a conversion factor of 0.9643 grams dry mass per gram as-received mass, which was used 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 using their own moisture determinations. A variability-in-moisture component is included in the uncertainties of both the certified and reference values, reported on a dry-mass basis, that are provided in this certificate.

Homogeneity Assessment

The homogeneity of selected elements was assessed at NIST by using the ICP-OES method described in section "NIST Analyses for Nutritive Elements". An analysis of variance using NIST's ICP-OES measurements of calcium, copper, iron, potassium, magnesium, manganese, sodium, phosphorus, and zinc did not show significant inhomogeneity for 0.5 g samples. Because inhomogeneity appeared to be significant relative to other sources of uncertainty, a prediction interval was used to establish the uncertainty on the certified value for cadmium. An analysis of variance using NIST's LC/UV measurements of caffeine (600 mg to 900 mg samples) and vitamins B_2 and B_6 (2 g samples) also did not show inhomogeneity. Other measurands were treated as though they were homogeneously distributed in the material, although homogeneity was not assessed.

Value Assignment

The equally weighted means from each set of data were used to calculate the assigned values unless otherwise stated in the tables. Results from collaborating laboratories were considered individual data sets except for the FPA FIACS interlaboratory comparison exercise, in which case an exercise mean was calculated from each of the laboratory means; this exercise mean, the NIST means, and the means of data from other collaborating laboratories (where available) were weighted equally to calculate the assigned values. In cases where NIST did not make measurements, the mean of the data set means became the assigned value.

Table 1. Certified Concentration Values for Ephedrine Alkaloids and Caffeine in SRM 3244[a]

Analyte	Mass Fraction (mg/g)		
Ephedrine[b,c,d,e,f,g,h,i]	0.242	±	0.038
Methylephedrine[b,c,d,h,i]	0.0075	±	0.0024
Pseudoephedrine[b,c,d,e,f,g,h,i]	0.0361	±	0.0086
Total Ephedrine Alkaloids[b,c,d,i]	0.296	±	0.067
Caffeine[b,d,g]	2.99	±	0.54

[a] Each certified concentration value, expressed as a mass fraction on a dry-mass basis, is the equally weighted mean of results from three to eight analytical methods carried out at NIST and at collaborating laboratories using the analytical methods listed in the footnotes below. The uncertainty in the certified values, calculated according to the method described in the ISO Guide [2,3], 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 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 approximately 95 % confidence for each analyte.
[b] NIST LC/UV
[c] NIST LC/MS
[d] NIST LC/MS/MS
[e] NIST CE
[f] FDA LC/MS/MS
[g] ChromaDex LC/UV
[h] NRCC LC/UV
[i] NRCC LC/MS/MS

Table 2. Certified Concentration Values for Selected Water-Soluble Vitamins in SRM 3244[a]

Analyte	Mass Fraction (mg/kg)		
Vitamin B_6[b]	34.1	±	2.2
Niacin	304	±	10

[a] Each certified concentration value, expressed as a mass fraction on a dry-mass basis, is the equally weighted mean of results from NIST and the mean of results provided by the FPA FIACS laboratories listed in Appendix A. Analytical methods used are provided in Appendix C. The uncertainty in the certified values, calculated according to the method described in the ISO Guide [2,3] 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 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 approximately 95 % confidence for each analyte.
[b] Pyridoxine hydrochloride

Table 3. Certified Concentration Values for Selected Elements in SRM 3244[a]

Element	Mass Fraction (%)		
Calcium	1.328	±	0.090
Phosphorus	1.220	±	0.088
Potassium	1.60	±	0.18

Element	Mass Fraction (mg/kg)		
Cadmium[b]	0.01266	±	0.00069
Copper	10.2	±	1.0
Lead[c]	0.0270	±	0.0027
Magnesium	3100	±	120
Manganese	30.0	±	1.4
Mercury[d]	0.000253	±	0.000033
Sodium	910	±	100
Zinc	126.4	±	7.7

[a] Each certified concentration value, expressed as a mass fraction on a dry-mass basis, is the equally weighted mean of results from analyses by NIST and collaborating laboratories. Analytical methods used for each element are provided in Appendix C. The uncertainty in the certified values, calculated according to the method described in the ISO Guide [2,3], 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 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 approximately 95 % confidence for each analyte.

[b] The certified concentration value for cadmium, 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 certified value, calculated according to the method described in the ISO Guide [2,3], is expressed as an expanded uncertainty, U. Because of concerns about possible inhomogenity of cadmium, a prediction interval is used to represent the expanded uncertainty. 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, drying, and possible inhomogeneity 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.

[c] Lead was determined by ID ICP-MS at NIST and NRCC, and by ICP-MS at FDA.

[d] The certified value for mercury, expressed as a mass fraction on a dry-mass basis, was determined using a definitive technique, ID ICP-MS, at NIST. The uncertainty in the certified value, calculated according to the method described in the ISO Guide [2,3], 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 95 % confidence for each analyte.

Table 4. Reference Concentration Values for Alkaloids in SRM 3244[a]

Analyte	Mass Fraction (mg/g)
Methylpseudoephedrine[b,c]	0.00028 ± 0.00011
Theobromine	0.762 ± 0.026
Theophylline	0.080 ± 0.003

[a] Each reference concentration value, expressed as a mass fraction on a dry-mass basis, is the equally weighted mean of results from one analytical method (LC/UV) at NIST. The uncertainty in the reference values, calculated according to the method described in the ISO Guide [2,3], 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 95 % confidence for each analyte.
[b] NIST LC/MS/MS
[c] NRCC LC/UV

Table 5. Reference Concentration Values for Selected Water-Soluble Vitamins in SRM 3244[a]

Analyte	Mass Fraction (mg/kg)
Vitamin C	890 ± 100
Vitamin B_1[b]	20.5 ± 3.6
Vitamin B_2	29.9 ± 2.3
Vitamin B_{12}	0.107 ± 0.017
Pantothenic Acid	172 ± 33
Biotin	4.36 ± 0.38
Folic Acid	5.4 ± 1.2
Choline Ion	1500 ± 600
Inositol	1550 ± 450

[a] Each reference concentration value, expressed as a mass fraction on a dry-mass basis, is the mean of results provided by the laboratories listed in Appendix A. The uncertainty in the reference values, calculated according to the method described in the ISO Guide [2,3] 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 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. Analytical methodology information is provided in Appendix C.
[b] Thiamine, **NOT** thiamine hydrochloride

Table 6. Reference Concentration Values for Selected Elements in SRM 3244[a]

Element	Mass Fraction (%)		
Carbon[c]	44.7	±	1.5
Hydrogen[c]	6.13	±	0.06
Silicon[c]	0.499	±	0.022
Sulfur[c]	0.650	±	0.010

Element	Mass Fraction (mg/kg)		
Arsenic[b]	0.0196	±	0.0027
Boron[c]	3.56	±	0.13
Chlorine[c]	800	±	48
Iron[d,e]	107	±	15

[a] Each reference concentration value, expressed as a mass fraction on a dry-mass basis, is the equally weighted mean of results provided by collaborating laboratories. The uncertainty in the reference values, calculated according to the method described in the ISO Guide [2,3], 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 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. Analytical methodology information is provided in footnotes below.

[b] NRCC HG-GFAAS

[c] FDA PGAA

[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 [2,3], 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 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.

[e] See methods provided in Appendix C.

Table 7. Reference Values for Proximates, Selected Fatty Acids (As Triglycerides), and Caloric Content in SRM 3244[a]

Analyte	Mass Fraction (%)		
Solids	96.4	±	1.2
Ash	9.11	±	0.36
Protein	66.1	±	1.3
Fat[b]	1.41	±	0.18
Carbohydrate (by difference)[b]	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[c]	(366.5	±	9.6) kcal/100 g

[a] Each reference concentration value, expressed as a mass fraction on a dry-mass basis, is the mean of results provided by the laboratories listed in Appendix A. The uncertainty in the reference values, calculated according to the method described in the ISO Guide [2,3], 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 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. Analytical methodology information is provided in Appendix C.

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

[c] The value for caloric content is the mean of individual caloric calculations from the laboratories listed in Appendix A. 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.

Table 8. Reference Concentration Values for Amino Acids in SRM 3244[a]

Analyte	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 a dry-mass basis, is the mean of results provided by the laboratories listed in Appendix A. The uncertainty in the reference values, calculated according to the method described in the ISO Guide [2,3], 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 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. Analytical methodology information is provided in Appendix C.

Table 9. Information Values for Ephedrine Alkaloids in SRM 3244[a]

Analyte	Mass Fraction (mg/g)
Norephedrine[b,c,d,e]	0.0030
Norpseudoephedrine[c,d,e,f]	0.0034

[a] Each information value, expressed as a mass fraction on a dry-mass basis, is an equally weighted mean of the results from two or four analytical methods carried out at NIST and at collaborating laboratories using the analytical methods listed in the footnotes below. Uncertainties, expressed as expanded uncertainties (U) about the mean ($\bar{x}$) following the ISO Guide [2,3], are too large to permit assignment of certified or reference values. Expanded uncertainties for these analytes ranged from 0.0028 mg/kg for norephedrine (a relative expanded uncertainty, REU, of 95 %) to 0.0025 mg/kg for norpseudoephedrine (REU of 73 %). 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 approximately 95 % confidence for each analyte.

[b] NIST LC/UV

[c] NIST LC/MS

[d] NIST LC/MS/MS

[e] NRCC LC/UV

[f] NRCC LC/MS/MS

REFERENCES

[1] 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*; NIST Special Publication 260-135; U.S. Government Printing Office: Washington, DC (2000); available at http://www.cstl.nist.gov/nist839/special_pubs/SP260136.pdf.

[2] ISO; *Guide to the Expression of Uncertainty in Measurement*; ISBN 92-67-10188-9, lst ed.; International Organization for Standardization: Geneva, Switzerland (1993); 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 http://physics.nist.gov/Pubs.

[3] 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).

[4] Taylor, B.N.; *Guide for the Use of the International System of Units (SI)*; NIST Special Publications 811, U.S. Government Printing Office: Washington, DC (1995); available at http://www.physics.nist.gov/Pubs.

[5] Gay, M.L.; White, K.D.; Obermeyer, W.R.; Betz, J.M.; Musser, S.M.; *Determination of Ephedrine Type Alkaloids in Dietary Supplements by LC/MS Using a Stable Isotope Labeled Internal Standard*; J. AOAC Int., Vol. 84, pp. 761–769 (2001).

[6] Roman, M.C.; *Determination of Ephedrine Alkaloids in Botanicals and Dietary Supplements by HPLC-UV*: *Collaborative Study*; J. AOAC Int., Vol. 87, pp. 1–14 (2004).

[7] Sander, L.C.; Sharpless, K.E.; Satterfield, M.B.; Ihara, T; Phinney, K.W.; Roman, M.; Yen. J.H.; Wise, S.A.; Gay, M.L.; Lam, J.W.; McCooeye, M.; Gardner, G.; Fraser, C.; Sturgeon, R.; Roman, M.; *Determination of Ephedrine Alkaloids in Dietary Supplement Standard Reference Materials*; Anal. Chem., Vol. 77, pp. 3101–3112 (2005).

[8] Phinney, K.W.; Ihara, T.; Sander, L.C.; *Determination of Ephedrine Alkaloid Stereoisomers in Dietary Supplements by Capillary Electrophoresis*; J. Chromatogr. A, Vol. 1077, pp. 90–97 (2005).

[9] Brown Thomas, J.M.; Yen, J.H.; Schantz, M.M.; Porter, B.J.; Sharplesss, K.E.; *Determination of Caffeine, Theobromine, and Theophylline in Standard Reference Material 2384 Baking Chocolate Using Reversed-Phase Liquid Chromatography*; J. Agric. Food Chem., Vol. 52, pp. 3259–3263 (2004).

[10] Murphy, K.E.; Beary, E.S.; Rearick, M.S.; Vocke, R.D.; *Isotope Dilution Inductively Coupled Plasma Mass Spectrometry (ID ICP-MS) for the Certification of Lead and Cadmium in Environmental Standard Reference Materials*; Fresenius' J. Anal. Chem., Vol. 368, pp. 362–370 (2000).

[11] Christopher, S.J.; Long, S.E.; Rearick, M.S.; Fassett, J.D.; *Development of Isotope Dilution Cold Vapor Inductively Coupled Plasma Mass Spectrometry and Its Application to the Certification of Mercury in NIST Standard Reference Materials*; Anal. Chem., Vol. 73, pp. 2190–2199 (2001).

Certificate Revision History: 10 August 2007 (Editorial changes); 20 March 2006 (Original certificate date).

Users of this SRM should ensure that the certificate in their possession is current. This can be accomplished by contacting the SRM Program at: telephone (301) 975-6776; fax (301) 926-4751; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.

APPENDIX A

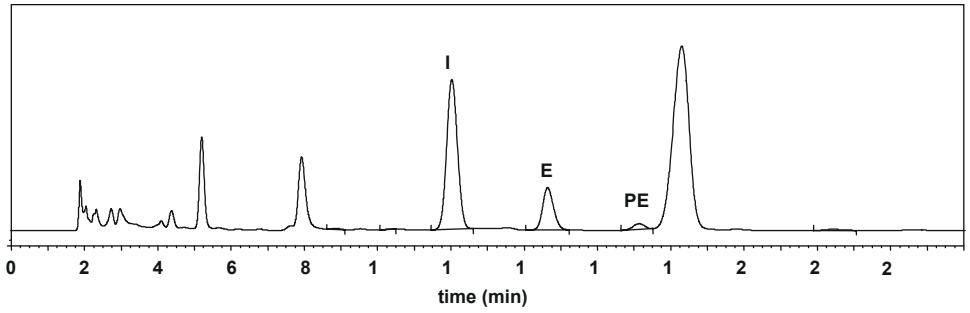
The laboratories listed below contributed to value assignment of SRM 3244 Ephedra-Containing Protein Powder as part of the FPA FIACS interlaboratory comparison exercise.

Campbell Soup Company (Camden, NJ)
Covance (Madison, WI)
Eurofins Scientific, Inc. (Memphis, TN)
General Mills, Inc. (Golden Valley, MN)
Hormel Foods Corporatio (Austin, MN)
Kraft East (East Hanover, NJ)
Kraft Foods (Glenview, IL; analyses performed by Silliker Laboratories)
Nestlé Foods Corporation (Dublin, OH)
Nestlé-Purina Pet Care (St. Louis, MO)
Novartis Nutrition Corporation (St. Louis Park, MN)
US Department of Agriculture, Nutrient Composition Laboratory (Beltsville, MD)

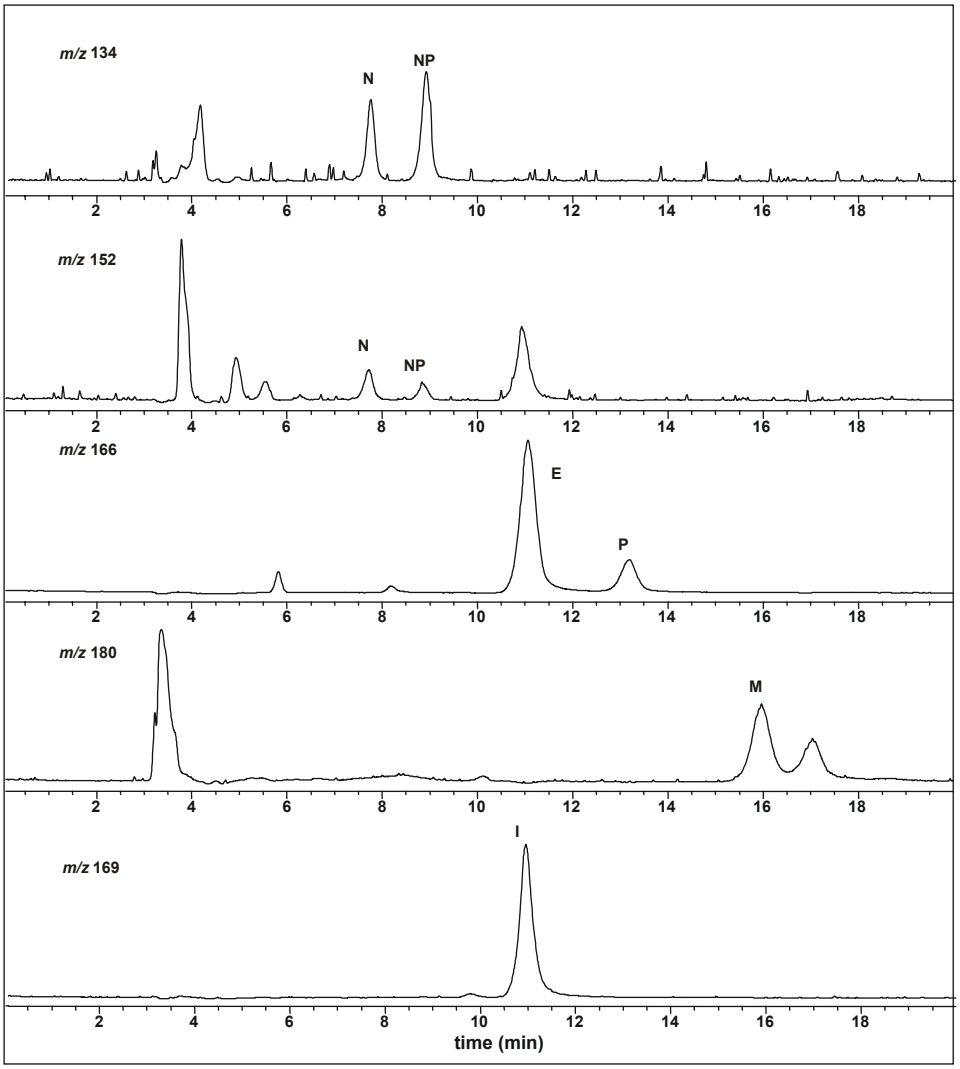
APPENDIX B

Typical chromatograms from the analysis of SRM 3244 by using a) LC/UV; b) LC/MS; c) LC/MS/MS; and d) CE. Components are identified as follows: norephedrine (NE); norpseudoephedrine (NPE); ephedrine (E); pseudoephedrine (PE); methylephedrine (ME); methylpseudoephedrine (MPE); internal standard (IS); (–)-ephedrine [(–)-E]; and (+)-pseudoephedrine [(+)-PE].

a



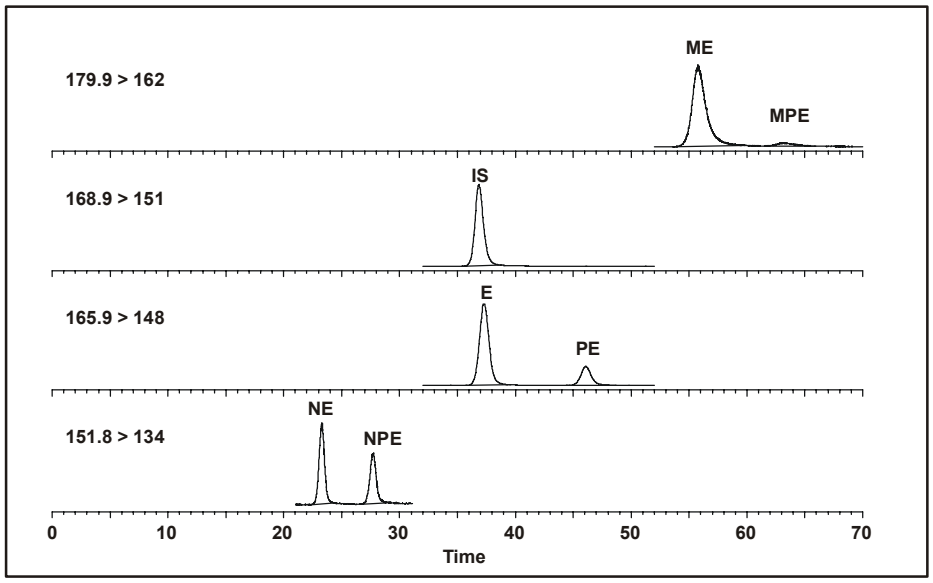
b)



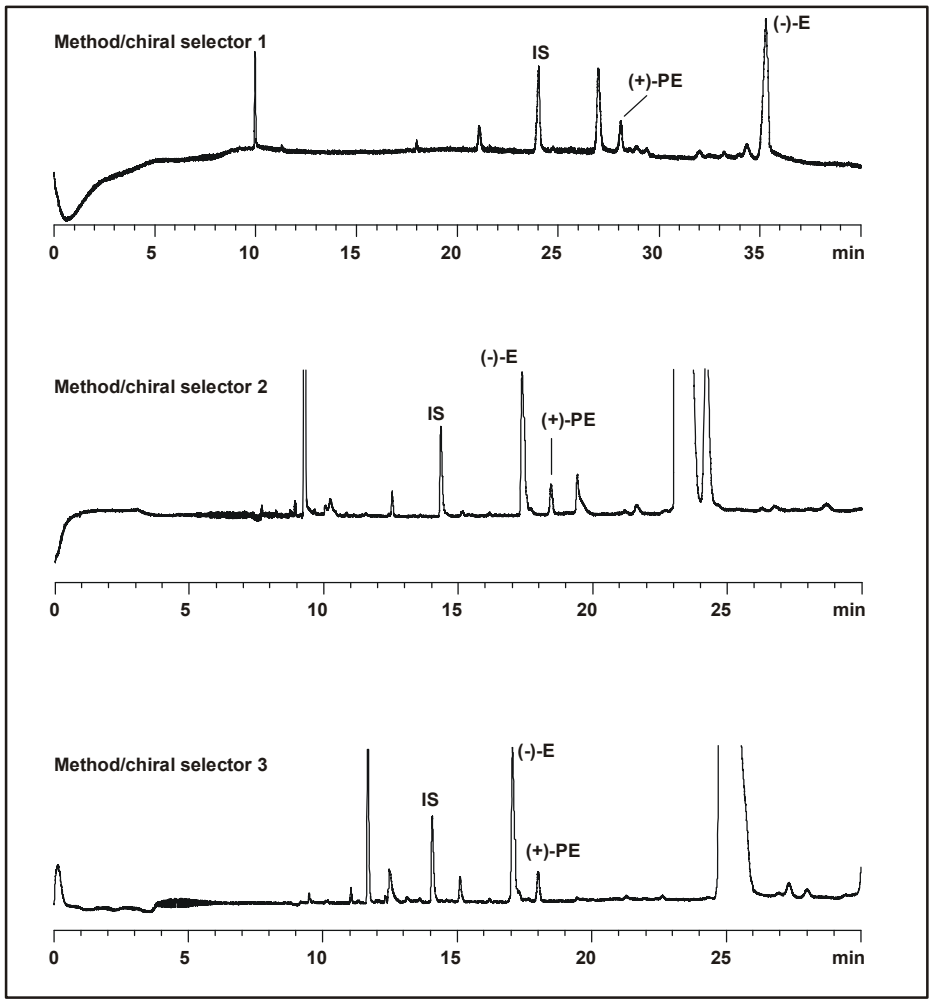
Appendix B (continued)

Typical chromatograms from the analysis of SRM 3244 by using a) LC/UV; b) LC/MS; c) LC/MS/MS; d) CE.

c)



d)



APPENDIX C

The methodological information reported by laboratories whose results were used for value assignments is summarized below. The number of laboratories using a particular method is provided in parentheses.

Proximates, Fatty Acids, Amino Acids and Calories

Solids	Moisture determined by mass loss after drying: Forced-air oven (2 + NIST) Vacuum oven (7) Freeze-dryer (NIST) Desiccator (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) Prompt gamma activation analysis (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, liquid chromatography (LC) (5) Amino acid analyzer (1)
Calories	Calculated; 9(fat) + 4(protein) + 4(carbohydrate)

Vitamins

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) RPLC – absorbance detection (NIST) Microbiological (4)
Total Vitamin B_{12}	Microbiological (6)
Niacin	Microbiological (6) RPLC – absorbance detection (NIST)
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)

Elements

Calcium	Direct current plasma atomic emission spectrometry (1) Inductively coupled plasma optical emission spectrometry (7 + NIST) Prompt gamma activation analysis (1)
Copper	Direct current plasma atomic emission spectrometry (1) Inductively coupled plasma optical emission spectrometry (6 + NIST)
Iron	Inductively coupled plasma optical emission spectrometry (7 + NIST)
Magnesium	Direct current plasma atomic emission spectrometry (1) Inductively coupled plasma optical emission spectrometry (7 + NIST)
Manganese	Inductively coupled plasma optical emission spectrometry (6 + NIST)
Phosphorus	Absorption spectrophotometry (3) Inductively coupled plasma optical emission spectrometry (9 + NIST) Prompt gamma activation analysis (1)
Potassium	Direct current plasma atomic emission spectrometry (1) Inductively coupled plasma optical emission spectrometry (6 + NIST)
Sodium	Direct current plasma atomic emission spectrometry (1) Inductively coupled plasma optical emission spectrometry (6 + NIST)
Zinc	Direct current plasma atomic emission spectrometry (1) Inductively coupled plasma optical emission spectrometry (7 + NIST)