National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material 3255

Green Tea (*Camellia sinensis*) Extract

This Standard Reference Material (SRM) is intended primarily for use in validating analytical methods for the determination of catechins, xanthines, and elements in extracts of green tea (*Camellia sinensis*) and similar matrices. SRM 3255 can also be used for quality assurance when assigning values to in-house control materials. A unit of SRM 3255 consists of five packets, each containing approximately 1 g of extract.

The development of SRM 3255 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: Certified mass fraction values of catechins, xanthines, and elements in SRM 3255, 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 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 [2–4].

Reference Mass Fraction Values: Reference mass fraction values for epigallocatechin methylgallate, gallic acid, theanine, theophylline, and additional elements in SRM 3255, reported on a dry-mass basis, are provided in Tables 3 and 4. 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 an uncertainty 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 3255** 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.

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).

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Steven J. Choquette, Director
Office of Reference Materials

Support for the development of SRM 3255 was provided in part by NIH-ODS and FDA CDER. Technical consultation was provided by J.M. Betz (NIH-ODS) and A. Nguyen Pho (FDA CDER). Acquisition of the material was coordinated by A. Nguyen Pho of FDA CDER and K.E. Sharpless of the NIST Special Programs Office.

Analytical measurements were performed by M. Bedner, K.E. Murphy, B.J. Porter, M.C. Tims, and L.J. Wood of the NIST Chemical Sciences Division and 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 3255 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 in the issuance of this SRM were coordinated through the NIST Office of Reference Materials.

NOTICE TO USERS: SRM 3255 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 unopened 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. The moisture conversion factor given below (see "Determination of Moisture") 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 6.

Determination of Moisture: Moisture content of SRM 3255 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 (0.9687 ± 0.0055) 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 (0.28 %) 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 3255 is a green tea extract of 90 % polyphenols. The material was prepared by first extracting dried green tea leaves in 50 % alcohol/water (volume fraction), filtering and vacuum evaporating. The next extraction step was in ethyl acetate followed again by vacuum extraction then spray drying. The material was blended and received as nominally 180 µm (80 mesh) particle size and was packaged

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

without additional grinding. The extract 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 3255 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 3255 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 and Xanthines using LC/UV: The mass fractions of catechins, caffeine and theobromine were measured by LC/UV in 20 mg to 75 mg test portions taken from each of six packets of SRM 3255. Test portions were combined with 7-(β-hydroxypropyl)theophylline (proxyphylline) as an internal standard and dissolved in methanol and water by shaking for 1 min. Samples were analyzed by LC/UV with a C18 column and absorbance detection at 210 nm. A typical separation is provided in Figure A1. 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 20 mg test portions taken from each of six packets of SRM 3255. Test portions were dissolved in methanol and water by ultrasonic agitation for 5 min. A C18 column was used with 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; 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 A1. 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/UV and LC/MS: The mass fractions of caffeine and theophylline were measured by LC/UV and LC/MS in duplicate 45 mg test portions taken from each of six packets of SRM 3255. 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. Selected ion monitoring was used for quantitation at *m/z* 198 for labeled caffeine, *m/z* 195 for caffeine, *m/z* 184 for labeled theophylline, and *m/z* 181 for theophylline. 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.

NIST Analyses for Xanthines and Theanine using LC/UV and LC/MS: The mass fractions of theobromine and theanine [7] were measured by LC/UV and LC/MS in duplicate 100 mg test portion sizes taken from each of six packets of SRM 3255. 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 C18 column and ESI in positive polarity were used. Selected ion monitoring was used for quantitation at *m/z* 187 for labeled theobromine, *m/z* 181 for theobromine, *m/z* 180 for labeled theanine, and *m/z* 175 for theanine. 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.

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).

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 3255. 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].

Homogeneity Assessment: The homogeneity of catechins, xanthines, and theanine was assessed at NIST using the methods and test portion sizes described above. Analysis of variance did not show statistically significant heterogeneity. 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 $U_{95\%}(x)/2$ [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 1. Certified Mass Fraction Values for Catechins and Xanthines in SRM 3255

	Mass Fraction (mg/g)
(+)-catechin [a,b,c,d]	9.17 ± 0.93
(–)-epicatechin [a,b,c,d]	47.3 ± 6.7
(–)-epicatechin gallate [a,b,d]	100.3 ± 7.8
(–)-epigallocatechin [a,b,d]	81.8 ± 6.5
(–)-epigallocatechin gallate [a,b,d]	422 ± 19
(–)-gallocatechin [a,b,d]	22.0 ± 1.7
(–)-gallocatechin gallate [a,b,d]	39.0 ± 2.0
Caffeine [a,b,c,d]	36.9 ± 2.7
theobromine [a,b,c]	0.867 ± 0.076

[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 $U_{95\%}(x)/2$ [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 kilogram).

Table 2. Certified Mass Fraction Values for Elements in SRM 3255

	Mass Fraction (mg/kg)
Arsenic (As)	0.1624 ± 0.0079
Lead (Pb)	0.0832 ± 0.0031

Reference Mass Fraction Values for Catechins, Xanthines, 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 [2–4]. The measurand is the mass fraction for analytes listed in Table 3, 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 3. Reference Mass Fraction Values for Epigallocatechin Methylgallate, Gallic Acid, Theanine, and Theophylline in SRM 3255

	Mass Fraction (mg/g)
(–)-epigallocatechin methylgallate[(a)]	6.87 ± 0.44
gallic acid[(a,b)]	3.231 ± 0.086
L-theanine[(b)]	0.340 ± 0.008
theophylline[(b)]	0.087 ± 0.002

[(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 [2–4]. The measurand is the mass fraction for each element listed in Table 4, 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 4. Reference Mass Fraction Values for Elements in SRM 3255

	Mass Fraction (mg/kg)
Aluminum (Al)	86.9 ± 9.6
Copper (Cu)	6.16 ± 0.10
Iron (Fe)	14.04 ± 0.52
Manganese (Mn)	58.4 ± 2.5
Zinc (Zn)	5.29 ± 0.41

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Certificate Revision History: 09 February 2018 (Addition of elemental values, change of expiration date, editorial changes); 30 December 2015 (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.

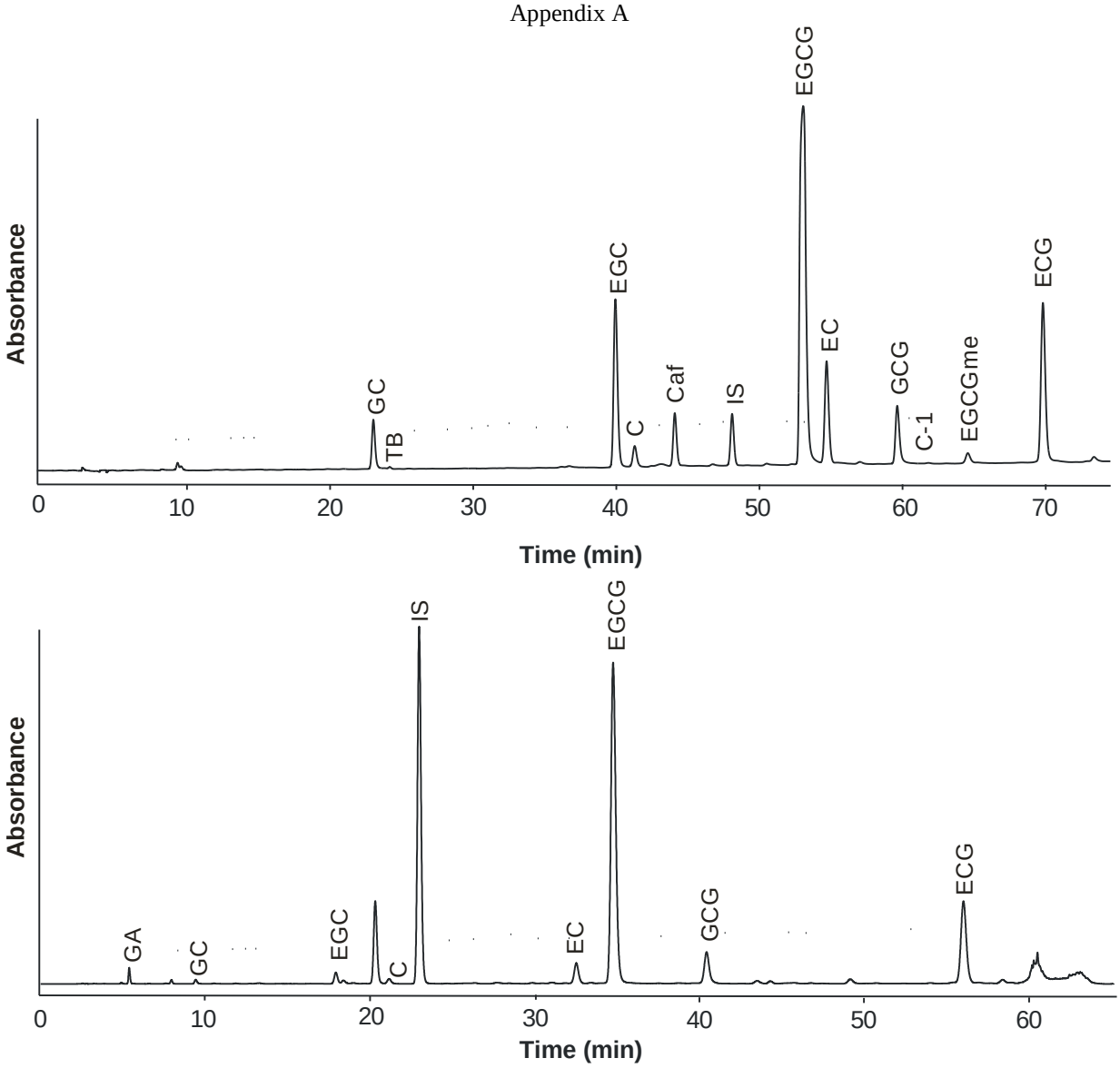

Figure A1. Chromatograms showing separation of catechins, gallic acid, and caffeine using Catechin Method 1 (top) and Catechin Method 2 (bottom). For Catechin Method 1, an Ace C18 ultra inert column (250 mm × 4.6 mm, 5 µm particle size; MAC-MOD Analytical, Chadds Ford, PA) was held at 23 °C. The separation was performed using a gradient consisting of water, acetonitrile, and methanol, each containing acetic 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: procyanidin trimer C1 (C-1), caffeine (Caf), catechin (C), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), epigallocatechin methylgallate (EGCGme), gallic acid (GA), gallocatechin (GC), gallocatechin gallate (GCG), proxyphylline (internal standard; IS), and theobromine (TB).