National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material 3256

Green Tea-Containing Solid Oral Dosage Form

This Standard Reference Material (SRM) is intended primarily for use in validating analytical methods for the determination of catechins, xanthines, and toxic elements in solid oral dosage forms containing green tea and in similar matrices. SRM 3256 can also be used for quality assurance when assigning values to in-house control materials. A unit of SRM 3256 consists of five packets, each containing approximately 2.5 g of powdered material.

Certified Mass Fraction Values: Certified mass fraction values of selected catechins, xanthines, and elements in SRM 3256, reported on a dry-mass basis, are provided in Tables 1 and 2. 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]. Analyses for value assignment were performed by NIST and collaborating laboratories. Certified values for catechins and xanthines in this material are the equally weighted means of the individual sets of results; certified values for elements are the equally weighted mean of the mean of NIST results and the median of collaborating laboratories' results. The associated uncertainties are expanded uncertainties at the 95 % level of confidence [2–4].

Reference Mass Fraction Values: Reference mass fraction values for gallocatechin gallate, theanine, and theophylline in SRM 3256, reported on a dry-mass basis, are provided in Table 3. A NIST reference value is a non-certified 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.

Expiration of Certification: The certification of **SRM 3256** is valid, within the measurement uncertainty specified, until **30 November 2029**, 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.

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 or register online) will facilitate notification.

Support for the development of SRM 3256 was provided in part by 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). Technical consultation was provided by J.M. Betz (NIH-ODS) and A. NguyenPho (FDA CDER).

Overall direction and coordination of the technical measurements leading to the certification of this SRM were performed by L.C. Sander and S.A. Wise of the NIST Chemical Sciences Division; and K.E. Sharpless of the NIST Special Programs Office.

Acquisition of the material was coordinated by K.E. Sharpless of the NIST Special Programs Office.

Carlos A. Gonzales, Chief
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Revision History on Last Page

Steven J. Choquette, Director
Office of Reference Materials

Analytical measurements at NIST were performed by M. Bedner, S.J. Christopher, K.E. Murphy, and L.J. Wood of the NIST Chemical Sciences Division and R.D. Day, B.J. Porter, and M.C. Tims formerly of NIST. 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), and by participants in an interlaboratory comparison exercise organized by the Community Reference Laboratory for Heavy Metals in Feeds and Food (M.B. de la Calle, International Measurement and Evaluation Program, Institute for Reference Materials and Measurements (IRMM), Geel, Belgium).

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

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

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

INSTRUCTIONS FOR STORAGE AND USE

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

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, minimum test portions of 60 mg to 100 mg for catechin analyses; 50 mg to 100 mg for xanthine analyses; and 0.5 g for element analysis should be used. 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.

Determination of Moisture: Moisture content of SRM 3256 was determined at NIST (see "Warning and Instructions for Storage and Use") by (1) freeze-drying to constant mass over 7 days and (2) drying over magnesium perchlorate in a desiccator at room temperature for 28 days. Unweighted results obtained using both techniques were averaged to determine a conversion factor of (0.9764 ± 0.0033) 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 (0.15 %) 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.

PREPARATION AND ANALYSIS[(1)]

Material Acquisition and Preparation: SRM 3256 was prepared from several different commercially available products (both tablets and capsules) that were purchased in the marketplace. The tablets and the contents of capsules were ground using a Teflon disc mill at room temperature. The powdered materials were transferred to High-Purity Standards (Charleston, SC) where they were 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 3256 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, Xanthines, and Theanine: Value assignment of the mass fractions of the catechins and xanthines in SRM 3256 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 by data provided by collaborating laboratories using LC with fluorescence detection (LC/FL) and LC/UV. NIST provided theanine measurements by using LC/MS.

NIST Analyses for Catechins: Catechins 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.

[(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 1. To establish the optimum test portion size for catechin analysis, test portions of varying masses (50 mg to 200 mg) from each of six packets were analyzed. Materials were individually combined with Ottawa sand (Sigma-Aldrich, Milwaukee, WI), 7-(β-hydroxypropyl)theophylline (proxyphylline; the internal standard), ethylenediaminetetracetic acid (EDTA), methanol, and water, and were extracted by inversion (rotation) for 2 h. Samples were centrifuged, the supernatant decanted, and re-extracted. Supernatants were combined and filtered prior to analysis by using LC/UV with a C18 column and absorbance detection at 210 nm. A typical separation is provided in Figure 1.

Catechin Method 2. Two 60 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 C18 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; IS), *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 50 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 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, and *m/z* 181 for theophylline were monitored.

Xanthine Method 3. Theobromine (and theanine) were measured in two 50 mg test portions from each of six packets. Test portions were individually combined with an internal standard solution containing $^{2}H_6$-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* 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.

Analytical Approach for Determination of Toxic Elements: Value assignment of the mass fractions of arsenic, cadmium, lead, and mercury in SRM 3256 was based on the combination of measurements from NIST using inductively coupled plasma-mass spectrometry (ICPMS) methods and data provided through an International Measurement Evaluation Program (IMEP) interlaboratory comparison exercise organized by IRMM [7].

NIST Analyses for Toxic Elements: Two 0.5 g test portions from six packets of SRM 3256 were digested in nitric acid in a microwave and analyzed by ICPMS. Arsenic, cadmium, and lead were measured using the collision cell mode (CCT-ICPMS) and the method of standard additions. Mercury was measured using isotope dilution and a cold-vapor introduction system (ID-CV-ICPMS) [8].

Collaborating Laboratories' Analyses: Results were reported by 54 laboratories representing 20 countries [7]. Laboratories used their usual methods of analysis.

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 for the organic compounds. 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 equally weighted means from the NIST data sets and the median of the lab means for the IMEP study were used to calculate assigned values for toxic elements.

Homogeneity Assessment: The homogeneity of catechins, xanthines, and theanine was assesses at NIST by using the LC/UV and LC/MS methods described above. The homogeneity of arsenic, cadmium, lead, and mercury was assessed at NIST by using ID-CV-ICPMS and CCT-ICPMS. An analysis of variance did not show inhomogeneity for the test portions analyzed.

Table 1. Certified Mass Fraction Values for Selected Catechins, Gallic Acid, and Xanthines in SRM 3256[(a)]

	Mass Fraction (mg/g, dry-mass basis)		
(+)-catechin	2.63	±	0.18
(–)-epicatechin	12.0	±	2.6
(–)-epicatechin gallate	17.1	±	2.6
(–)-epigallocatechin	30.7	±	5.7
(–)-epigallocatechin gallate	71.1	±	6.6
(–)-gallocatechin	7.55	±	0.28
gallic acid	13.10	±	0.49
caffeine	70.0	±	2.6
theobromine	1.04	±	0.15

[(a)] Each certified mass fraction value is the combined, equally weighted mean of results from each set of analyses by NIST and the individual means of collaborating laboratories' data where available. In cases where data were provided by using UV and MS detectors in series, the average was treated as a single method mean when it was combined with other data. 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 $U_{95\%}(x)/2$ [2–4]. The measurand is the total mass fraction for each analyte listed in Table 1 on a dry-mass basis. Metrological traceability is to the SI derived unit for mass fraction (expressed as milligrams per gram).

Table 2. Certified Mass Fraction Values for Toxic Elements in SRM 3256[(a)]

	Mass Fraction (mg/kg, dry-mass basis)		
Arsenic	0.269	±	0.019
Cadmium	0.025	±	0.002
Lead	0.316	±	0.030
Mercury	0.014	±	0.002

[(a)] Each certified mass fraction, value is an equally weighted mean of results provided by NIST and the IMEP median. 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 $U_{95\%}(x)/2$ [2–4]. The measurand is the total mass fraction for each element listed in Table 2 on a dry-mass basis. Metrological traceability is to the SI derived unit for mass fraction (expressed as milligrams per gram).

Table 3. Reference Mass Fraction Values for Gallocatechin Gallate, Theanine, and Theophylline in SRM 3256[a]

	Mass Fraction (mg/g, dry-mass basis)
(–)-gallocatechin gallate	4.6 ± 1.8
L-theanine	3.7 ± 1.2
theophylline[b]	0.060 ± 0.002

[a] Each reference mass fraction value, is an equally weighted mean of results provided by using LC/UV and LC/MS. In cases where data were provided using both detectors in the method in which UV and MS detectors were arranged in series, the average was treated as a single method mean when it was combined with other data. Values are expressed as $x \pm U_{95\%}(x)$, where x is the non-certified value and $U_{95\%}(x)$ is the expanded uncertainty of the non-certified value. The method-specific value of the analyte lies within the interval $x \pm U_{95\%}(x)$ with 95 % confidence. The uncertainty incorporates within-method uncertainty and Type B uncertainty components related to the analysis, as well as a component related to moisture correction. The measurands are the total mass fractions for each analyte reported in Table 3 as determined by the methods indicated. Metrological traceability is to the SI derived unit for mass fraction (expressed as milligrams per gram).

[b] This reference value 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. The uncertainty incorporates within-method uncertainty and Type B uncertainty components related to the analysis, as well as a component related to moisture correction.

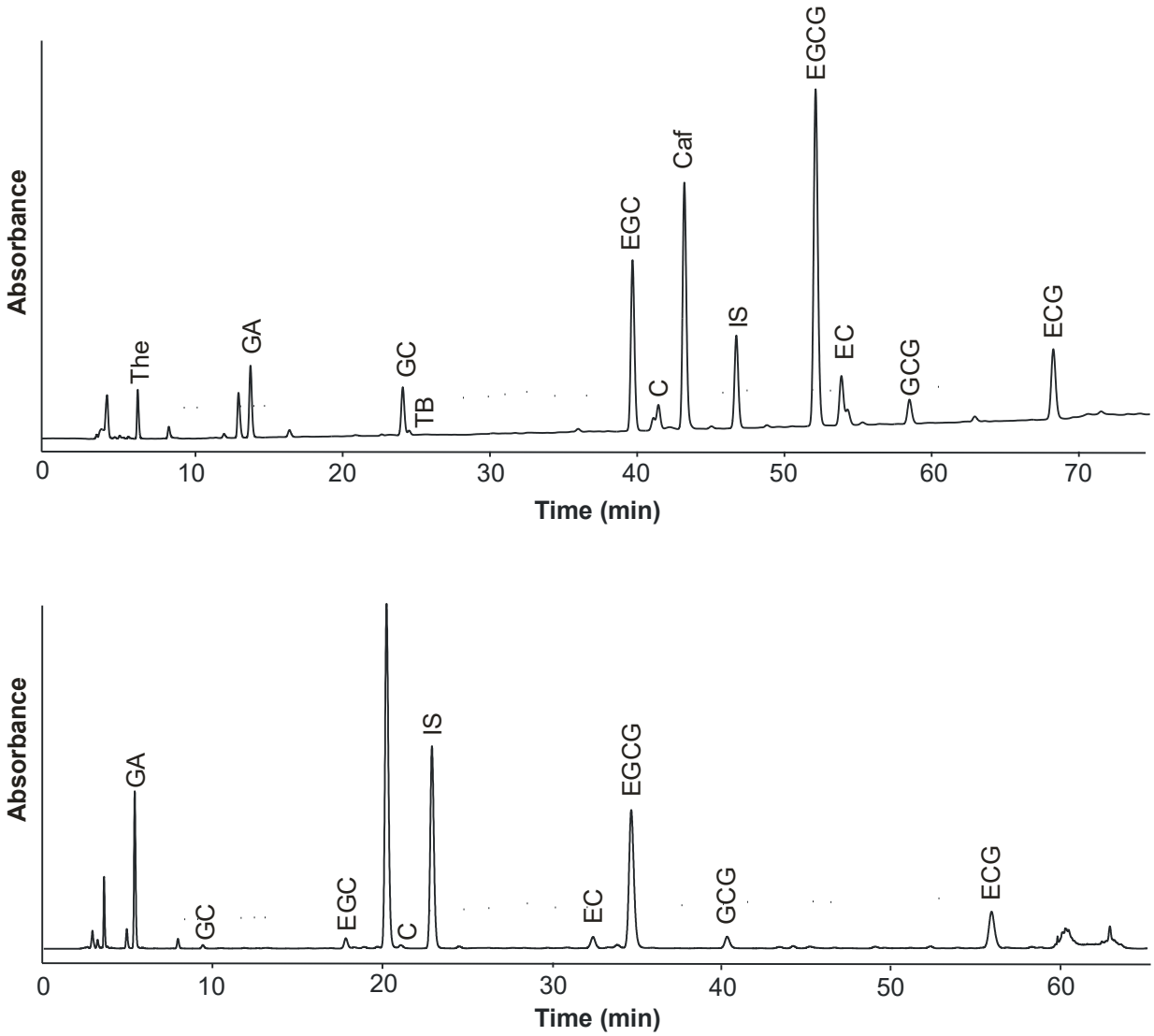

Figure 1. Chromatograms showing separation of catechins, gallic acid, and caffeine using Catechin Method 1 (top) and Catechin Method 2 (bottom). For Catechin Method 1, an Ace C_{18} Ultra Inert column (250 mm × 4.6 mm, 5 µm particle size; MAC-MOD Analytical, Chadds Ford, PA) was held at 23 °C. The separation was performed using a gradient consisting of water, acetonitrile, and methanol, each containing phosphoric acid. The solvent composition reached full elution strength at 75 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), theanine (The), and theobromine (TB).

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Certificate Revision History: 12 March 2021 (Change of expiration date; 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; e-mail srminfo@nist.gov; or via the Internet at https://www.nist.gov/srm.