National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material 3254

Camellia sinensis (Green Tea) Leaves

This Standard Reference Material (SRM) is intended primarily for use in validating analytical methods for the determination of catechins and xanthines in the leaves of *Camellia sinensis* (green tea) and similar matrices. SRM 3254 can also be used for quality assurance when assigning values to in-house control materials. A unit of SRM 3254 consists of five packets, each containing approximately 3 g of leaf powder.

The development of SRM 3254 was a collaboration among the National Institute of Standards and Technology (NIST), the National Institutes of Health Office of Dietary Supplements (NIH-ODS), and the Food and Drug Administration Center for Drug Evaluation and Research (FDA CDER).

Certified Mass Fraction Values: The certified mass fraction values of selected catechins and xanthines are provided in Table 1. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account [1]. Values were derived from the combination of results provided by NIST using two independent methods and by two collaborating laboratories. The certified values in this material are the equally weighted means of the individual sets of results; the associated uncertainties are expanded uncertainties at the 95 % level of confidence [2–4]. Values are reported on a dry-mass basis in mass fraction units [5].

Reference Mass Fraction Values: Reference mass fraction values for catechin, gallocatechin, gallic acid, and theanine are provided in Table 2. 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 Certification: The certification of **SRM 3254** is valid, within the measurement uncertainties specified, until **30 November 2016**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Warning and Instructions for Storage and Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

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

Support for the development of SRM 3254 was provided in part by NIH-ODS and FDA CDER. Technical consultation was provided by J.M. Betz (NIH-ODS) and A. NguyenPho (FDA CDER).

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

Acquisition of the material was coordinated by A. NguyenPho of FDA CDER and K.E. Sharpless of the NIST Analytical Chemistry Division.

Stephen A. Wise, Chief
Analytical Chemistry Division

Gaithersburg, MD 20899
Certificate Issue Date: 15 November 2010

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

Analytical measurements at NIST were performed by M. Bedner, J.L. Molloy, K.E. Murphy, B.J. Porter, M.C. Tims, and L.J. Wood of the NIST Analytical Chemistry Division. Results were also provided by analysts at two collaborating laboratories: M. Payne at Hershey Foods Corporation (Hershey, PA) and M. Roman at Tampa Bay Analytical Research, Inc. (Largo, FL).

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

Support aspects involved with the certification and issuance of this SRM were coordinated through the NIST Measurement Services Division.

WARNING AND INSTRUCTIONS FOR STORAGE AND USE

Warning: For laboratory use only. Not for human consumption.

Storage: The material should be stored at controlled room temperature (20 °C to 25 °C), in an unopened packet, until needed.

Use: Prior to removal of a test portion for analysis, the contents of a packet of material should be mixed thoroughly. For certified values to be valid, test portions of the powder equal to or greater than 400 mg for catechin analyses and 70 mg to 100 mg for xanthine analyses should be used. The stability of catechins, xanthines, and theanine in opened packets has not been investigated. Test portions should be analyzed as received and results converted to a dry-mass basis by determining moisture content (described below) on a separate test portion.

PREPARATION AND ANALYSIS[1]

Material Acquisition and Preparation: The material for production of SRM 3254 was received as nominally 250 µm (60 mesh) particles and was further ground and sieved to 180 µm (80 mesh). The sieved material was transferred to High-Purity Standards (Charleston, SC) where it was blended, aliquotted, and heat-sealed inside nitrogen-flushed 4 mil polyethylene bags, which were then sealed inside nitrogen-flushed aluminized plastic bags along with two packets of silica gel each. Following packaging, SRM 3254 was irradiated (Neutron Products, Inc., Dickerson, MD) to an absorbed dose of 7.9 kGy to 9.5 kGy.

Analytical Approach for Determination of Catechins, Gallic Acid, Xanthines, and Theanine: Value assignment of the concentrations of the catechins, gallic acid, and xanthines in SRM 3254 was based on the combination of measurements from two different liquid chromatography (LC) methods with different detection, i.e., ultraviolet absorbance detection (UV) and mass spectrometry (MS) and results provided by collaborating laboratories using LC/UV and LC with fluorescence detection (LC/FL). NIST provided theanine measurements by using LC/MS.

NIST Analyses for Catechins and Gallic Acid: Catechins and gallic acid were measured by using two LC methods with UV or MS detection. Calibrants were prepared gravimetrically, and a single internal standard solution was used for the calibrants and samples. For Catechin Method 1, below, a series of three calibrants containing varying analyte levels was used. For Catechin Method 2, below, four calibrants were prepared at levels approximating the values expected in the SRMs.

Catechin Method 1. To establish the optimum test portion size for catechin analysis, test portions of varying masses (100 mg to 400 mg) from each of six packets were analyzed. Materials were individually combined with diatomaceous earth (Hydromatrix, Isco, Lincoln, NE) in polypropylene tubes. 7-(β-Hydroxypropyl)theophylline (proxyphylline; the internal standard), ethylenediaminetetracetic acid (EDTA), carbohydrases, and cellulase were added, and the samples were placed in a heated ultrasonicating bath for 6 h. A proteinase was then added, the samples were incubated and centrifuged. Filtrates were extracted into acetone and water (20 % and 80 % volume fractions, respectively) using pressurized-fluid extraction. Samples were analyzed by using LC/UV with a C_{18} column and absorbance detection at 210 nm. A typical separation is provided in Figure 1.

[1] Certain commercial equipment, instruments, or materials are identified in this certificate in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology or the other named parties, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Catechin Method 2. Two 90 mg test portions from each of six packets were combined with proxyphylline (internal standard) and extracted by ultrasonic agitation for 90 min. The extraction process was repeated using fresh solvent, and supernatants were combined. Supernatants were syringe-filtered prior to analysis by using LC/UV and LC/MS. A C_{18} column (different brand than that used for Catechin Method 1) was used with an absorbance detector (detection at 280 nm) and a mass spectrometer (electrospray ionization source; ESI) connected in series. Selected ion monitoring was used for quantitation at *m/z* 171 for gallic acid (GA), *m/z* 239 for proxyphylline (internal standard), *m/z* 291 for catechin (C) and epicatechin (EC), *m/z* 307 for gallocatechin (GC) and epigallocatechin (EGC), *m/z* 443 for epicatechin gallate (ECG), and *m/z* 459 for gallocatechin gallate (GCG) and epigallocatechin gallate (EGCG). A typical separation is provided in Figure 1.

NIST Analyses for Xanthines: Xanthines were measured by using LC/UV and LC/MS. Calibrants were prepared gravimetrically, at levels intended to approximate the levels of the xanthines in the SRM. A single internal standard solution was used for the calibrants and samples.

Xanthine Method 1. Caffeine (Caf) and theobromine (TB) were measured simultaneously with the catechins measured using Catechin Method 1, above.

Xanthine Method 2. Caffeine and theophylline were measured in two 70 mg test portions from each of six packets. Test portions were individually combined with an internal standard solution containing trimethyl-$^{13}C_3$-caffeine and $^{13}C^{15}N_2$-theophylline, methanol, and water. Materials were extracted using ultrasonic agitation for 2 h. Samples were syringe-filtered prior to LC/MS analysis. A C_{18} column and ESI in positive polarity were used, and ions at *m/z* 198 for labeled caffeine, *m/z* 195 for caffeine, *m/z* 184 for labeled theophylline, and *m/z* 181 for theophylline were monitored.

Xanthine Method 3. Theobromine (and theanine) were measured in two 100 mg test portions from each of six packets. Test portions were individually combined with an internal standard solution containing $^2H_6$-theobromine and $^2H_5$-L-theanine, methanol, and phosphate buffer in water. Materials were extracted using ultrasonic agitation for 2 h. Samples were syringe-filtered prior to LC/MS analysis. A C_{18} column and ESI in positive polarity were used, and ions at *m/z* 187 for labeled theobromine, *m/z* 181 for theobromine, *m/z* 180 for labeled theanine, and *m/z* 175 for theanine were monitored.

NIST Analyses for Theanine: Theanine was measured simultaneously with theobromine (Xanthine Method 3) using LC/MS [6]. Calibrants were prepared gravimetrically, at levels intended to approximate the levels of the theanine in the SRM. A single internal standard solution was used for the calibrants and samples.

Collaborating Laboratories' Analyses: Hershey Foods analyzed 250 mg test portions from each of six packets for catechin, epicatechin, caffeine, and theobromine using sonication, and analyzed extracts by using LC/FL (catechins) or LC/UV at 280 nm (xanthines). Tampa Bay Analytical Research analyzed 150 mg test portions in triplicate from each of five packets for catechins and caffeine using sonication. Extracts were analyzed by using LC/UV.

NIST Analyses for Toxic Elements: Two 0.5 g test portions from a single packet of SRM 3254 were screened for arsenic, cadmium, and lead using inductively coupled plasma with mass spectrometric detection (ICP-MS). Three 0.05 g test portions from a single packet were screened for mercury using a mercury analyzer. The material contains an arsenic mass fraction of approximately 140 ng/g, a cadmium mass fraction of approximately 40 ng/g, a lead mass fraction of approximately 1700 ng/g, and a mercury mass fraction of approximately 10 ng/g.

Determination of Moisture: Moisture content of SRM 3254 was determined at NIST (see "Warning and Instructions for Storage and Use") by (1) freeze-drying to constant mass over 7 days; (2) drying over magnesium perchlorate in a desiccator at room temperature for 28 days; and (3) drying for 2 h in a forced-air oven at 80 °C. Unweighted results obtained using all three techniques were averaged to determine a conversion factor of (0.9481 ± 0.0029) gram dry mass per gram as-received mass, which was used to convert data from an as-received to a dry-mass basis; the uncertainty shown on this value is an expanded uncertainty. An uncertainty component for the conversion factor (0.14 %) obtained from the moisture measurements is incorporated in the uncertainties of the certified and reference values, reported on a dry-mass basis, that are provided in this certificate.

Homogeneity Assessment: The homogeneity of catechins, xanthines, and theanine was assessed at NIST by using the LC/UV and LC/MS methods described above. An analysis of variance did not show inhomogeneity for the test portions analyzed; see "Warning and Instructions for Storage and Use."

Value Assignment: The equally weighted mean of results provided by LC/UV, LC/MS, and the individual means of collaborating laboratories' data, where available, were used to calculate assigned values. In cases where NIST data were provided using two detectors in series, the average was treated as a single method mean when it was combined with other data.

Table 1. Certified Mass Fraction Values for Selected Catechins and Xanthines in SRM 3254[(a)]

	Mass Fraction (mg/g, dry-mass basis)			k
(–)-epicatechin	9.0	±	1.6	3.01
(–)-epicatechin gallate	12.7	±	1.2	2.00
(–)-epigallocatechin	25.2	±	4.5	2.00
(–)-epigallocatechin gallate	52.0	±	2.2	2.00
(–)-gallocatechin gallate	0.99	±	0.21	2.00
caffeine	23.5	±	1.8	2.63
theobromine	0.463	±	0.052	2.00

[(a)] Each certified value, expressed as a mass fraction, is an equally weighted mean of results provided by using LC/UV, LC/MS, and the individual means of collaborating laboratories' data where available. In cases where NIST data were provided using UV and MS detectors arranged in series, the average was treated as a single method mean when it was combined with other data. The uncertainty provided with each value is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence; it incorporates Type B uncertainty components related to the analyses, as well as a component related to moisture correction, and expresses both the observed difference between the results from the methods and their respective uncertainties, consistently with the ISO Guide and its Supplement 1 [2–4]. The expanded uncertainty is calculated as $U = ku_c$, where u_c is the combined uncertainty, and k is a coverage factor corresponding to approximately 95 % confidence for each analyte.

Table 2. Reference Mass Fraction Values for Additional Catechins, Gallic Acid, and L-Theanine in SRM 3254[(a)]

	Mass Fraction (mg/g, dry-mass basis)			k
(+)-catechin	1.01	±	0.41	3.14
(–)-gallocatechin	2.4	±	1.1	2.00
gallic acid	1.12	±	0.61	2.00
L-theanine[(b)]	2.130	±	0.054	2.02

[(a)] Each reference value, expressed as a mass fraction, is an equally weighted mean of results provided by using LC/UV, LC/MS, and the individual means of collaborating laboratories' data where available. In cases where NIST data were provided using UV and MS detectors arranged in series, the average was treated as a single method mean when it was combined with other data. The uncertainty provided with each value is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence, consistent with the ISO Guide [2]. The expanded uncertainty is calculated as $U = ku_c$, where u_c is the combined uncertainty, and k is a coverage factor corresponding to approximately 95 % confidence for each analyte. The uncertainty incorporates within-method uncertainty and Type B uncertainty components related to the analysis, as well as a component related to moisture correction.

[(b)] This reference value, expressed as a mass fraction, was obtained by using only LC/MS. The uncertainty provided is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence, consistent with the ISO Guide [2]. The expanded uncertainty is calculated as $U = ku_c$, where u_c is the combined uncertainty, and k is a coverage factor corresponding to approximately 95 % confidence for each analyte. The uncertainty incorporates within-method uncertainty and Type B uncertainty components related to the analysis, as well as a component related to moisture correction.

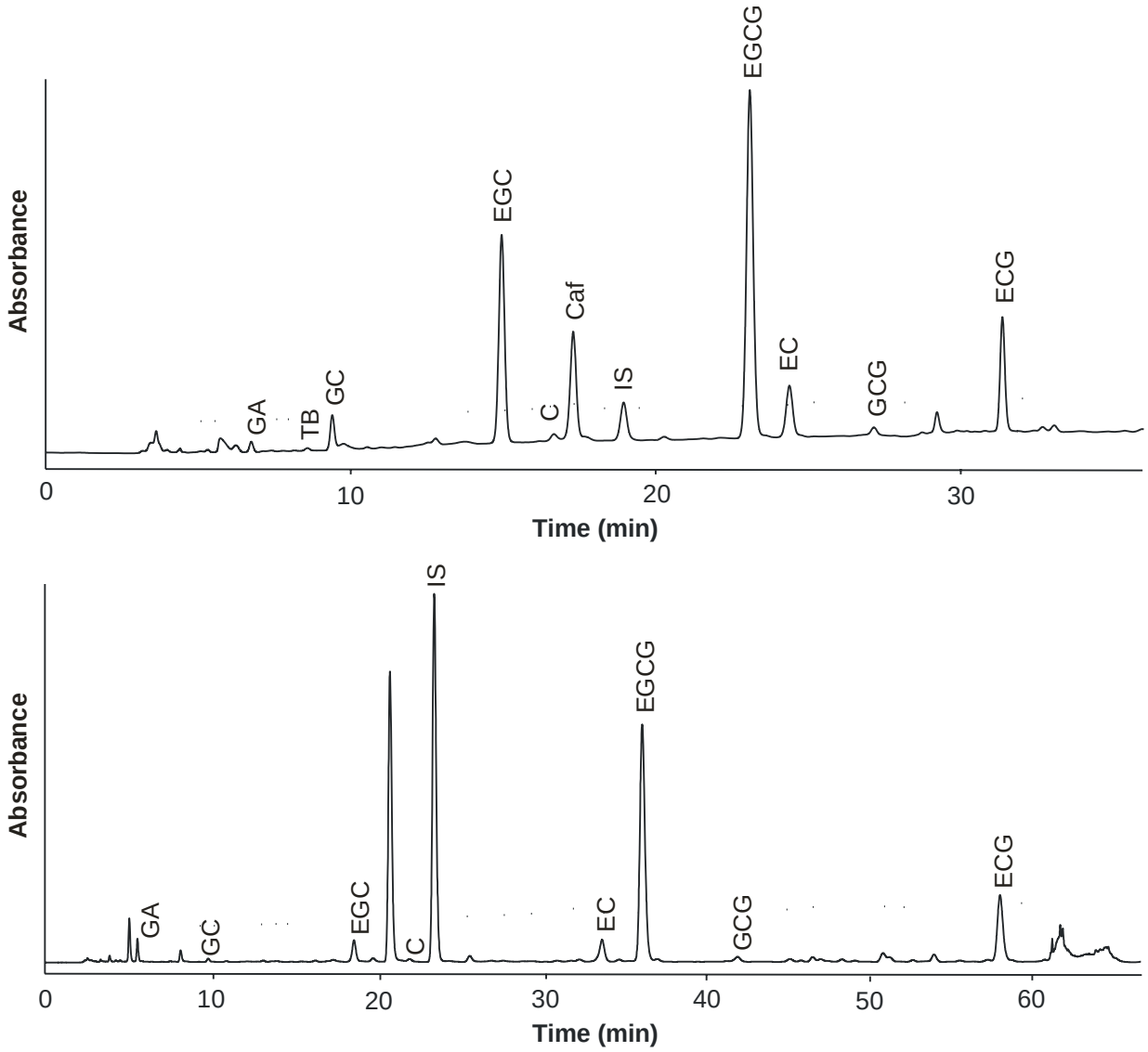

Figure 1. Chromatograms showing separation of catechins, gallic acid, and caffeine using Catechin Method 1 (top) and Catechin Method 2 (bottom). For Catechin Method 1, an Ace C_{18} ultra inert column (250 mm × 4.6 mm, 5 µm particle size; MAC-MOD Analytical, Chadds Ford, PA) was held at 23 °C. The separation was performed using a gradient consisting of water, acetonitrile, and methanol, each containing phosphoric acid. The solvent composition reached full elution strength at 35 min. Absorbance detection was at 210 nm. For Catechin Method 2, a Zorbax Eclipse XDB-C18 column (250 mm × 4.6 mm, 5 µm particle size; Agilent Technologies, Palo Alto, CA) was used. The separation was performed using a gradient of water and acetonitrile, both containing 0.1 % formic acid (volume fraction). Absorbance detection was at 280 nm; data were also generated using MS with ESI in positive polarity in series with the absorbance detector (chromatograms not shown). Abbreviations: caffeine (Caf), catechin (C), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), gallic acid (GA), gallocatechin (GC), gallocatechin gallate (GCG), proxyphylline (internal standard; IS), and theobromine (TB).

REFERENCES

[1] May, W.; Parris, R.; Beck II, C.; Fassett, J.; Greenberg, R.; Guenther, F.; Kramer, G.; Wise, S.; Gills, T.; Colbert, J.; Gettings, R.; MacDonald, B.; *Definition of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements*; NIST Special Publication 260-136 (2000); available at http://ts.nist.gov/MeasurementServices/ReferenceMaterials/PUBLICATIONS.cfm (accessed Nov 2010).

[2] JCGM 100:2008; *Evaluation of Measurement Data — Guide to the Expression of Uncertainty in Measurement* (ISO GUM 1995 with Minor Corrections); Joint Committee for Guides in Metrology (2008); available at http://www.bipm.org/utils/common/documents/jcgm/JCGM_100_2008_E.pdf (accessed Nov 2010); 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://www.nist.gov/physlab/pubs/index.cfm (accessed Nov 2010).

[3] JCGM 101:2008; *Evaluation of measurement data – Supplement 1 to the Guide to Expression of Uncertainty in Measurement*; Propagation of Distributions Using a Monte Carlo Method; Joint Committee for Guides in Metrology (BIPM, IEC, IFCC, ILAC, ISO, IUPAC, IUPAP and OIML), International Bureau of Weights and Measures (BIPM), Sèvres, France (2008); available at http://www.bipm.org/utils/common/documents/jcgm/JCGM_101_2008_E.pdf (accessed Nov 2010).

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

[5] Thompson, A.; Taylor, B.N.; *Guide for the Use of the International System of Units (SI)*; NIST Special Publication 811; U.S. Government Printing Office: Washington, DC (2008); available at http://ts.nist.gov/WeightsAndMeasures/Metric/mpo_pubs.cfm (accessed Nov 2010).

[6] Bedner, M.; Sander, L.C.; Sharpless, K.E.; *An LC-ESI/MS Method for Determining Theanine in Green Tea Dietary Supplements*; Anal. Bioanal. Chem., Vol. 397, pp. 1773–1777 (2010).

Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 926-4751; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.