National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 2385

Slurried Spinach

This Standard Reference Material (SRM) is intended primarily for use in validating methods for determining proximates, calories, carotenoids, vitamins, and elements in spinach and similar matrices. This SRM can also be used for quality assurance when assigning values to in-house control materials. A unit of SRM 2385 consists of four jars of slurried spinach containing approximately 70 g each.

Certified Concentration Values: The certified concentration values of elements and carotenoids in SRM 2385 are provided in Tables 1 and 2. Values were derived from the combination of results provided by NIST and collaborating laboratories. 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 by NIST [1]. The certified values in this material are the equally weighted means of the mean NIST result(s) and the mean of the measurements made by collaborating laboratories; the associated uncertainties are expanded uncertainties at the 95 % level of confidence [2,3]. Values are reported on an as-received (not dry-mass) basis in mass fraction units [4].

Reference Concentration Values: Reference concentration values for proximates, energy content, total dietary fiber, vitamins, and additional carotenoids are provided in Tables 3 through 5. Reference values are noncertified values that are the best estimate of the true values based on available data; however, the values do not meet the NIST criteria for certification [1] and are provided with associated uncertainties that may reflect only measurement reproducibility, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods.

Expiration of Value Assignment: The value assignment of this SRM is valid until **30 September 2010**, 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. Return of the attached registration card will facilitate notification.

Coordination of the technical measurements leading to the certification of this SRM was performed by K.E. Sharpless of the NIST Analytical Chemistry Division, H.B. Chin of the National Food Processors Association (NFPA, Dublin, CA), and D.W. Howell (NFPA, Washington, DC).

Analytical measurements at NIST were performed by J.M. Brown Thomas, B.J. Porter, K.E. Sharpless, and L.J. Wood of the NIST Analytical Chemistry Division. Analyses for value assignment were also performed by the laboratories listed in Appendices A and B.

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

Willie E. May, Chief
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Certificate Issue Date: 09 December 2003

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The support aspects involved with the certification and issuance of this SRM were coordinated through the NIST Standard Reference Materials Program by J.C. Colbert and B.S. MacDonald of the NIST Measurement Services Division.

NOTICE AND WARNING TO USERS

Storage: The spinach should be refrigerated at 4 °C 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 jar of spinach should be mixed thoroughly and homogenized (e.g., using a rotor/stator-type blender) for 1 min prior to removal of a test portion. Test portions used for NIST analyses described below were 1.5 g for carotenoids and 7 g for elements.

PREPARATION AND ANALYSIS

Preparation: SRM 2385 was prepared from a spinach crop that was held under refrigeration following harvesting, then was washed lightly in a wash reel with cold water and blanched at 93 °C. It was then pureed by passing it through a Bertocchi cold extractor 0.635-cm (0.250-inch) screen. The puree was heated to 90.5 °C in a batch tank and the consistency was adjusted to 3 to 5 Bostwick units with water. The puree was then passed through a Fitzmill 0.084-cm (0.033-inch) screen, filled into 2688 jars to contain approximately 70 g (2.5 oz) of slurried spinach each, and capped. The spinach was processed in two retort loads. The jars were retorted at 121 °C and 207 kPa for 35 min.

NIST Analyses for Elements: Calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc were measured using inductively coupled plasma optical emission spectrometry (ICP-OES). Two 7 g portions were taken from each of five jars of spinach and digested with nitric acid using a high-pressure microwave sample preparation system. 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 7 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.

NIST Analyses for Carotenoids: Carotenoids (lutein and β-carotene) were measured using combinations of two sample preparation methods and two liquid chromatographic (LC) methods as described below.

Sample Preparation 1. Carotenoids were measured in duplicate test portions taken from six jars of SRM 2385 on each of two days (n = 12) using a C_{18} analytical column (for homogeneity assessment) and a C_{30} analytical column; LC methods are described below. Single samples from six different jars were analyzed using the same C_{18} column several months later. For all analyses, an entire jar of spinach was homogenized using a rotor/stator-type tissue homogenizer for 1 min, then approximately 1.5 g of material was combined with calcium carbonate, an ethanolic internal standard solution, tetrahydrofuran (THF), and methanol, and the mixture was homogenized for 1 min. This mixture was then vacuum-filtered, and aqueous sodium chloride solution was added to the filtrate. The analytes were extracted into a mixture of hexane and diethyl ether. This solution was washed with water, and the organic phase was then evaporated under nitrogen. Dried extracts were reconstituted with 1 mL ethanol, and portions of the resultant solution were injected into the appropriate LC system.

Sample Preparation 2. Carotenoids were measured in individual test portions from four jars of SRM 2385 on a single day using a C_{30} analytical column. Carotenoids were measured in duplicate test portions from three different jars of SRM 2385 on a single day using a C_{18} column. For all analyses, an entire jar of spinach was homogenized for 1 min, then approximately 1.5 g of the spinach was combined with an ethanolic internal standard solution, THF, and methanol. The mixture was homogenized for approximately 45 s, and the beaker containing the mixture was placed in a 40 °C water bath. Methanolic potassium hydroxide was added, and the mixture was saponified for 30 min. Glacial acetic acid was then added to neutralize any remaining potassium hydroxide. Aqueous sodium chloride solution was added, and the analytes were extracted into a mixture of hexane and diethyl ether. The organic phase was washed with water, and the organic solvents were evaporated under nitrogen. The residue was redissolved in 500 µL ethanol.

Chromatographic Analysis.

C_{18} Column. Lutein, *trans*-β-carotene, and total β-carotene were measured using a C_{18} analytical column and a gradient consisting of acetonitrile, methanol, and ethyl acetate [5,6]. A programmable UV/visible absorbance detector with a tungsten lamp was used to measure the carotenoids at 450 nm.

C_{30} Column. Lutein, *trans*-β-carotene, and total β-carotene were measured using a C_{30} analytical column and a gradient consisting of methanol, water, and methyl *tert*-butyl ether [7,8]. A programmable UV/visible absorbance detector with a tungsten lamp was used for measurement of the carotenoids at 450 nm.

Analyses by Collaborating Laboratories: Data from four additional sources were used for value assignment of this material: an interlaboratory comparison exercise organized by the NFPA's Food Industry Analytical Chemists Subcommittee (FIACS; 14 participating laboratories, listed in Appendix A); and three separate interlaboratory comparison exercises organized by NIST in which carotenoids and vitamins were measured by 19 laboratories (Appendix B). Not every laboratory measured every analyte. The laboratories listed in Appendix A were asked to use AOAC methods or their equivalent, to make single measurements from each of two jars, and to report the analytical method that was used. The laboratories listed in Appendix B were asked to use their usual methods to make single measurements of carotenoids and/or vitamins in each of two or three jars. A summary of the methodological information and the number of laboratories using a particular analytical technique is provided in Appendix C. The methods used by NIST are included in this listing as well.

Homogeneity Assessment: The homogeneity of calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc, and carotenoids was assessed at NIST using the methods described above. Carotenoids and iron, manganese, phosphorus, sodium, and zinc were found to be homogeneous. A component of inhomogeneity has been added to the uncertainty for the following elements: calcium (2.2 %), copper (5.7 %), magnesium (3.0 %), and potassium (2.4 %). Other measurands (e.g., ash, carbohydrates, etc.) were treated as though they were homogeneous, although homogeneity was not assessed.

Value Assignment: The laboratories listed in Appendix A reported values from one to eight analyses. The laboratories listed in Appendix B reported values from two to six analyses. The mean for each laboratory was determined from these values, and a mean of laboratory means was calculated. In the case of the elements and solids, where NIST also made measurements, the NIST mean was averaged with the mean of the laboratory means to obtain the assigned value. (In the case of sodium, only NIST's result was used to assign the reference value.) In the case of the carotenoids, where four analytical methods were employed by NIST, each method mean was equally weighted with the mean of the combined interlaboratory comparison exercises. In cases where NIST did not make measurements, the mean of the laboratory means became the assigned value.

Table 1. Certified Concentration Values for Elements[a]

	Mass Fraction (mg/kg)		
Calcium	624	±	40
Iron	17.1	±	1.9
Magnesium	368	±	30
Manganese	3.81	±	0.10
Phosphorus	323.7	±	6.6
Potassium	3650	±	250
Zinc	8.37	±	0.37

[a] Each certified concentration value, expressed as a mass fraction on an as-received basis, is the weighted mean of results provided by laboratories listed in Appendix A and NIST. 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 and within-laboratory 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 2. Certified Concentration Values for Carotenoids[a]

	Mass Fraction (mg/kg)		
Total Lutein (includes esters)	32.9	±	6.5
Total β-Carotene	19.2	±	2.9

[a] Each certified concentration value, expressed as a mass fraction on an as-received basis, is the weighted mean of results provided by laboratories listed in Appendices A and B and NIST. 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 and within-laboratory 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 3. Reference Values for Proximates and Energy Content[a]

	Mass Fraction (%)		
Solids[b]	5.28	±	0.10
Ash	0.97	±	0.05
Fat[c]	0.20	±	0.06
Protein	1.42	±	0.13
Carbohydrate (by difference)	2.73	±	0.18
Total Dietary Fiber	1.55	±	0.28
Energy[d]	(18.16	±	0.50) kcal/100 g

[a] Each reference value, expressed as a mass fraction on an as-received basis, is the weighted mean of results provided by laboratories listed in Appendix A. The uncertainty in the reference values, calculated according to the method described in the ISO Guide [2], 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 and within-laboratory 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. (The certified values for fat are provided in Table 1.)

[b] The reference value, expressed as a mass fraction on an as-received basis, is the weighted mean of results provided by laboratories listed in Appendix A and NIST. 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 and within-laboratory 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. Analytical methodology information is provided in Appendix C.

[c] As the sum of fatty acids

[d] The value for energy 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, protein, and carbohydrate, respectively, the mean caloric content is 18.4 kcal/100 g.

Table 4. Reference Concentration Values for Selected Elements

	Mass Fraction (mg/kg)		
Copper[a]	0.90	±	0.16
Sodium[b]	47	±	1

[a] The reference concentration value for copper, expressed as a mass fraction on an as-received basis, is the weighted mean of results provided by laboratories listed in Appendix A and NIST. 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 and within-laboratory 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. Analytical methodology information is provided in Appendix C.

[b] The reference concentration value for sodium, expressed as a mass fraction on an as-received basis, is the mean result of analyses performed by NIST using one analytical technique. The expanded uncertainty, U, is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined standard uncertainty calculated according to the ISO Guide [2]. 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 5. Reference Concentration Values for Selected Vitamins and *Trans*-β-Carotene[a]

	Mass Fraction (mg/kg)		
Vitamin B_2	0.73	±	0.05
Niacin	2.99	±	0.43
Trans-β-Carotene[b]	15.1	±	3.1

[a] Each reference concentration value, expressed as a mass fraction on an as-received basis, is the weighted mean of results provided by laboratories listed in Appendices A and B. The uncertainty in the reference values, calculated according to the method described in the ISO Guide [2], 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 and within-laboratory 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] A reference value is provided for this analyte because the concentration may be affected by isomerization of β-carotene during sample preparation. The reference concentration value, expressed as a mass fraction on an as-received basis, is the weighted mean of results provided by laboratories listed in Appendices A and B and NIST. 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 and within-laboratory 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.

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 260-136, U.S. Government Printing Office: Washington, DC (2000).

[2] *Guide to the Expression of Uncertainty in Measurement*; ISBN 92-67-10188-9, 1st Ed.; ISO, 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.L.; 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 Publication 811, U.S. Government Printing Office: Washington, DC, 1994 Ed., (1994); available at http://physics.nist.gov/Pubs.

[5] Epler, K.S.; Ziegler, R.G.; Craft, N.E.; *Liquid Chromatographic Method for the Determination of Carotenoids, Retinoids, and Tocopherols in Human Serum and in Food*; J. Chromatogr. (Biomed. Applications) Vol. 619, pp. 37-48 (1993).

[6] Brown Thomas, J.; Kline, M.C.; Schiller, S.B.; Ellerbe, P.M.; Sniegoski, L.T.; Duewer, D.L.; Sharpless, K.E.; *Certification of Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum: Standard Reference Material 968b*; Fresenius' J. Anal. Chem. Vol. 356, pp. 1-9 (1996).

[7] Sander, L.C., Epler, K.S., Sharpless, K.; Craft, N.E.; Wise, S.A.; *Development of Engineered Stationary Phases for the Separation of Carotenoid Isomers*; Anal. Chem. Vol. 66, pp. 1667-1674 (1994).

[8] Sharpless, K.E.; Brown Thomas, J.; Sander, L.C.; Wise, S.A.; *Liquid Chromatographic Determination of Carotenoids in Human Serum Using an Engineered C_{30} and a C_{18} Stationary Phase*; J. Chromatogr., B Vol. 678, pp. 187-195 (1996).

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 http://www.nist.gov/srm.

APPENDIX A

The laboratories listed below performed measurements as part of the NFPA FIACS exercise that contributed to the value assignment of SRM 2385 Slurried Spinach.

Campbell Soup Company; Camden, NJ, USA
Centro de Investigación y Asistencia Técnica a la Industria; Provincia de Río Negro, Argentina
Covance, Inc.; Madison, WI, USA
Del Monte Foods; Walnut Creek, CA, USA
General Mills, Inc.; Golden Valley, MN, USA
Gerber Products Company; Fremont, MI, USA
Hormel Foods Corporation; Austin, MN, USA
Kraft Foods, Inc.; Glenview, IL, USA
Nabisco, Inc.; East Hanover, NJ, USA
National Food Laboratory; Dublin, CA, USA
Nestlé Food Corporation; Dublin, OH, USA
Novartis Nutrition Technical Center; St. Louis Park, MN, USA
TPC Labs/Pillsbury; St. Paul, MN, USA
U.S. Department of Agriculture (USDA), Food Composition Laboratory; Beltsville, MD, USA

APPENDIX B

The laboratories listed below performed vitamin and carotenoid measurements that contributed to the value assignment of SRM 2385 Slurried Spinach

AgriQuality; Auckland, New Zealand
Anchor Products; Waitoa, New Zealand
Harvard School of Public Health; Boston, MA, USA
Inspectorate for Health Protection and Veterinary Public Health; Maastricht, The Netherlands
Institute of Nutrition, Directorate of Fisheries; Bergen, Norway
Institut National de la Recherche Agronomique; Saint-Genès Champanelle, France
Laboratoire Marcel Merieux; Lyon, France
Livsmedelsverket; Uppsala, Sweden
Metametrix Clinical Laboratory, Inc.; Norcross, GA, USA
Nestlé Research Center; Lausanne, Switzerland
New Zealand Dairy Research Institute; Palmerston North, New Zealand
Puerta de Hierro; Madrid, Spain
Roche Vitamins Ltd.; Basel, Switzerland
TNO Nutrition and Food Research; Zeist, The Netherlands
University of Illinois at Chicago; Chicago, IL, USA
University of Ulster; Coleraine, County Londonderry, Northern Ireland
USDA, BHNRC; Beltsville, MD, USA
USDA – Human Nutrition Research Center on Aging at Tufts University; Boston, MA, USA
U.S. Food and Drug Administration; College Park, MD, USA

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, Total Dietary Fiber, and Calories

Solids	Moisture determined by mass loss after oven-drying: Forced-air oven (1) Vacuum oven (11) Freeze-drying (NIST)
Ash	Mass loss after ignition in muffle furnace (12)
Fat	Summation of individual fatty acids as determined by hydrolysis followed by gas chromatography (8)
Nitrogen	Kjeldahl (5) Thermal conductivity (2) Pyrolysis, gas chromatography (2) Pyrolysis, thermal conductivity (1) Pyrolysis, thermal conductivity, gas chromatography (1)
Protein	Calculated; a factor of 6.25 was used to calculate protein from nitrogen results
Carbohydrate	Calculated; [solids – (protein + fat as the sum of fatty acids + ash)]
Total Dietary Fiber	Enzymatic – gravimetry (5)
Calories	Calculated; [9(fat) + 4(protein) + 4(carbohydrate)]

Vitamins and Carotenoids

Vitamin B_2	Extraction – reversed-phase liquid chromatography (RPLC) – fluorescence detection (8) Microbiological (2) Digestion – fluorescence detection (1)
Niacin	Microbiological (7) Extraction – RPLC – absorbance detection (1) Acid digestion – absorbance detection (2)
Carotenoids	Extraction – RPLC – absorbance detection (7 + NIST) Saponification – RPLC – absorbance detection (7 + NIST) Extraction – normal-phase liquid chromatography (NPLC) – absorbance detection (1) Saponification – NPLC – absorbance detection (1)

Elements

Calcium	Flame atomic absorption spectrometry (3) Direct current plasma atomic emission spectrometry (1) Inductively coupled plasma optical emission spectrometry (8 + NIST)
Copper	Flame atomic absorption spectrometry (4) Direct current plasma atomic emission spectrometry (1) Inductively coupled plasma optical emission spectrometry (7 + NIST)
Iron	Flame atomic absorption spectrometry (4) Direct current plasma atomic emission spectrometry (1) Inductively coupled plasma optical emission spectrometry (7 + NIST)
Magnesium	Flame atomic absorption spectrometry (4) Direct current plasma atomic emission spectrometry (1) Inductively coupled plasma optical emission spectrometry (8 + NIST)
Manganese	Flame atomic absorption spectrometry (4) Direct current plasma atomic emission spectrometry (1) Inductively coupled plasma optical emission spectrometry (6 + NIST)
Phosphorus	Absorption spectrophotometry (3) Flame atomic absorption spectrometry (1) Inductively coupled plasma optical emission spectrometry (10 + NIST)
Potassium	Flame atomic absorption spectrometry (3) Flame atomic emission spectrometry (1) Direct current plasma atomic emission spectrometry (1) Inductively coupled plasma optical emission spectrometry (8 + NIST)
Sodium	Inductively coupled plasma optical emission spectrometry (NIST)
Zinc	Flame atomic absorption spectrometry (4) Direct current plasma atomic emission spectrometry (1) Inductively coupled plasma optical emission spectrometry (7 + NIST)