National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material 3254

Green Tea (*Camellia sinensis*) Leaves

This Standard Reference Material (SRM) is intended primarily for use in validating analytical methods for the determination of catechins, xanthines and elements in the leaves of green tea (*Camellia sinensis*) and similar matrices. SRM 3254 can also be used for quality assurance when assigning values to in-house control materials. This SRM has also been characterized for its DNA sequence. 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). The addition of genetic information was accomplished through collaboration among NIST, NIH-ODS, the U.S. Department of Agriculture (USDA) Agricultural Research Service (ARS), AuthenTechnologies (Richmond, CA), and American Herbal Pharmacopoeia (Scotts Valley, CA).

Taxonomic Identification: The taxonomic identity is *Camellia sinensis*, established through identification by a trained botanist using an herbarium specimen from original material and from associated DNA sequence analysis from botanically authenticated *Camellia sinensis.* The associated DNA sequences are available in companion FASTA-formatted files [1]. The uncertainty associated with each nucleotide in the sequence, and in turn the uncertainty associated with the DNA sequence as an identifier of species, is expressed in an ordinal scale that represents the confidence estimates of the assigned value (Tables 1 and 2) [2]. These DNA sequences are used as a source of identity data for *Camellia sinensis.*

Certified Mass Fraction Values: Certified mass fraction values of catechins, xanthines, and elements in SRM 3254, reported on a dry-mass basis, are provided in Tables 3 and 4. 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 [3]. Analyses for value assignment were performed by NIST and collaborating laboratories. Certified values of catechins and xanthines are the equally weighted means of the individual sets of results; certified values for elements were calculated as the mean of the mean values from NIST methods and the median of the means of results provided by collaborating laboratories, where appropriate. The associated uncertainties are expressed at an approximately 95 % level of confidence [4-6].

Reference Mass Fraction Values: Reference mass fraction values for catechin, gallocatechin, gallic acid, theanine, and additional elements in SRM 3254, reported on a dry-mass basis, are provided in Tables 5 and 6. A NIST reference value is a noncertified value that is the best estimate of the true value 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. The reference mass fraction values were derived from results reported by NIST or collaborating laboratories.

Expiration of Certification: The certification of **SRM 3254** is valid, within the measurement uncertainty specified, until **30 June 2027**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Instructions for Storage and Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

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Gaithersburg, MD 20899
Certificate Issue Date: 07 February 2018
Certificate Revision History on Page 8

Steven J. Choquette, Director
Office of Reference Materials

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

Coordination of the technical measurements leading to the certification of this SRM was performed by C.A. Rimmer, L.J. Wood, L.C. Sander, and S.A. Wise of the NIST Chemical Sciences Division; K.E. Sharpless of the NIST Special Programs Office; and W. Koshute and B. St. Amant of the Grocery Manufacturers Association (GMA, Washington, DC).

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). Acquisition of the material was coordinated by A. NguyenPho of FDA CDER and K.E. Sharpless of the NIST Special Programs Office.

Analytical measurements were performed by M. Bedner, J.L. Molloy, K.E. Murphy, B.J. Porter, M.C. Tims, and L.J. Wood of the NIST Chemical Sciences Division; M. Payne at Hershey Foods Corporation (Hershey, PA); and M. Roman at Tampa Bay Analytical Research, Inc. (Largo, FL).

Coordination of the distribution of materials and reporting of measurement results for an interlaboratory comparison exercise were performed by M.M. Phillips and L.J. Wood of the NIST Chemical Sciences Division and by W. Koshute and B. St. Amant of the GMA. Analysts at the following laboratories performed measurements that contributed to the value assignment of elements in SRM 3254 as part of a GMA Food Industry Analytical Chemists Share Group (FIACSG) interlaboratory comparison exercise: Campbell Soup Company (Camden, NJ); ConAgra Foods (Omaha, NE); Covance Inc. (Battle Creek, MI); Covance Inc. (Madison, WI); Covance (Asia) Pte. Ltd. (The Synergy, Singapore); Covance Inc. (Harrogate North Yorkshire, UK); Del Monte Foods (Walnut Creek, CA); Eurofins Central Analytical Laboratories (Metairie, LA); Eurofins Frontier Global Sciences (Bothell, WA); Eurofins Scientific (Des Moines, IA); Eurofins WEJ Contaminants GmbH (Hamburg, Germany); Land O' Lakes (Arden Hills, MN); and NSF International (Ann Arbor, MI).

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

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

NOTICE TO USERS: SRM 3254 IS INTENDED FOR RESEARCH USE; NOT FOR HUMAN CONSUMPTION.

INSTRUCTIONS FOR STORAGE AND USE

Storage: The SRM should be stored at controlled room temperature (20 °C to 25 °C), in the original packet, until needed. For elemental analyses, the packet can be re-sealed and test portions removed and analyzed until the material reaches its expiration date. The stability of catechins, xanthines, and theanine in opened packets has not been investigated.

Use: Before use, the contents of a packet of material should be mixed thoroughly. To relate analytical determinations to the certified values in this Certificate of Analysis, the test portion mass indicated in the description of the NIST analyses for each group of analytes below should be used (see "Source, Preparation, and Analysis"). 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. The moisture conversion factor can be used for the sample(s) when using an unopened packet for the first time. If using a previously opened and resealed packet, moisture must be determined using one of the recommended techniques (see "Determination of Moisture"). Analytical results should include their own estimates of uncertainty and can be compared to the certified and reference values using procedures described in reference 7.

Determination of Moisture: Moisture content of SRM 3254 was determined at NIST by (1) freeze-drying to constant mass over 7 days; (2) drying over magnesium perchlorate in a desiccator at room temperature for 28 d; 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 (k = 2) to represent a 95 % level of confidence. 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.

SOURCE, PREPARATION AND ANALYSIS[1]

Source and Preparation: The material for production of SRM 3254 was received as nominally 250 µm (60 mesh) particles and was further ground and sieved at NIST to 180 µm (80 mesh). The sieved material was transferred to High-Purity Standards (Charleston, SC) where it was blended, aliquoted, 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 mass fractions of the catechins, gallic acid, and xanthines in SRM 3254 was based on the combination of measurements provided by NIST using liquid chromatography (LC) with ultraviolet absorbance detection (UV) and LC with mass spectrometry (MS), and by data provided by collaborating laboratories using LC with fluorescence detection (LC/FL) and LC/UV. NIST provided theanine measurements using LC/MS.

NIST Analyses for Catechins, Gallic Acid, and Xanthines using LC/UV: The mass fractions of catechins, gallic acid, caffeine and theobromine were measured by LC/UV in 100 mg to 400 mg test portions taken from each of six packets of SRM 3254. Test portions were combined with diatomaceous earth (Hydromatrix, Isco, Lincoln, NE), 7-(β-Hydroxypropyl) theophylline (proxyphylline) as an internal standard, ethylenediaminetetracetic acid (EDTA), carbohydrases, and cellulase in polypropylene tubes. The samples were placed in a heated ultrasonicating bath for 6 h. A proteinase was then added and 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 C18 column and absorbance detection at 210 nm. A typical separation is provided in Figure C1 (Appendix C). Calibrants were prepared gravimetrically, and a single internal standard solution was used for the calibrants and samples. A series of three calibrants containing varying analyte levels was used.

NIST Analyses for Catechins using LC/UV and LC/MS: The mass fractions of catechins were measured by LC/UV and LC/MS in duplicate 90 mg test portions taken from each of six packets of SRM 3254. Test portions 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 C18 column was used with an absorbance detection at 280 nm and a mass spectrometer with 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 C1 (Appendix C). Calibrants were prepared gravimetrically, and a single internal standard solution was used for the calibrants and samples. A series of four calibrants were prepared at levels approximating the values expected in the SRM.

NIST Analyses for Xanthines using LC/MS: The mass fractions of caffeine, theophylline, theobromine, and theanine were measured by LC/MS in duplicate 0.07 g to 0.1 g test portions taken from each of six packets of SRM 3254. Test portions were combined with internal standard solutions containing trimethyl-$^{13}C_3$-caffeine and $^{13}C^{15}N_2$-theophylline, methanol, and water or $^{2}H_6$-theobromine and $^{2}H_5$-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 C18 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, *m/z* 181 for theophylline, *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. 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.

Analyses for Elements: For analytes that were measured by NIST, duplicate 0.5 g test portions from a single packet of SRM 3255 were analyzed using inductively coupled plasma mass spectrometry (ICP-MS). Samples were digested in a microwave sample preparation system using HNO_3 and HF. The GMA FIACC laboratories prepared samples using a microwave sample preparation system with analyses by either ICP-MS or atomic absorption spectroscopy (AAS).

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

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. The GMA FIACC laboratories were asked to use their usual methods to make single measurements of elements on test portions taken from each of two packets of SRM 3254. Because of variability among data provided by laboratories participating in an interlaboratory comparison exercise, the median of laboratory means was used, with the uncertainty estimated using the median absolute deviation (MADe) [8].

ASSIGNMENT OF IDENTITY

Molecular Approach for Species Identity: Sanger sequencing was used on two independent chloroplast gene regions, *psbA-trnH* intergenic region [9,10], and the nuclear ribosomal gene internal transcribed spacer (ITS) sequence for authentication of SRM 3254. Complete sequencing of the strands of DNA for the two gene regions was performed on triplicate samples, the sequence reads were independently hand-curated, and the confidence values were estimated as described below. DNA sequences are available in companion FASTA-formatted files [1]. To certify the species identity, validation studies were performed based on the "Probability of Identification (POI)" model using an Inclusivity and Exclusivity Panel [11]. Multiple samples of authenticated herbarium vouchers, botanical identity reference materials, and published literature were used to comprise the panels from the target and most closely related species (see Appendix A for more information on the reference samples). These samples were analyzed a minimum of ten times to ensure consistency in methods from replicate to replicate. Maximum Likelihood (ML) phylogenetic analyses were performed using the phylogenetic estimation using ML (PhyML) algorithm, with a General Time Reversible (GTR) substitution model, a fixed transition-transversion ratio, and 1,000 bootstrap replicates. ML phylogenies, and the DNA aligned matrices, which can be found in Appendix B, were used to determine the species identity of SRM 3254 and to estimate the confidence (as described below). The confidence level for the *psbA-trnH* intergenic region was "Ambiguous" (3).

The taxonomic identification is *Camellia sinensis* and the associated chloroplast DNA sequences from the *psbA-trnH* intergenic spacer and from the nuclear ribosomal gene internal transcribed spacer (ITS) sequence are available in companion FASTA-formatted files [1]. The confidence associated with each nucleotide in the sequence, and in turn the confidence associated with the DNA sequence as an identifier of species, is expressed in an ordinal scale that represents the strength of the belief in the assigned value (Table 1) [2]. In the absence of a fully developed metrology for identity, these DNA sequences are used as a source of identity data for *Camellia sinensis.* Chloroplast and nuclear ribosomal DNA sequences from botanically authenticated *Camellia sinensis* samples are used to establish inclusivity; chloroplast and nuclear ribosomal DNA sequences from close relatives are used to establish exclusivity.

Nucleotide Identity: A set of heuristic, experience-based, rules (see Table 2) were used to establish confidence estimates for the nucleotides comprising the DNA sequences obtained from SRM 3254, and their use in identifying the species (see Table 1). The DNA sequences with curated confidence estimates are available in companion FASTA-formatted files [1]. The DNA aligned matrix used to determine the species identity and their confidence estimates are provided in Appendix B. The confidence associated with each nucleotide in the sequence in the FASTA files provided [1], and in turn the confidence associated with the DNA sequence as an identifier of species, is expressed in an ordinal scale that represents the strength of the belief in the assigned value (Table 2) [2]. Characteristics of sequence data and phylogenetic data used for species identity associated with the levels of the ordinal scale are described in Tables 1 and 2. The confidence estimates for the *psbA-trnH* and ITS sequences are available in companion FASTA-formatted files [1].

Table 1. Definitions of Heuristic Rules for Confidence Estimates of Species Identity

Confidence Level	Species Identity
Most Confident (0)	Have very well-supported and well-resolved phylogeny and/or multiple diagnostic nucleotides differentiating species from closest relatives; have data from multiple samples of both an inclusivity and exclusivity panel; data from multiple independent gene regions agree.
Very Confident (1)	Have reasonably well-supported and well-resolved phylogeny and/or a few diagnostic nucleotides differentiating species from close relatives; have data from multiple samples of both an inclusivity and exclusivity panel; data from one gene, or data from multiple independent gene regions agree.
Confident (2)	Have reasonably well-supported and well-resolved phylogeny and/or one or a few diagnostic nucleotides differentiating species from close relatives; have data from a few samples of both an inclusivity and exclusivity panel; data from one gene, or data from multiple independent gene regions generally agree.
Ambiguous (3)	Have a poorly supported and poorly resolved phylogeny and/or no diagnostic nucleotides differentiating species from close relatives; have data from a few or multiple samples of both an inclusivity and exclusivity panel; data from one gene, or data from multiple independent gene regions generally disagree.

Table 2. Definitions of Heuristic Rules for Confidence Estimates of DNA Nucleotide Identity

Confidence Level	Nucleotide Identity
Most Confident (0)	Have good answers (fully reliable, unambiguous base calls) on both strands; all data from both strands agree.
Very Confident (1)	Have good answer on one strand; poor answer (less than fully reliable, potentially ambiguous base call) on the second/alternate strand; base calls from both strands typically agree, and there is biochemical context that explains the anomalous sequence data.
Confident (2)	Have good answer on one strand; anomalous sequence data that may give rise to a conflicting base call on the alternate strand; judgment required to resolve anomaly.
Ambiguous (3)	No clear mutually supporting results; unambiguous base calls disagree; or no unambiguous base calls on either strand; data from the two opposing strands could not be authoritatively reconciled.

Homogeneity Assessment: The homogeneity of catechins, xanthines, and theanine was assessed at NIST by using the LC/UV and LC/MS methods described above. Analysis of variance did not show statistically significant heterogeneity at an approximately 95 % level of confidence. Other analytes have been treated as though they are homogeneously distributed in the material although the homogeneity of only the catechins, xanthines, and theanine was assessed.

Value Assignment: For calculation of assigned values for catechins, xanthines, and theanine, the equally weighted mean of results provided by NIST, and the individual means of collaborating laboratories' data, where available, were used to calculate assigned values. In cases where data were provided using two detectors in series, the average was treated as a single method mean when it was combined with other data. The GMA FIACC laboratories reported the individual results for each of their analyses for a given analyte. The mean of each laboratory's results was then determined. For calculation of assigned values for analytes that were measured only by the collaborating laboratories, the median of the laboratory means was used. For analytes that were also measured by NIST, the median of the individual collaborating laboratory means and the mean of the individual sets of NIST data were averaged, as appropriate.

Certified Mass Fraction Values for Catechins and Xanthines: Each certified mass fraction value is the combined mean from each set of analyses by NIST and the mean of results provided by collaborating laboratories. Values are expressed as $x \pm U_{95\%}(x)$, where x is the certified value and $U_{95\%}(x)$ is the expanded uncertainty of the certified value. The true value of the analyte is believed to lie within the interval $x \pm U_{95\%}(x)$ with 95 % confidence. To propagate this uncertainty, treat the certified value as a normally distributed random variable with mean x and standard deviation $U_{95\%}(x)/2$ [4–6]. The measurand is the total mass fraction for each analyte listed in Table 3 on a dry-mass basis. Metrological traceability is to the SI derived unit for mass fraction (expressed as milligrams per gram).

Table 3. Certified Mass Fraction Values for Catechins and Xanthines in SRM 3254

	Mass Fraction (mg/g)		
(–)-epicatechin [a,b,c,d]	9.0	±	1.6
(–)-epicatechin gallate [a,b,d]	12.7	±	1.2
(–)-epigallocatechin [a,b,d]	25.2	±	4.5
(–)-epigallocatechin gallate [a,b,d]	52.0	±	2.2
(–)-gallocatechin gallate [a,b,d]	0.99	±	0.21
Caffeine [a,b,c,d]	23.5	±	1.8
Theobromine [a,b,c]	0.463	±	0.052

[a] NIST LC/UV
[b] NIST LC/MS
[c] Collaborating Laboratories LC/FL
[d] Collaborating Laboratories LC/UV

Certified Mass Fraction Values for Elements: Each certified mass fraction value is the combined mean from each set of analyses by NIST using ICP-MS and the median of the mean of results provided by collaborating laboratories. Values are expressed as $x \pm U_{95\%}(x)$, where x is the certified value and $U_{95\%}(x)$ is the expanded uncertainty of the certified value. The true value of the analyte is believed to lie within the interval $x \pm U_{95\%}(x)$ with 95 % confidence. To propagate this uncertainty, treat the certified value as a normally distributed random variable with mean x and standard deviation $U_{95\%}(x)/2$ [4–6]. The measurand is the total mass fraction for each element listed in Table 4 on a dry-mass basis. Metrological traceability is to the SI derived unit for mass fraction (expressed as milligrams per kilogram).

Table 4. Certified Mass Fraction Values for Elements in SRM 3254

	Mass Fraction (mg/kg)		
Arsenic (As)	0.150	±	0.011
Cadmium (Cd)	0.037	±	0.002
Mercury (Hg)	0.014	±	0.001
Lead (Pb)	1.73	±	0.19

Reference Mass Fraction Values for Catechins, Gallic Acid, and Theanine: Each reference mass fraction value is the combined mean from the means of results from each set of analyses by NIST. Values are expressed as $x \pm U_{95\%}(x)$, where x is the estimated value and $U_{95\%}(x)$ is the expanded uncertainty of the value. The method-specific true value of the analyte is believed to lie within the interval $x \pm U_{95\%}(x)$ with about a 95 % confidence [4-6]. The measurand is the mass fraction for each analyte listed in Table 5, on a dry-mass basis, as determined by the methods indicated. Metrological traceability is to mass fraction (expressed as milligrams per gram), as realized by the methods used.

Table 5. Reference Mass Fraction Values for Additional Catechins, Gallic Acid, and L-Theanine in SRM 3254

	Mass Fraction (mg/g)		
(+)-catechin[a,b]	1.01	±	0.41
(–)-gallocatechin[b]	2.4	±	1.1
gallic acid[b]	1.12	±	0.61
L-theanine[b]	2.130	±	0.054

[a] NIST LC/UV
[b] NIST LC/MS

Reference Mass Fraction Values for Elements: Each reference mass fraction value is the median of the mean results provided by collaborating laboratories using ICP-MS or AAS. Values are expressed as $x \pm U_{95\%}(x)$, where x is the estimated value and $U_{95\%}(x)$ is the expanded uncertainty of the value. The method-specific true value of the analyte is believed to lie within the interval $x \pm U_{95\%}(x)$ with about a 95 % confidence [4–6]. The measurand is the mass fraction for each element listed in Table 6, on a dry-mass basis, as determined by the method indicated. Metrological traceability is to mass fraction (expressed as milligrams per kilogram) as realized by the method used.

Table 6. Reference Mass Fraction Values for Elements in SRM 3254

	Mass Fraction (mg/kg)		
Aluminum (Al)	1089	±	59
Copper (Cu)	11.34	±	0.46
Iron (Fe)	273	±	17
Manganese (Mn)	943	±	36
Zinc (Zn)	24.7	±	1.2

REFERENCES

[1] Certified data can be downloaded at https://www-s.nist.gov/srmors/view_detail.cfm?srm=3254.
— The certified confidence estimates for every base of *psbA-trnH* intergenic region sequence data file is:
SRM3254 *pbsA-trnH* Nucleotide Confidence_v1.TXT.
— The certified sequence data file for ITS sequence is:
SRM3254 ITS Sequence_v1.FASTA.
— The certified confidence estimates for every base of ITS sequence data file is:
SRM3254 ITS Nucleotide Confidence_v1.TXT.

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Certificate Revision History: 07 February 2018 (Added certified and reference elemental mass fraction values, added DNA taxonomic identity, change of expiration date, editorial changes); 12 January 2016 (Editorial changes); 04 September 2015 (Change of expiration date; editorial changes); 15 November 2010 (Original certificate date)

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

Appendix A

Reference Samples and Sequences Used in the Specificity Validation Study for SRM 3254

Table A1. Inclusivity Panel for *Camellia sinensis*

Camellia sinensis	Sample Source[a]	Voucher Information or GenBank Accession #	ITS[b], *psbA-trnH*[c]
1	UC	1340867, Japan	+, +
2	UC	316309, China	+, –
3	UCBG	unknown	+, +
4	UC	M237139, Japan	+, +
5	UC	1376032, Sri Lanka	+, +
6	UC	312722, Indonesia	+, +
7	UC	M087161, Japan	+, +
8	UCBG	82.0215.235, China	?, +
9	UCBG	82.0215.235, China	?, +
10	UCBG	50.0958.245, China	?, +
11	UCBG	82.0215.235, China	?, +
12	UC	354679, China	?, +

[a] UC = University Herbarium, University of California, Berkeley; UCBG = University of California Botanical Garden.
[b] Internal Transcribed Spacer Sequence is included when a plus sign (+) is present or unknown when a (?) is present.
[c] Intergenic spacer sequence is included when a plus sign (+) is present or not included when a minus (–) is present.

Table A2. Exclusivity Panel for *Camellia sinensis*

Species	Sample Source[a] or Reference	Voucher Information or GenBank Accession #	ITS[b], *psbA-trnH*[c]
Camellia sinensis forma macrophylla	UC	1777598	+, +
Camellia sinensis var. takasagomontana	UC	M307894	+, +
Camellia sinensis var. assamica	UC	466362	?, +
Camellia fraterna	UCBG	82.0211	?, +
Camellia grijsii	UCBG	81.0743.235	?, +
Camellia irrawadensis	UCBG	75.0680.222B	?, +
Camellia japonica var. macrocarpa	UCBG	72.0639.220B	?, +
Camellia oleifera	UCBG	82.0184	?, +
Camellia yunnanensis	UCBG	80.0055.237	?, +

[a] UC = University Herbarium, University of California, Berkeley; UCBG = University of California Botanical Garden.
[b] Internal Transcribed Spacer Sequence is included when a plus sign (+) is present or unknown when a (?) is present.
[c] Intergenic spacer sequence is included when a plus sign (+) is present.

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SRM 3254                        TGGATAAGACTTTGGTCTTAGTGTATACTCGTTTTTAAAAGTAAAGGAGCAATAACCAAT
Camellia sinensis               TGGATAAGACTTTGGTCTTAGTGTATACTCGTTTTTAAAAGTAAAGGAGCAATAACCAAT
C. sinensis assamica            TGGATAAGACTTTGGTCTTAGTGTATACTCGTTTTTAAAAGTAAAGGAGCAATAACCAAT
C. sinensis macrophylla         TGGATAAGACTTTGGTCTTAGTGTATACTCGTTTTTAAAAGTAAAGGAGCAATAACCAAT
C. sinensis takasagomontana     TGGATAAGACTTTGGTCTTAGTGTATACTCGTTTTTAAAAGTAAAGGAGCAATAACCAAT
Camellia oleifera               TGGATAAGACTTTGGTCTTAGTGTATACTCGTTTTTAAAAGTAAAGGAGCAATAACCAAT
Camellia yunnanensis            TGGATAAGACTTTGGTCTTAGTGTATACTCGTTTTTAAAAGTAAAGGAGCAATAACCAAT
Camellia irrawadensis           TGGATAAGACTTTGGTCTTAGTGTATACTCGTTTTTAAAAGTAAAGGAGCAATAACCAAT
Camellia japonica               TGGATAAGACTTTGGTCTTAGTGTATACTCGTTTTTAAAAGTAAAGGAGCAATAACCAAT
Camellia fraterna               TGGATAAGACTTTGGTCTTAGTGTATACTCGTTTTTAAAAGTAAAGGAGCAATAACCAAT
Camellia grijsii                TGGATAAGACTTTGGTCTTAGTGTATACTCGTTTTTAAAAGTAAAGGAGCAATAACCAAT

SRM 3254                        TTCTTGTTCTATCAGGAAGGCGTTATTGTTCCTTTACT--TTTTTTTTTTTTACATATCC
Camellia sinensis               TTCTTGTTCTATCAGGAAGGCGTTATTGTTCCTTTACT--TTTTTTTTTTTTACATATCC
C. sinensis assamica            TTCTTGTTCTATCAGGAAGGCGTTATTGTTCCTTTACT--TTTTTTTTTTTTACATATCC
C. sinensis macrophylla         TTCTTGTTCTATCAGGAAGGCGTTATTGTTCCTTTACT--TTTTTTTTTTTTACATATCC
C. sinensis takasagomontana     TTCTTGTTCTATCAGGAAGGCGTTATTGTTCCTTTACT---TTTTTTTTTTTACATATTC
Camellia oleifera               TTCTTGTTCTATCAGGAAGGCGTTATTGTTCCTTTACTTTTTTTTTTTTTTACATATCC
Camellia yunnanensis            TTCTTGTTCTATCAGGAAGGCGTTATTGTTCCTTTACT--TTTTTTTTTTTTACATATCC
Camellia irrawadensis           TTCTTGTTCTATCAGGAAGGCGTTATTGTTCCTTTACT-TTTTTTTTTTTTTACATATCC
Camellia japonica               TTCTTGTTCTATCAGGAAGGCGTTATTGTTCCTTTACT---TTTTTTTTTTTACATATCC
Camellia fraterna               TTCTTGTTCTATCAGGAAGGCGTTATTGTTCCTTTACT--TTTTTTTTTTTTACATATCC
Camellia grijsii                TTATTGTTCTATCAGGAAGGCGTTATTGTTCCTTTACT-TTTTTTTTTTTTTACATATCT

SRM 3254                        TTTTTTCGTTACAAGAAAAAGATTCGTATGGGTAAAAAGAAAAGGATTTTTATGGGTTAT
Camellia sinensis               TTTTTTCGTTACAAGAAAAAGATTCGTATGGGTAAAAAGAAAAGGATTTTTATGGGTTAT
C. sinensis assamica            TTTTTTCGTTACAAGAAAAAGATTCGTATGGGTAAAAAGAAAAGGATTTTTATGGGTTAT
C. sinensis macrophylla         TTTTTTCGTTACAAGAAAAAGATTCGTATGGGTAAAAAGAAAAGGATTTTTATGGGTTAT
C. sinensis takasagomontana     TTTTTTCGTTACAAGAAAAAGATTCGTATGGGTAAAAAGAAAAGGATTTTTATGGGTTAT
Camellia oleifera               TTTTTTCGTTACAAGAAAAAGATTCGTATGGGTAAAAAGAAAAGGATTTTTATGGGTTAT
Camellia yunnanensis            TTTTTTCGTTACAAGAAAAAGATTCGTATGGGTAAAAAGAAAAGGATTTTTATGGGTTAT
Camellia irrawadensis           TTTTTTCGTTACAAGAAAAAGATTCGTATGGGTAAAAAGAAAAGGATTTTTATGGGTTAT
Camellia japonica               TTTTTTCGTTACAAGAAAAAGATTCGTATGGGTAAAAAGAAAAGGATTT-------TTAT
Camellia fraterna               TTTTTTCGTTACAAGAAAAAGATTCGTATGGGTAAAAAGAAAAGTATTTTTATGGGTTAT
Camellia grijsii                TTTTTTCGTTACAAGAAAAAGATTCGTATGGGTAAAAAGAAAAGGATTTTTATGGGTTAT

SRM 3254                        GGGTTGGTTCATCATTGAGTATCGTCTTTTTGTTATGTATTAATTTAGAATTTATATACC
Camellia sinensis               GGGTTGGTTCATCATTGAGTATCGTCTTTTTGTTATGTATTAATTTAGAATTTATATACC
C. sinensis assamica            GGGTTGGTTCATCATTGAGTATCGTCTTTTTGTTATGTATTAATTTAGAATTTATATACC
C. sinensis macrophylla         GGGTTGGTTCATCATTGAGTATCGTCTTTTTGTTATGTATTAATTTAGAATTTATATACC
C. sinensis takasagomontana     GGGTTGGTTCATCATTGAGTATCGTCTTTTTGTTATGTATTAATTTAGAATTTATATACC
Camellia oleifera               GGGTTGGTTCATCATTGAGTATCGTCTTTTTGTTATGTATTAATTTAGAATTTATATACC
Camellia yunnanensis            GGGTTGGTTCATCATTGAGTATCGTCTTTTTGTTATGTATTAATTTAGAATTTATATACC
Camellia irrawadensis           GGGTTGGTTCATCATTGAGTATCGTCTTTTTGTTATGTATTAATTTAGAATTTATATACT
Camellia japonica               GGGTTGGTTCATCATTGAGTATCGTCTTTTTGTTATGTATTAATTTAGAATTTATATACC
Camellia fraterna               GGGTTGGTTCATCATTGAGTATCGTCTTTTTGTTATGTATTAATTTAGAATTTATATACC
Camellia grijsii                GGGTTGGTTCGTCATTGAGTATCGTCTTTTTGTTATGTATTAATTTAGAATTTATATACC

SRM 3254                        TTTGTGAAATTGTTATTTTCCATTTAAAATAAAAGATAAAATTTTGAATTTTTGCTTACT
Camellia sinensis               TTTGTGAAATTGTTATTTTCCATTTAAAATAAAAGATMAAATTTTGAATTTTTGCTTACT
C. sinensis assamica            TTTGTGAAATTGTTATTTTCCATTTAAAATAAAAGATAAAATTTTGAATTTTTGCTTACT
C. sinensis macrophylla         TTTGTGAAATTGTTATTTTCCATTTAAAATAAAAGATAAAATTTTGAATTTTTGCTTACT
C. sinensis takasagomontana     TTTGTGAAATTGTTATTTTCCATTTAAAATAAAAGATAAAATTTTGAATTTTTGCTTACT
Camellia oleifera               TTTGTGAAATTGTTATTTTCCATTTAAAATAAAAGATAAAATTTTGAATTTTTGCTTACT
Camellia yunnanensis            TTTGTGAAATTGTTATTTTCCATTTAAAATAAAAGATAAAATTTTGAATTTTTGCTTACT
Camellia irrawadensis           TTTGTGAAATTGTTATTTTCCATTTAAAATAAAAGATAAAATTTTGAATTTTTGCTTACT
Camellia japonica               TTTGTGAAATTGTTATTTTCCATTTAAAATAAAAGATAAAATTTTGAATTTTTGCTTACT
Camellia fraterna               TTTGTGAAATTGTTATTTTCCATTTAAAATAAAAGATAAAATTTTGAATTTTTGCTTACT
Camellia grijsii                TTTGTGAAATTGTTATTTTCCATTTAAAATAAAAGATAAAATTTTGAATTTTTGCTTACT

SRM 3254                        ATTTGTATCTCAAAAATAAGAGAAGAAAGAAATAA-------TCATGAATGGTTGAATTT
Camellia sinensis               ATTTGTATCTCAAAAATAAGAGAAGAAAGAAATAA-------TCATGAATGGTTGAATTT
C. sinensis assamica            ATTTGTATCTCAAAAATAAGAGAAGAAAGAAATAA-------TCATGAATGGTTGAATTT
C. sinensis macrophylla         ATTTGTATCTCAAAAATAAGAGAAGAAAGAAATAA-------TCATGAATGGTTGAATTT
C. sinensis takasagomontana     ATTTGTATCTCAAAAATAAGAGAAGAAAGAAATAATCATGAATCATGAATGGTTGAATTT
Camellia oleifera               ATTTGTATCTCAAAAATAAGAGAAGAAAGAAATAA-------TCATGAATGGTTGAATTT
Camellia yunnanensis            ATTTGTATCTCAAAAATAAGAGAAGAAAGAAATAA-------TCATGAATGGTTGAATTT
Camellia irrawadensis           ATTTGTATCTCAAAAATAAGAGAAGAAAGAAATAA-------TCATGAATGGTTGAATTT
Camellia japonica               ATTTGTATCTCAAAAATAAGAGAAGAAAGAAATAA-------TTATGAATGGTTGAATTT
Camellia fraterna               ATTTGTATCTCAAAAATAAGAGAAGAAAGAAATAA-------TCATGAATGGTTGAATTT
Camellia grijsii                ATTTGTATCTCAAAAATAAGAGAAGAAAGAAATAA-------TCATGAATGGTTGAATTT
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Figure B1. *psbA-trnH* DNA Aligned Matrix for *Camellia sinensis* and Relatives. The results from the chloroplast region demonstrates that this region does distinguish this species from its relatives and does authenticate *Camellia sinensis.* The individual bases are represented as A = Adenine, T = Thymine, G = Guanine, C= Cystosine, and U = Uracil. Polymorphic bases are represented by standard IUPAC codes with R = A/G, W = A/T, M = A/c, Y = C/T, S = G/C, K = G/T, and N = missing data. The confidence estimate for the species identification of SRM 3254 as *Camellia sinensis* is Ambiguous (3).

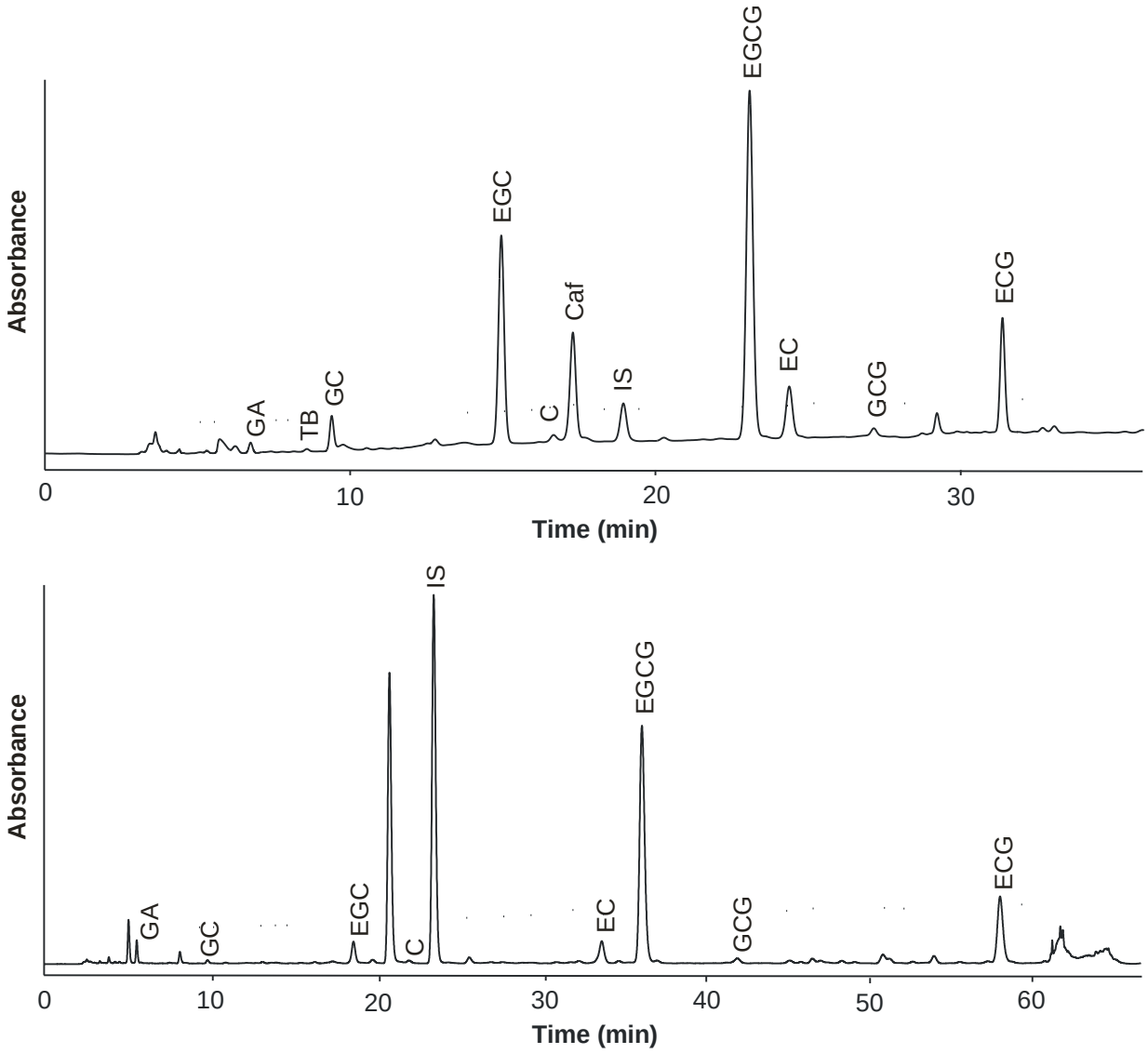

Figure C1. Chromatograms showing separation of catechins, gallic acid, and caffeine using Catechin Method 1 (top) and Catechin Method 2 (bottom). For Catechin Method 1, an Ace C18 ultra inert column (250 mm × 4.6 mm, 5 µm particle size; MAC-MOD Analytical, Chadds Ford, PA) was held at 23 °C. The separation 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).