Development and Certification of Green Tea-Containing Standard Reference Materials†

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Abstract

A suite of three green tea-containing Standard Reference Materials (SRMs) has been issued by the National Institute of Standards and Technology (NIST). The materials are characterized for catechins, xanthine alkaloids, theanine, and toxic elements. As many as five methods were used in assigning certified and reference values to the constituents, with measurements carried out at NIST and at collaborating laboratories. The materials are intended for use in the development and validation of new analytical methods, and for use as control materials as a component in the support of claims of measurement traceability.

Introduction

Tea is a popular beverage that has been consumed in some cultures for centuries. Green tea (*Camellia sinensis* L.) is converted to oolong and black teas by enzyme catalyzed fermentation; this process alters the chemical composition by the oxidation and polymerization of monomeric catechins (also known as tannins) to theaflavins and thearubigins [1]. Increased interest in this beverage has resulted from perceived health benefits that may be associated with its consumption, and it is now commonly used in dietary supplement formulations. The latter materials are typically marketed as aids for weight loss and as stimulants to promote energy. Catechins represent about 10% to 15% of the mass of dried green tea leaves and dietary supplement formulations. Extracts of *Camellia sinensis* are typically used in the manufacture of dietary supplements to achieve product consistency and greater potency; levels of catechins in these commercial extracts can approach 75% mass fraction.

A number of instrumental methods have been published for the separation of green tea catechins. Liquid chromatographic (LC) methods most commonly utilize reversed-phase (RP) separations with gradient elution and absorbance detection [2-11], mass spectrometric detection [12-16], coulometric detection [17,18], and Fourier transform infrared detection [19,20]. The separation of catechin standards is readily achieved by RPLC with octadecylsilane (ODS) columns; however, separation of these compounds from matrix interferences is more challenging [21]. Several LC methods have been reported with run times less than ≈20 minutes [1,2,17,22-26] and recently Spacil et al. described an ultra-high performance liquid chromatography (UHPLC) tandem mass spectrometry method with resolution of 8 catechins in less than 2.5 minutes [27]. Others have developed methods with longer run times in an effort to resolve matrix interferences [14,28-32]. Catechins are also easily resolved by micellar electrokinetic chromatography in ≈20 minutes [33-40] although

this approach is less commonly employed than RPLC. Other aspects of catechin assays such as extraction conditions [6,41-47], and catechin stability and interconversion of species [36,45,46,48], can directly affect the accuracy of measurements.

Unlike pharmaceutical drugs, manufacturers of dietary supplements are not required to demonstrate safety or effectiveness of their products prior to sale. Adverse reactions to dietary supplements have been reported, and recently a consumer protection organization recommended against the consumption of dietary supplements that contain certain generic ingredients [49]. Although laws pertaining to pharmaceutical drugs may not apply to dietary supplements, prior to 1994 these products were regulated as foods. In 1994, the U.S. Congress passed the Dietary Supplement Health and Education Act (DSHEA), which provided a separate legal status for dietary supplements and imposed certain requirements for the manufacture and sale of these materials [50]. More recently, the Food and Drug Administration issued current good manufacturing practices (cGMPs) which in part require manufacturers to characterize the chemical composition of their ingredients and products [51]. Other regulations may also apply to specific dietary supplements. The National Institute of Standards and Technology (NIST) is working in collaboration with the National Institutes of Health, Office of Dietary Supplements (NIH-ODS) and the Food and Drug Administration (FDA) to develop certified reference materials (CRMs) to support the analysis of dietary supplements. A NIST-produced CRM is termed a "Standard Reference Material (SRM™). These SRMs are being developed as suites to provide a close match for sample matrices with different complexity and analytical challenges. For example, the current suite of green tea-containing SRMs consists of dried and ground leaves of *Camellia sinensis*, a commercial powdered extract of *Camellia sinensis*, and a blend of green tea-containing dietary supplements. These SRMs are not intended to be archetypes for product formulation, but instead to provide similar analytical challenges that may be encountered by the analyst.

CRMs are produced by national metrology institutes (NMIs) and other providers of higher-order reference materials to assist in the development of new analytical methods, to improve the accuracy of measurements, and for use in establishing measurement traceability. Traceability has been defined as a "property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty" [52]. In principle, measurements that are traceable to the same stated reference are also comparable over time and for different methods and analysts. SRMs can help to establish measurement traceability when used as calibrants or control materials, depending on their intended purposes. By providing SRMs with known

composition, a major source of measurement uncertainty is eliminated leading to improved measurement accuracy.

A suite of three green tea-containing SRMs has been developed to support the analysis of green tea-containing dietary supplements: SRM 3254 *Camellia sinensis* (Green Tea) Leaves, SRM 3255 *Camellia sinensis* (Green Tea) Extract, and SRM 3256 Green Tea-Containing Solid Oral Dosage Form. This report describes the certification of seven catechins and gallic acid, three xanthine alkaloids, theanine, and toxic elements in the three green tea-containing SRMs. As many as five methods were used in the value assignment of these SRMs, with measurements performed by NIST and by collaborating laboratories.

Experimental Section[‡]

Reagents. Catechin reference standards were obtained from Blaze Scientific Industries (BSI; Lawndale, CA) and Sigma-Aldrich (St. Louis, MO) and were used as received without further purification (see Figure 1). Caffeine, theobromine, theophylline, theanine and proxyphylline (7-(β-hydroxypropyl)-theophylline) were obtained from Sigma-Aldrich. Isotopically labeled caffeine, (trimethyl-$^{13}C_3$-caffeine), and labeled theophylline ($^{13}C^{15}N_2$-theophylline) were obtained from Cambridge Isoptope Laboratories (Andover, MA, USA). Gallic acid was obtained from MP Biomedicals (Solon, OH). Reagent purities were determined by multiple methods including liquid chromatography, Karl Fischer moisture analysis, and ^{1}H-nuclear magnetic resonance spectroscopy, and corrections were made for purity.

SRM Preparation. Green tea leaves and extract were obtained from Sinochem Ningbo in China by Modern Nutrition and Biotech, Inc. (Appleton, WI). The material for production of SRM 3254 *Camellia sinensis* (Green Tea) Leaves was received as a grey-green powder with nominal particle size of 425 μm (40 mesh) and was further ground and sieved to 180 μm (80 mesh). The material for SRM 3255 *Camellia sinensis* (Green Tea) Extract was received as a light brown powder with a nominal particle size of 180 μm

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

(80 mesh screen size) and was used without further processing. SRM 3256 Green Tea-Containing Solid Oral Dosage Form was prepared by combining commercially available green tea-containing dietary supplements from four manufacturers. These products were labeled as containing green tea and dandelion, eleuthero, fo ti root, garcinia, ginger root, ginseng, gotu kola, guarana, kelp, passion flower, various vitamins, and yerba mate. Each of the individual materials was ground using a Teflon disk mill. The resulting powders were passed through 45 mesh, 60 mesh, and 80 mesh screens. Materials with particles larger than ≈180 μm (80 mesh screen size) were reground and sieved as necessary.

The sieved materials were transferred to High-Purity Standards (Charleston, SC) where they were 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, the candidate SRMs were gamma irradiated to prevent mold growth (Neutron Products, Inc., Dickerson, MD) at an absorbed dose of 7.9 kGy to 9.5 kGy.

Moisture Determination. Moisture content of the three green tea-containing SRMs was determined by forced air drying, freeze-drying, and desiccator drying studies. Twelve samples of each SRM were dried at 80 °C for 2 h in a drying oven, and the mass loss upon drying was recorded. A second set of twelve samples were dried in a desiccator over magnesium perchlorate. Samples were removed and masses were recorded over a 42 day period. A third set of twelve samples for each SRM were freeze-dried for 20 days to 26 days, until a constant mass was achieved. Levels of all constituents are reported on a dry mass basis by applying a correction for moisture content as determined by the mean of the drying studies. Uncertainty in the moisture correction is included in the expanded uncertainty provided with each assigned value.

Analytical Methods.

Method 1. Determination of Catechins and Gallic Acid by Liquid Chromatography with Mass Spectrometric Detection (LC/MS) (NIST). Duplicate test portions from each of six packets were combined with 5 mL portions of a solution containing proxyphylline (the internal standard) and extracted with ultrasonic agitation for 90 min. A solution of 30 % methanol, 0.05 % formic acid in water (all mixed solvent compositions in the method descriptions are expressed as volume fractions) was used to prepare all solutions and was also used as the extraction solvent for the green tea samples. The extraction process was repeated using 5 mL portions of solvent, and supernatants were combined. Sample sizes were

approximately 90 mg for SRM 3254, 20 mg for SRM 3255, and 60 mg for SRM 3256. Samples of SRM 3255 were dissolved in a single 7 mL portion of the extraction solvent. Supernatants were syringe-filtered prior to analysis. An LC with a mass spectrometric (MS) detector and electrospray ionization source (ESI) was used to determine all analytes. A 250 mm x 4.6 mm, 5 μm particle size Zorbax Eclipse XDB-C18 column (Agilent Technologies, Palo Alto, CA) was used with a Security Guard C18, 4 mm x 3.0 mm guard cartridge (Phenomenex, Torrance, CA). The mobile phase consisted of solvent A 0.1 % formic acid in water and B 0.1 % formic acid in acetonitrile. The following gradient conditions were used: time 0 min 93 % A/ 7 % B; time 55 min 83 % A/17 % B; time 60 min 20 % A/80 % B; time 61 min 93 % A/7 % B. The mobile phase was delivered at a flow rate of 1.0 mL/min, and a post-run time of 10 min was incorporated into the method to allow the column to equilibrate to the initial conditions of the gradient. MS detection was achieved using electrospray ionization (ESI) in positive polarity. ESI/MS detection conditions included: fragmentor, 130 V; drying gas flow, 12 L/min; drying gas temperature, 350 °C; nebulizer pressure, 55 psi; capillary voltage, 3500 V. 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 dynamic quantitation model based on polynomial data-fitting was used to reduce the variability in the MS data[53]. A typical separation is provided in Figure 2; corresponding separations of SRM 3255 and SRM 3256 are provided in Figures S1 and S2.

Method 2. Determination of Catechins and Gallic Acid by Liquid Chromatography with Absorbance Detection (LC/Abs) (NIST). A second set of data was collected simultaneously with Method 1 by using absorbance detection at 280 nm (Figure 2). As with Method 1, proxyphylline was used as an internal standard for all analytes.

Method 3. Determination of Catechins and Gallic Acid by LC/Abs (alternate LC/Abs methods) (NIST). An alternate LC absorbance method was developed for comparison of data with that from the other methods. Chromatographic conditions were modified for each of the three SRMs to address challenges provided by different matrix interferences. In all cases, analyses were performed using a liquid chromatograph consisting of a reciprocating piston pump, autosampler, and variable wavelength absorbance detector with detection at 210 nm. A 250 mm x 4.6 mm, 5 μm particle size Ace C18 column (MAC-MOD Analytical, Inc., Chadds Ford, PA) was used with a 10 mm x 3.0 mm 5 μm particle size ACE C18 guard column. 7-(β-Hydroxypropyl)theophylline (proxyphylline) was used as an internal standard. Representative separations are illustrated in Figure 3.

SRM 3254. Analyses were performed using test portions of 100 mg to 400 mg from each of six packets. Materials were individually combined with diatomaceous earth (Hydromatrix, Isco, Lincoln, NE) in polypropylene tubes. Proxyphylline, ethylenediaminetetracetic acid (EDTA), carbohydrases, and cellulase were added, and the samples were placed in a heated ultrasonic bath for 6 h. A proteinase was then added, the samples were incubated, and decanted. Residues were further extracted with acetone and water (20 % and 80% volume fractions, respectively) using pressurized-fluid extraction, and the extracts were combined. A different mobile phase gradient from that used with SRM 3255 was used to reduce interferences from acetic acid. The mobile phase consisted of solvent A (0.2 % aqueous 85 % phosphoric acid), solvent B (a 0.2 % solution of 85% phosphoric acid in acetonitrile), and solvent C (a 0.2 % solution of 85 % phosphoric acid in methanol). The gradient profile is as follows: time 0 min, 4 % B/6 % C; time 10 min, 8 % B/12 % C; time 20 min 12 % B/13 % C; time 25 min 16 % B/15 % C; time 35 min 16 % B/15 %C; time 37 min 50 % B/50 % C; time 42 min 50 % B/50 % C; time 60 min 4 % B /6 % C. The flow rate was 0.9 mL/min and the column temperature was 23 °C.

SRM 3255. Test portions (20 mg to 70 mg) from each of six packets were analyzed. Samples were spiked with the internal standard, and dissolved in 10 mL 30 % methanol in water. The mobile phase consisted of solvent A (0.01 % aqueous acetic acid,) and solvent B (methanol:acetonitrile, 2:1 and 0.01 % acetic acid), with linear gradient conditions as follows: 0 min, 3% B to 32% B in 75 min at a flow rate of 1.0 mL/min and column temperature of 25 °C.

SRM 3256. Test portions (50 mg to 200 mg) were extracted with 10 mL portions of water containing 0.1 % EDTA. Ottawa sand (Sigma-Aldrich, Milwaukee WI) was added to the slurry to disperse the sample during the extraction. Samples were continuously agitated in sealed polypropylene tubes by inversion (rotation) at ≈377 rad/min (60 rpm) for 2 h. The slurries were centrifuged and filtered with 0.45 µm PTFE filters (Alltech, Deerfield IL). The mobile phase consisted of solvent A (0.1% aqueous 85% phosphoric acid) and solvent B (methanol:acetonitrile, 2:1 and 0.1 % 85 % phosphoric acid), with linear gradient conditions as follows: 0 min, 3 % B to 32 % B in 75 min under a flow rate of 1.0 mL/min and column temperature of 25 °C.

Method 4. Determination of Catechins and Caffeine by Liquid Chromatography with Absorbance Detection (LC/Abs) (Tampa Bay Analytical Research, Inc.). Analyses were performed using a liquid chromatograph consisting of a reciprocating piston pump, autosampler, photodiode array detector with

detection at 278 nm, and a 100 mm x 3.0 mm, 3 µm particle size Ascentis Phenyl column (Supelco, Atlanta, GA, USA). The mobile phase consisted of solvent A (5 % acetonitrile in water, with 0.1 % H_3PO_4, and 0.1 mg/mL EDTA) and solvent B (35 % acetonitrile in water, with 0.1 % H_3PO_4, and 0.1 mg/mL EDTA). A linear gradient from 0 % B to 100 % B in 9 min was used, with 5 min re-equilibration. The flow rate was 0.96 mL/min and column temperature was 35 °C. Test portion sizes were as follows: SRM 3264, ≈150 mg; SRM 3255, ≈400 mg; and SRM 3256 ≈1000 mg. Test portions were weighed into 100-mL volumetric flasks and diluted with 60 mL of water containing 100 mg/L EDTA and 1 mL H_3PO_4 ("diluent"). Samples were sonicated for 15 min with occasional shaking. After cooling, the solutions were diluted to volume with the diluent. Solutions of SRM 3255 and 3256 were further diluted (≈1:100 volume fraction) prior to analysis.

Method 5. Determination of Catechins and Xanthines by Liquid Chromatography with Fluorescence Detection (LC/FL) and Absorbance Detection (Hershey Foods). Catechins were determined using 250 mg test portions from each of 6 samples for SRM 3254 and SRM 3256 and 50 mg test portions from each of 6 samples for SRM 3255. After the addition of the internal standard, samples were extracted with 5 mL portions of 50 % methanol/water containing 0.1 % acetic acid by ultrasonic extraction at 50 °C for 10 min. The extraction was repeated, and the extracts combined. The extracts were filtered through a PTFE filter prior to analysis. Separate test samples were analyzed for caffeine and theobromine with sample sizes of 500 mg (for SRM 3254 and 3256), and 200 mg (for SRM 3255) being used. Samples were added to 100 mL water and shaken for 1 h, at 85 °C. After cooling, 25 mL of the extract was diluted to 100 mL with water. The samples were further diluted by adding 2 mL extract to 4 mL of the mobile phase.

Catechins were analyzed by LC with fluorescence detection. Separations were carried out with a Luna Phenyl-hexyl 250 mm x 4.6 mm column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of solvent A (water with 0.05 % trifluoroacetic acid) and solvent B (methanol with 0.05 % trifluoroacetic acid)). A linear gradient from solvent A to solvent B in 18 minutes was used with a flow rate of 1 mL/min and column temperature of 30 °C. Excition and emission wavelengths of 280 nm and 315 nm, respectively, were used. Caffeine and theobromine were determined separately by LC/abs with detection at 280 nm. Separations were carried out isocratically, with mobile phase composition 75 % water, 25 % methanol, (each containing 1 % acetic acid) with a run time of 20 min.

Method 6. Isotope dilution liquid chromatography mass spectrometric method (ID-LC/MS) for xanthines and theanine (NIST). Caffeine, theophylline, theobromine and theanine were measured in two 70 mg to 100 mg test portions from each of six packets using isotope dilution LC/MS. Caffeine and theophylline were determined separately from theobromine and theanine; however, sample preparation and analysis were similar for each pair of analytes. Test samples were individually combined with an internal standard solution containing trimethyl-$^{13}C_3$-caffeine and $^{13}C^{15}N_2$-theophylline, or $^{2}H_6$-theobromine and $^{2}H_5$-L-theanine at similar levels to each corresponding analyte. After the addition of internal standards, samples of SRMs 3254 and 3256 were extracted with ≈5 mL of 30 % methanol in water with 0.05 % formic acid using ultrasonic agitation for 90 min. Samples were centrifuged at 37700 rad/min (6000 rpm) for 10 min and decanted. This process was repeated and the extracts were combined. For SRM 3255 samples, the tubes were vortex-mixed for 10 s then sonicated for 5 min until the material was completely dissolved. All samples were filtered with a 25 mm, 0.45 μm pore size, cellulose acetate syringe filter with a GD/X prefilter prior to analysis. Separations were carried out using an Ace C18 column (15 cm x 3.0 mm, 3 μm particle size) with gradient elution. Solvent A was 0.05% trifluoroacetic acid (TFA) in water; solvent B was 0.05 % TFA in acetonitrile. Initial conditions were 100% solvent A with a hold for 2 min; this was followed by an 8 min linear gradient to 70% A/30% B with a hold for 10 minutes. At the end of each run, the initial conditions were restored and equilibrated for 5 minutes. The mobile phase flow rate was 0.4 mL/min. MS detection was achieved using ESI in positive polarity. ESI/MS detection conditions included: fragmentor, 80 V; drying gas flow, 9 L/min; drying gas temperature, 350 °C; nebulizer pressure, 40 psi; capillary voltage, 3000 V. Selected ion monitoring was used for quantitation at *m/z* 181 for theophylline and theobromine, *m/z* 184 for $^{13}C^{15}N_2$-theophylline, *m/z* 195 for caffeine, *m/z* 198 for trimethyl-$^{13}C_3$-caffeine, *m/z* 181 for theobromine, *m/z* 187 for $^{2}H_6$-theobromine, *m/z* 175 for theanine, and *m/z* 180 for $^{2}H_5$-L-theanine. Representative separations are illustrated in Figure 4.

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Method 7. Elemental Analysis by ICP/MS (NIST). SRMs 3254 and 3255 were screened for arsenic, cadmium, and lead using inductively coupled plasma-mass spectrometry (ICP/MS) operated in the standard mode. Two test portions (approximately 0.5 g) from a single packet of each SRM were utilized. Concentrations for As in both test portions and for Pb and Cd in one test portion were quantified by external calibration with standards containing 0.2 ng/g, 2 ng/g and 10 ng/g of each element after correction for signal drift using ^{115}In internal standard. Concentrations for Cd and Pb in the second test portion were quantified by isotope dilution using enriched ^{111}Cd and ^{206}Pb isotopes. Spiked test portions

were digested by microwave using concentrated nitric acid and a small amount of concentrated hydrofluoric acid. Samples were evaporated to near-dryness to remove the HF and diluted to 10 g with 2 % weight/volume nitric acid prior to ICP-MS analysis. Signal intensities at *m/z* 75, 111, 112, 114, 115, 204, 206, 207, and 208 were measured.

Arsenic, cadmium, and lead were determined quantitatively in SRM 3256 by inductively coupled plasma-mass spectrometry (ICP/MS) using collision cell mode (CCT-ICP/MS) and the method of standard additions. Approximately 0.5 g samples (n=7 portions of material) of SRM 3256 were spiked with aliquots of an internal standard solution containing ^{103}Rh and ^{197}Au (0.4 g aliquot, 1 mg/kg) and standard addition spike samples (3 of 7 aliquots of material) were spiked with a calibrant solution containing the analytes of interest. This calibrant solution was derived from SRM 3100 series of NIST spectrometric solution SRMs., ,Nutraceutical SRMs 3243 Ephedra-Containing Solid Oral Dosage Form and SRM 3244 Ephedra-Containing Protein Powder were analyzed as control samples using two point standard additions calibration curves Samples were digested by microwave digestion using concentrated nitric acid. The ICP/MS was operated in the collision cell mode using 8 % hydrogen in helium (mass fraction) to minimize the $^{40}Ar^{35}Cl+$ interference on As at *m/z* 75.

Information values for Hg in SRM 3254 and SRM 3255 were obtained using direct combustion analysis according to ASTM method D6722 [54]. Ni sample boats containing approximately 0.05 g samples were directly inserted into a furnace. The sample was dried at 180 ºC for 60 s, and decomposed at 750 ºC under an oxygen atmosphere for 300 s. Molecular Hg species collected on a catalyst, causing conversion to elemental Hg vapor which was collected on a gold amalgamator. After the system was flushed with O_2 gas for 45 s, the amalgamator was flash heated to 900 ºC. The collected Hg was released and detected through the use of atomic absorption measurements across a 15 cm path length cuvette. SRM 3241 (*Ephedra sinica* Stapf Native Extract) and SRM 1633b (Trace Elements in Coal Fly Ash) were used for instrument calibration.

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For SRM 3256, mercury was determined using isotope dilution and a cold vapor introduction system (ID-CV-ICP/MS) [55]. Approximately 0.5 g samples of each SRM were spiked with ^{201}Hg internal standard solution, and digested by microwave digestion with nitric acid. The mercury in each sample solution was reduced to elemental Hg using a reductant solution of 10 % $SnCl_2$ in 7 % HCl (mass fractions). A gas-liquid separator was used to strip the Hg^0 from solution using a stream of Ar gas (≈250 mL/min), which was directed into the injector of the ICP/MS. Spike calibration was achieved via reverse isotope dilution using calibrants prepared using SRM 3133 Mercury Standard Solution.

Results and Discussion

SRMs 3254 through 3256 were characterized by multiple analytical methods, and the results of individual measurements were combined to produce certified and reference values with associated uncertainties. Certified values are reported for measurements in which NIST places the highest level of confidence, with good agreement among methods and good measurement precision. Reference values are reported when the data do not meet the requirements for certification. For example, similar methods may not have sufficient independence for certification, and/or a lack of sufficient agreement among methods may preclude certification. As many as five methods were used in assigning these values, with measurements carried out at NIST and at collaborating laboratories. By utilizing multiple methods with substantial independence, confidence in the accuracy of the measurements is gained and a realistic estimate of measurement uncertainty can be assigned.

Organic Constituents. Levels of organic constituents determined by each method are summarized in Table 1, and plots of individual measurements for SRMs 3254 to 3256 are provided in Figures S3 to S5, respectively. Not all constituents were determined by each method. In general, excellent agreement was achieved among the methods and few if any indications of method biases are apparent. Method precision is likewise excellent, with percent relative standard deviations (RSD) typically ranging from 2 % to 5 % for most of the analytes. The best measurement precision was obtained for LC/MS measurements that utilized an isotopically labeled internal standard, i.e., Method 6 for caffeine, theobromine, and theanine. The utility of labeled internal standards for improving the precision of LC/MS quantitation has been reported [56] and this effect is readily apparent in the current work by comparing the results of Methods 1 and 2. Because isotopically labeled catechins were not available for the current effort, measurements by Method 1 (and Method 2) were based on a single, unlabeled internal standard, proxyphylline. Method 2 data were collected simultaneously with Method 1, with absorbance detection at 280 nm. A comparison of the RSD values for each of the analytes reveals that better precision resulted for absorbance detection compared with mass spectrometric detection. In most cases, RSD values for absorbance detection were about 25 % smaller than RSD values for mass spectrometric detection. This is perhaps not unexpected, since UV spectrophotometry exhibits a high degree of stability whereas mass spectrometry is influenced by fluctuations in source pressure and analyte ionization that occur on a short time scale. These response variations are not compensated by a single internal standard that is resolved from the analytes, unlike isotope dilution approaches to quantitation, for which the internal standard(s) co-elute with the analytes.

To achieve an additional measure of method independence, levels of catechins were also determined by LC/Abs using a different column and mobile phase conditions with detection at 210 nm, and samples were prepared using an alternate extraction approach (Method 3). SRM 3254, the green tea leaves, was treated with carbohydrases, cellulase, and proteinase with ultrasonic agitation to promote release of the catechins from the botanical matrix. The residue was further extracted by pressurized-fluid extraction, and the extracts were combined. Samples of SRM 3255, the green tea extract, were dissolved in 30 % methanol in water prior to analysis. For SRM 3256, the solid oral dosage form blend, samples were extracted with water containing 0.1 % EDTA. These slurries were mixed with Ottawa sand and were continuously resuspended by inverting (rotating) the sample tubes over the course of the extraction. A comparison of the separations resulting from Method 2 and Method 3 reveals a change in elution order for EGCG and EC. A survey of the literature indicates that differences in selectivity among catechin methods are common; about an equal proportion of methods give the elution order of Method 2 (i.e., EC eluting prior to EGCG) [1,14,17,25,31,57] or Method 3 (EGCG eluting before EC) [2,22,24,26,27,29,30,58]. This change in selectivity appears to result from the choice of methanol or acetonitrile as the mobile phase modifier. Although Methods 2 and 3 are not fully independent, the differences in sample processing and chromatographic selectivity provide an approach for evaluating potential method specific biases. Measurement precision was slightly improved for Method 2 compared with Method 3, probably due to increased sample processing associated with Method 3. RSDs for most of the analytes range from about 3 % to 9 %. The agreement between the two methods is excellent, and instances of disagreement could usually be attributed to matrix interferences or, in the case of EGCG, detector overload (data for EGCG, Method 3 was not used in certification). The agreement between Methods 2 and 3 suggests that the additional processing steps utilized with Method 3 (i.e., the use of enzymatic hydrolysis and re-extraction steps) are unnecessary for quantitative recovery of catechins from the different types of sample matrices.

SRMs 3254 through 3256 were also analyzed by Tampa Bay Analytical Research (TBAR, Method 4), and Hershey Foods Laboratories (Method 5). Each laboratory used their usual methods for analytes they chose to measure. Both laboratories utilized phenyl stationary phase based columns rather than C18 columns. LC/Abs detection was employed with Method 4, and LC fluorescence detection was employed with Method 5. These method differences provide additional independence that support an assessment of potential biases among methods. In most cases, remarkably good agreement was achieved for catechins and caffeine among the various methods. Two exceptions are GC and GCG; levels of these catechins were significantly higher for Method 4 than Methods 1, 2, and 3. These analytes were not determined with Method 5. Certified values were determined as the unweighted mean of method averages, except for

data excluded as outliers. Because of the lack of independence between Method 1 and Method 2, these data were averaged for use as a combined method in the calculation of certified values.

Catechins constitute a significant fraction of the mass of green tea-containing samples. This class of compounds is also present in other botanicals and notably, in chocolate. Levels of catechin and epicatechin are certified in SRM 2384 Baking Chocolate. Approximately 50 % of the mass of this material consists of fat (i.e, the sum of fatty acids), and different extraction approaches may be required compared with dried botanical or other processed green tea-containing materials. Xanthine alkaloids are also present in a variety of foods and dietary supplements. Levels of caffeine, theobromine and theophylline have been determined in several complex matrix SRMs (see Table 2). An IDMS method for caffeine has been reported [56]; in the course of the certification of the green tea-containing SRMs, this IDMS method was expanded to include theobromine, theophylline, and theanine [59]. The different SRMs listed in Table 2 offer a choice in the selection of an appropriate matrix, for use as a control material.

Samples of the three green tea-containing SRMs were also screened for the presence of chlorinated pesticides by GC/MS. Quantifiable concentrations of hexachlorocyclohexanes (HCHs), chlordanes, nonachlors, dieldrin, mirex, heptachlors, mirex, DDT and associated metabolites were not present in any of the materials. SRM 3254 *Camellia sinensis* Leaves was further screened for the presence of chlorinated pesticides by two-dimensional gas chromatography mass spectrometry (GCxGC/MS). No evidence of pesticide residues was observed with this alternate method.

Inorganic Constituents. In addition to organic constituents, each of the three green tea-containing SRMs was screened for the presence of the toxic elements arsenic, cadmium, lead, and mercury. Levels for these elements were low, less than 0.5 µg/g in all cases except for Pb in SRM 3254 (see Table 3), and information values are provided for SRM 3254 and 3255. Because insufficient information exists to assign an estimate of uncertainty, information values are typically provided as mass fractions without an expanded uncertainty, and their use in quantitative comparisons is not recommended. In the case of SRM 3256, reference values were assigned and the material was distributed for use in an International Measurement Evaluation Programme (IMEP) interlaboratory comparison exercise (Method 8) [60]. The results of this study are summarized in Table 3. Excellent overall agreement was obtained between the results of the IMEP intercomparison and NIST ICP/MS measurements, and certified values were assigned through combination of the data.

Homogeniety, stability, and traceabilty. Each of the green tea-containing SRMs is provided in dual-barrier, single-use packages that are designed to exclude moisture and oxygen in a format that is also compatible with gamma irradiation. Although the bulk materials were blended prior to packaging, the mass of the subsamples taken for analysis should be similar to those used in certification for the reported uncertainty estimates to be valid. In general, accelerated stability studies are not carried out as part of the certification protocol at NIST. The long-term stability of SRMs are periodically assessed at NIST though stability studies and through use of the SRMs as control materials. The frequency of stability assessment is based on reported or suspected analyte instability. In cases for which constituent levels have changed, values are recertified (or removed from certification), and users are notified. To achieve traceability to the amount-of-substance units of the International System of Units (SI), the measurement result must be related to an unbroken chain of calibrations with associated uncertainties. As part of the certification of the green-tea containing SRMs, purities of the reference standards used in instrument calibration were evaluated with multiple methods.

Conclusions

SRMs 3254 through 3256 are provided by NIST as part of a continuing effort to support the chemical metrology of foods and dietary supplements. This suite of reference materials represent the first green tea-containing reference materials with certified values for catechins These complex-matrix reference materials are intended for use in the development and validation of new analytical methods, and for use as control materials for quality assurance. Proper use of reference materials can also assist in establishing claims of measurement traceability. It is anticipated that use of the reference materials will improve measurement precision and accuracy of measurements of constituents in green tea.

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Reference List

1. Neilson, A. P.; Green, R. J.; Wood, K. V.; Ferruzzi, M. G. High-throughput analysis of catechins and theaflavins by high performance liquid chromatography with diode array detection. *J. Chromatogr. A* **2006,** *1132* (1-2), 132-140.

2. Hu, B.; Wang, L.; Zhou, B.; Zhang, X.; Sun, Y.; Ye, H.; Zhao, L. Y.; Hu, Q. H.; Wang, G. X.; Zeng, X. X. Efficient procedure for isolating methylated catechins from green tea and effective simultaneous analysis of ten catechins, three purine alkaloids, and gallic acid in tea by high-performance liquid chromatography with diode array detection. *J. Chromatogr. A* **2009,** *1216* (15), 3223-3231.

3. Hussain, I.; Khan, H.; Saleem, M.; Marwat, G. A.; Iqbal, Y. Investigation of organic constituents of commercial tea brands and fresh tea leaves by high-performance liquid chromatography. *Journal of the Chemical Society of Pakistan* **2008,** *30* (4), 571-576.

4. Mizukami, Y.; Sawai, Y.; Yamaguchi, Y. Simultaneous analysis of catechins, gallic acid, strictinin, and purine alkaloids in green tea by using catechol as an internal standard. *J. Agric. Food Chem.* **2007,** *55* (13), 4957-4964.

5. Saito, S. T.; Froehlich, P. E.; Gosmann, G.; Bergold, A. M. Full validation of a simple method for determination of catechins and caffeine in Brazilian green tea (Camellia sinensis var. assamica) using HPLC. *Chromatographia* **2007,** *65* (9-10), 607-610.

6. Blahova, E.; Lehotay, J. Sample preparation and HPLC determination of catechins in green tea. *Chemia Analityczna* **2006,** *51* (5), 795-807.

7. Repolles, C.; Herrero-Martinez, J. M.; Rafols, C. Analysis of prominent flavonoid aglycones by high-performance liquid chromatography using a monolithic type column. *J. Chromatogr. A* **2006,** *1131* (1-2), 51-57.

8. Hong, S. B.; Kim, J. I.; Row, K. H. Preparative separation of EGCG from Korean green tea by high performance liquid chromatography. *Separation Science And Technology* **2002,** *37* (7), 1631-1640.

9. Dalluge, J. J.; Nelson, B. C.; Thomas, J. B.; Sander, L. C. Selection of column and gradient elution system for the separation of catechins in green tea using high-performance liquid chromatography. *J. Chromatogr. A* **1998,** *793* (2), 265-274.

10. Amarowicz, R.; Shahidi, F. A rapid chromatographic method for separation of individual catechins from green tea. *Food Research International* **1996,** *29* (1), 71-76.

11. Goto, T.; Yoshida, Y.; Kiso, M.; Nagashima, H. Simultaneous analysis of individual catechins and caffeine in green tea. *J. Chromatogr. A* **1996,** *749* (1-2), 295-299.

12. Pongsuwan, W.; Bamba, T.; Harada, K.; Yonetani, T.; Kobayashi, A.; Fukusaki, E. High-Throughput Technique for Comprehensive Analysis of Japanese Green Tea Quality Assessment Using Ultra-performance Liquid Chromatography with Time-of-Flight Mass Spectrometry (UPLC/TOF MS). *J. Agric. Food Chem.* **2008,** *56* (22), 10705-10708.

13. Mata-Bilbao, M. D. L.; ndres-Lacueva, C.; Roura, E.; Jauregui, O.; Torre, C.; Lamuela-Raventos, R. M. A new LC/MS/MS rapid and sensitive method for the determination of green tea catechins and their metabolites in biological samples. *J. Agric. Food Chem.* **2007,** *55* (22), 8857-8863.

14. Masukawa, Y.; Matsui, Y.; Shimizu, N.; Kondou, N.; Endou, H.; Kuzukawa, M.; Hase, T. Determination of green tea catechins in human plasma using liquid chromatography-electrospray ionization mass spectrometry.

Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences **2006,** *834* (1-2), 26-34.

15. Frauen, M.; Rode, T.; Rapp, C.; Steinhart, H. Determination of green-tea catechins in cosmetic formulations and in in vitro skin extracts by high-performance liquid chromatography coupled with electrospray ionization mass spectrometry. *Chromatographia* **2002,** *55* (1-2), 43-48.

16. Dalluge, J. J.; Nelson, B. C.; Thomas, J. B.; Welch, M. J.; Sander, L. C. Capillary liquid chromatography electrospray mass spectrometry for the separation and detection of catechins in green tea and human plasma. *Rapid Commun. Mass Spectrom.* **1997,** *11* (16), 1753-1756.

17. Chu, K. O.; Wang, C. C.; Rogers, M. S.; Choy, K. W.; Pang, C. P. Determination of catechins and catechin gallates in biological fluids by HPLC with coulometric array detection and solid phase extraction. *Anal. Chim. Acta* **2004,** *510* (1), 69-76.

18. Lee, M. J.; Prabhu, S.; Meng, X. F.; Li, C.; Yang, C. S. An improved method for the determination of green and black tea polyphenols in biomatrices by high-performance liquid chromatography with coulometric array detection. *Anal. Biochem.* **2000,** *279* (2), 164-169.

19. Chen, Q. S.; Zhao, J. W.; Chaitep, S. P.; Guo, Z. M. Simultaneous analysis of main catechins contents in green tea (Camellia sinensis (L.)) by Fourier transform near infrared reflectance (FT-NIR) spectroscopy. *Food Chemistry* **2009,** *113* (4), 1272-1277.

20. Robb, C. S.; Geldart, S. E.; Seelenbinder, J. A.; Brown, P. R. Analysis of green tea constituents by HPLC-FTIR. *J. Liq. Chromatogr. Relat. Technol.* **2002,** *25* (5), 787-801.

21. Dalluge, J. J.; Nelson, B. C. Determination of tea catechins. *J. Chromatogr. A* **2000,** *881*, 411-424.

22. Bonoli, M.; Pelillo, M.; Toschi, T. G.; Lercker, G. Analysis of green tea catechins: comparative study between HPLC and HPCE. *Food Chemistry* **2003,** *81* (4), 631-638.

23. Chu, K. O.; Wang, C. C.; Chu, C. Y.; Rogers, M. S.; Choy, K. W.; Pang, C. P. Determination of catechins and catechin gallates in tissues by liquid chromatography with coulometric array detection and selective solid phase extraction. *J. Chromatogr. B* **2004,** *810* (2), 187-195.

24. Pelillo, M.; Bonoli, M.; Biguzzi, B.; Bendini, A.; Toschi, T. G.; Lercker, G. An investigation in the use of HPLC with UV and MS-electrospray detection for the quantification of tea catechins. *Food Chemistry* **2004,** *87* (3), 465-470.

25. Sharma, V.; Gulati, A.; Ravindranath, S. D.; Kumar, V. A simple and convenient method for analysis of tea biochemicals by reverse phase HPLC. *J. Food Compos. Anal.* **2005,** *18* (6), 583-594.

26. Wang, H. F.; Provan, G. J.; Helliwell, K. HPLC determination of catechins in tea leaves and tea extracts using relative response factors. *Food Chemistry* **2003,** *81* (2), 307-312.

27. Spacil, Z.; Novakova, L.; Solich, P. Comparison of positive and negative ion detection of tea catechins using tandem mass spectrometry and ultra high performance liquid chromatography. *Food Chemistry* **2010,** *123* (2), 535-541.

28. Peng, Z. K.; Hayasaka, Y.; Iland, P. G.; Sefton, M.; Hoj, P.; Waters, E. J. Quantitative analysis of polymeric procyanidins (tannins) from grape (Vitis vinifera) seeds by reverse phase high performance liquid chromatography. *J. Agric. Food Chem.* **2001,** *49* (1), 26-31.

29. Kotani, A.; Miyashita, N.; Kusu, F. Determination of catechins in human plasma after commercial canned green tea ingestion by high-performance liquid chromatography with electrochemical detection using a

microbore column. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2003,** *788* (2), 269-275.

30. Nishitani, E.; Sagesaka, Y. M. Simultaneous determination of catechins, caffeine and other phenolic compounds in tea using new HPLC method. *J. Food Compos. Anal.* **2004,** *17* (5), 675-685.

31. Yang, D. J.; Hwang, L. S.; Lin, J. T. Effects of different steeping methods and storage on caffeine, catechins and gallic acid in bag tea infusions. *J. Chromatogr. A* **2007,** *1156* (1-2), 312-320.

32. Wang, D. M.; Lu, J. L.; Miao, A. Q.; Xie, Z. Y.; Yang, D. P. HPLC-DAD-ESI-MS/MS analysis in leaves of 22 tea of polyphenols and purine alkaloids cultivars in China. *J. Food Compos. Anal.* **2008,** *21* (5), 361-369.

33. Aucamp, J. P.; Hara, Y.; Apostolides, Z. Simultaneous analysis of tea catechins, caffeine, gallic acid, theanine and ascorbic acid by micellar electrokinetic capillary chromatography. *J. Chromatogr. A* **2000,** *876* (1-2), 235-242.

34. Bonoli, M.; Colabufalo, P.; Pelillo, M.; Toschi, T. G.; Lercker, G. Fast determination of catechins and xanthines in tea beverages by micellar electrokinetic chromatography. *J. Agric. Food Chem.* **2003,** *51* (5), 1141-1147.

35. Nelson, B. C.; Brown Thomas, J. M.; Wise, S. A.; Dalluge, J. J. The Separation of Green Tea Catechins by Micellar Electrokinetcis Capillary Chromatography. *J. Microcol. Sep.* **1998,** *10*, 671-679.

36. Stach, D.; Schmitz, O. J. Decrease in concentration of free catechins in tea over time determined by micellar electrokinetic chromatography. *J. Chromatogr. A* **2001,** *924* (1-2), 519-522.

37. Watanabe, T.; Nishiyama, R.; Yamamoto, A.; Nagai, S.; Terabe, S. Simultaneous analysis of individual catechins, caffeine, and ascorbic acid in commercial canned green and black teas by micellar electrokinetic chromatography. *Analytical Sciences* **1998,** *14* (2), 435-438.

38. Weiss, D. J.; Anderton, C. R. Determination of catechins in matcha green tea by micellar electrokinetic chromatography. *J. Chromatogr. A* **2003,** *1011* (1-2), 173-180.

39. Worth, C. C. T.; Wiessler, M.; Schmitz, O. J. Analysis of catechins and caffeine in tea extracts by micellar electrokinetic chromatography. *Electrophoresis* **2000,** *21* (17), 3634-3638.

40. Weiss, D. J.; Austria, E. J.; Anderton, C. R.; Hompesch, R.; Jander, A. Analysis of green tea extract dietary supplements by micellar electrokinetic chromatography. *J. Chromatogr. A* **2006,** *1117* (1), 103-108.

41. Li, Z. B.; Huang, D. N.; Tang, Z. X.; Deng, C. H. Microwave-assisted extraction followed by CE for determination of catechin and epicatechin in green tea. *J. Sep. Sci.* **2010,** *33* (8), 1079-1084.

42. Gu, X. G.; Cai, J. B.; Zhang, Z. Z.; Su, Q. D. Dynamic ultrasound-assisted extraction of catechins and caffeine in some tea samples. *Annali di Chimica* **2007,** *97* (5-6), 321-330.

43. Friedman, M.; Levin, C. E.; Choi, S. H.; Kozukue, E.; Kozukue, N. HPLC analysis of catechins, theaflavins, and alkaloids in commercial teas and green tea dietary supplements: Comparison of water and 80% ethanol/water extracts. *J. Food Sci.* **2006,** *71* (6), C328-C337.

44. Cheong, W. J.; Park, M. H.; Kang, G. W.; Ko, J. H.; Seo, Y. J. Determination of catechin compounds in Korean green tea infusions under various extraction conditions by high performance liquid chromatography. *Bulletin of the Korean Chemical Society* **2005,** *26* (5), 747-754.

45. Pineiro, Z.; Palma, M.; Barroso, C. G. Determination of catechins by means of extraction with pressurized liquids. *J. Chromatogr. A* **2004,** *1026* (1-2), 19-23.

46. Song, G. Q.; Lin, J. M.; Qu, F.; Huie, C. W. Extraction of catechins and caffeine from different tealeaves and comparison with micellar electrokinetic chromatography. *Chinese Science Bulletin* **2003,** *48* (22), 2438-2443.

47. Chang, C. J.; Chiu, K. L.; Chen, Y. L.; Yang, P. W. Effect of ethanol content on carbon dioxide extraction of polyphenols from tea. *J. Food Compos. Anal.* **2001,** *14* (1), 75-82.

48. Suematsu, S.; Hisanobu, Y.; Saigo, H.; Matsuda, R.; Hara, K.; Komatsu, Y. Effects of Ph on Stability of Constituents in Canned Tea Drinks (Studies on Preservation of Constituents in Canned Drinks .1.). *Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi* **1992,** *39* (2), 178-182.

49. Internet Communication 2010.

50. Dietary Supplement Health and Education Act. Public Law 103-417 [S.784]; Oct 25. 1994.

51. Food and Drug Administration 21 CFR Part 111 Current Good Manufacturing Practice in Manufacturing, Packaging, Labeling, or Holding Operations for Dietary Supplements; Final Rule. *Fed. Regist.* **2007,** *72* (121), 34751-34958.

52. JCGM *International Vocabulary of Metrology - Basic and General Concepts and Associated Terms, VIM;* 3rd edition ed.; Bureau International des Poids et Mesures: 2008.

53. Bedner, M.; Duewer, D. L. A Dynamic Calibration Approach for Determining Catechins and Gallic Acid in Green Tea. Analytical Chemistry . 2011.

54. *Standard Test Method for Total Mercury in Coal and Coal Combustion Residues by Direct Combustion Analysis*;ASTM Standard D6722-01, 2006; ASTM International: West Conshohocken, PA, 10.

55. Christopher, S. J.; Long, S. E.; Rearick, M. S.; Fassett, J. D. Development of isotope dilution cold vapor inductively coupled plasma mass spectrometry and its application to the certification of mercury in NIST standard reference materials. *Anal. Chem.* **2001,** *73* (10), 2190-2199.

56. Sander, L. C.; Putzbach, K.; Nelson, B. C.; Rimmer, C. A.; Bedner, M.; Thomas, J. B.; Porter, B.; Wood, L. J.; Schantz, M. M.; Murphy, K. E.; Sharpless, K. E.; Wise, S. A.; Yen, J. H.; Siitonnen, P. H.; NguyenPho, A.; Roman, M. C.; Betz, J. M. Certification of Standard Reference Materials Containing Bitter Orange. *Anal. Bioanal. Chem.* **2008,** *391* (3), 2023-2034.

57. Peng, L.; Song, X. H.; Shi, X. G.; Li, J. X.; Ye, C. X. An improved HPLC method for simultaneous determination of phenolic compounds, purine alkaloids and theanine in Camellia species. *J. Food Compos. Anal.* **2008,** *21* (7), 559-563.

58. Pelillo, M.; Biguzzi, B.; Bendini, A.; Toschi, T. G.; Vanzini, M.; Lercker, G. Preliminary investigation into development of HPLC with UV and MS-electrospray detection for the analysis of tea catechins. *Food Chemistry* **2002,** *78* (3), 369-374.

59. Bedner, M.; Sander, L. C.; Sharpless, K. E. An LC-ESI/MS method for determining theanine in green tea dietary supplements. *Anal. Bioanal. Chem.* **2010,** *397* (5), 1773-1777.

60. Baer, I.; de la Calle Guntinas, M. B.; Sander, L. C.; long, s.; Christopher, S. J.; Day, R. D.; Murphy, K. E.; Verbist, I.; Vendelbo, D.; Emteborg, H.; Taylor, P. *IMEP-28: Total Arsenic, Cadmium, Lead and Mercury in Food Supplements*;JRC55721; 09.

Table 1. Means, standard deviations (s) and relative standard deviations (RSD) of "N" measurements of catechins, xanthines, and other constituents of SRMs 3254, 3255, and 3256 for different analytical methods. Values are reported as mass fractions, on a dry-mass basis (mg/g).

SRM 3254	Certified and Reference Values			1. NIST LC/MS			2. NIST LC/Abs (A)			3. NIST LC/Abs (B)			4. Tampa Bay Analytical Research			5. Hershey Laboratories			6. NIST LC/MS Xanthines/Theanine		
Analyte				**Mean**	**s**	**N**	**Mean**	**s**	**N**	**Mean**	**s**	**N**	**Mean**	**s**	**N**	**Mean**	**s**	**N**	**Mean**	**s**	**N**
Catechin	1.01	±	0.41[b]	0.76	0.01	6	1.12	0.00	6	0.77	0.03	6	1.38	0.04	15	0.94	0.06	6			
Epicatechin	8.9	±	1.6[a]	7.46	0.04	6	9.30	0.05	6	8.21	0.08	6	8.84	0.12	15	10.51	0.69	6			
Epicatechin gallate	12.7	±	1.2[a]	11.7	0.1	6	12.8	0.1	6	12.0	0.2	6	13.8	0.2	15						
Gallocatechin	2.4	±	1.1[b]	2.99	0.02	6	2.87	0.04	6	1.83	0.03	6									
Epigallocatechin	25.2	±	4.5[a]	25.8	0.1	6	29.5	0.4	6	21.1	0.5	6	26.9	0.2	15						
Gallocatechin gallate	0.99	±	0.21[a]	0.84	0.02	6	1.26	0.02	6	0.93	0.02	6									
Epigallocatechin gallate	52.0	±	2.2[a]	51.9	0.5	6	51.0	0.3	6	50.1	0.8	6	54.4	0.3	15						
Gallic acid	1.12	±	0.61[b]	0.84	0.01	6	0.80	0.01	6	1.42	0.05	6									
Theanine	2.13	±	0.054[b]																2.13	0.02	6
Caffeine	23.5	±	1.8[a]							21.9	0.2	6	24.4	0.1	15	23.2	0.3	6	24.56	0.02	6
Theobromine	0.463	±	0.052[a]							0.41	0.02	6				0.48	0.01	6	0.50	0.00	6
Procyanidin C-1																0.563	0.022	6			
Polyphenols																165.249	5.215	6			

SRM 3255	Certified and Reference Values			1. NIST LC/MS			2. NIST LC/Abs (A)			3. NIST LC/Abs (B)			4. Tampa Bay Analytical Research			5. Hershey Laboratories			6. NIST LC/MS Xanthines/Theanine		
Analyte				**Mean**	**s**	**N**	**Mean**	**s**	**N**	**Mean**	**s**	**N**	**Mean**	**s**	**N**	**Mean**	**s**	**N**	**Mean**	**s**	**N**
Catechin	9.17	±	0.93[a]	9.77	0.13	6	10.07	0.07	6	9.27	0.09	6	8.40	0.19	15	9.10	0.42	6			
Epicatechin	47.3	±	6.7[a]	46.5	0.6	6	45.7	0.3	6	42.3	0.4	6	47.9	0.3	14	52.8	2.5	6			
Epicatechin gallate	100.3	±	7.8[a]	95.9	1.8	6	93.9	1.5	6	97.7	1.3	6	108.4	0.4	14						
Gallocatechin	22.0	±	1.7[a]	23.4	0.4	6	22.4	0.2	6	21.2	0.5	6									
Epigallocatechin	81.8	±	6.5[a]	86.3	0.8	6	86.5	0.5	6	82.6	1.1	6	76.3	0.2	14						
Gallocatechin gallate	39.0	±	2[a]	38.2	0.9	6	38.4	0.6	6	39.8	1.5	6									
Epigallocatechin gallate	422	±	19[a]	406	5	6	419	5	6				431	3	14						
Gallic acid	3.23	±	0.008[b]	3.26	0.02	6	3.20	0.02	6												
Theanine	0.34	±	0.008[b]																0.34	0.00	6
Caffeine	36.9	±	2.7[a]							35.5	0.3	6	37.9	0.1	14	35.2	0.5	6	39.0	0.1	6
Theobromine	0.867	±	0.076[a]							0.84	0.05	6				0.84	0.02	6	0.93	0.00	6
Theophylline	0.087	±	0.002[b]																0.087		6
Procyanidin C-1										2.235	0.229	6				1.03		6			
Polyphenols																774.2	8.8	6			

SRM 3256	Certified and Reference Values			1. NIST LC/MS			2. NIST LC/Abs (A)			3. NIST LC/Abs (B)			4. Tampa Bay Analytical Research			5. Hershey Laboratories			6. NIST LC/MS Xanthines/Theanine		
Analyte				**Mean**	**s**	**N**	**Mean**	**s**	**N**	**Mean**	**s**	**N**	**Mean**	**s**	**N**	**Mean**	**s**	**N**	**Mean**	**s**	**N**
Catechin	2.63	±	0.18[a]	2.37	0.01	6	2.93	0.02	6	2.46	0.53	4	2.66	0.06	15	2.75	0.12	6			
Epicatechin	12.0	±	2.6[a]	9.61	0.07	6	10.85	0.04	6	11.16	0.30	4	14.03	0.13	15	12.63	0.57	6			
Epicatechin gallate	17.1	±	2.6[a]	16.4	0.2	6	17.4	0.1	6	14.9	0.5	4	19.6	0.2	15						
Gallocatechin	7.55	±	0.28[a]	7.63	0.05	6	7.49	0.05	6	7.53	0.12	4									
Epigallocatechin	30.7	±	5.7[a]	28.4	0.3	6	32.7	0.2	6	26.9	0.5	4	34.6	0.3	15						
Gallocatechin gallate	4.6	±	1.8[b]	5.50	0.04	6	5.46	0.04	6	3.65	0.67	4									
Epigallocatechin gallate	71.1	±	6.6[a]	73.7	0.7	6	72.3	0.3	6	62.7	2.4	4	77.6	0.6	15						
Gallic acid	13.10	±	0.49[a]	13.5	0.1	6	13.0	0.1	6	12.9	0.1	4									
Theanine	3.7	±	1.2[b]							3.08	0.19	4							4.24	0.03	6
Caffeine	70.0	±	2.6[a]							69.2	1.0	4	70.3	0.6	15	68.3	0.6	6	72.2	0.1	6
Theobromine	1.04	±	0.15[a]							0.96	0.02	4							1.12	0.01	6
Theophylline	0.060	±	0.002[b]																0.060	0.001	6
Procyanidin C-1																0.734	0.031	6			
Polyphenols																237.3	4.1	6			

(a) Each certified value (shown in bold) is an equally weighted mean of the results from two to five analytical methods carried out at NIST and at collaborating laboratories. In cases where data were provided using UV and MS detectors 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.

(b) Reference values. 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 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.

Table 2. Certified levels of Catechin, Epicatechin, Caffeine, Theobromine, and Theophylline in dietary supplement and food matrix SRMs. Values are reported as mass fractions, on a dry-mass basis (mg/g)

	Catechin	Epicatechin	Caffeine	Theobromine	Theophylline[a]
SRM 2384 Baking Chocolate	0.25 ± 0.051	1.22 ± 0.24	1.06 ± 0.05	11.6 ± 1.1	0.151 ± 0.003
SRM 3243 Ephedra-Containing Solid Oral Dosage Form			76.5 ± 4.1		
SRM 3244 Ephedra-Containing Protein Powder			2.99 ± 0.54	0.762 ± 0.026[a]	0.080 ± 0.003
SRM 3254 Camellia sinensis (Green Tea) Leaves	1.01 ± 0.41[a]	8.9 ± 1.6	23.5 ± 1.8	0.463 ± 0.052	
SRM 3255 Camellia sinensis (Green Tea) Extract	9.17 ± 0.93	47.3 ± 6.7	36.9 ± 2.7	0.867 ± 0.076	0.087 ± 0.002
SRM 3256 Green Tea-Containing Solid Oral Dosage Form	2.63 ± 0.18	12.0 ± 2.6	70.0 ± 2.6	1.04 ± 0.15	0.060 ± 0.002
SRM 3260 Bitter Orange-Containing Solid Oral Dosage Form			64.3 ± 1.2		

(a) Reference values. 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 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.

Table 3. Means, standard deviations (s) and relative standard deviations (RSD) of "N" measurements of toxic elements of SRMs 3254, 3255, and 3256 for different analytical methods. Values are reported as mass fractions, on a dry-mass basis (μg/g).

SRM 3254	**Information Values**				**7. NIST ICP/MS or direct combustion[c] atomic absorption[c]**										
Analyte					**Mean**	**s**	**N**	**%RSD**							
Arsenic		0.15			0.147	0.008	2	5.8%							
Cadmium		0.04			0.038	0.001	2	2.8%							
Lead		1.8			1.823	0.073	2	4.0%							
Mercury[c]		0.01			0.0136	0.0005	3	4.0%							
SRM 3255	**Information Values**				**7. NIST ICP/MS or direct combustion[c] atomic absorption[c]**										
Analyte					**Mean**	**s**	**N**	**%RSD**							
Arsenic		0.16			0.163	0.009	2	5.7%							
Cadmium		0.006			0.0064	0.0003	2	4.8%							
Lead		0.085			0.085	0.001	2	1.2%							
Mercury[c]		0.004			0.0037	0.0004	3	9.6%							
SRM 3256	**Certified Values[a]**				**7. NIST ICP/MS**					**8. IMEP Intercomparison**					
Analyte					**Mean**	**s**	**N**	**%RSD**		**Mean**	**s**	**Median**	**MAD[b]**	**N**	**%RSD**
Arsenic	0.269	±	0.019		0.279	0.019	7	7.0%		0.278	0.022	0.259	0.072	28	7.9%
Cadmium	0.025	±	0.002		0.0266	0.0010	7	3.8%		0.0264	0.0012	0.0240	0.0045	28	4.5%
Lead	0.316	±	0.030		0.314	0.060	7	19.2%		0.314	0.069	0.318	0.064	30	22.0%
Mercury	0.014	±	0.002		0.0130	0.0009	14	7.1%		0.0129	0.0026	0.015	0.006	22	20.2%

(a) Each certified value, expressed as a mass fraction, is an equally weighted mean of results provided by NIST and the IMEP median. 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.

(b) A measure of variability of the laboratory means based on the median absolute deviation.

(c) For SRM 3254 and SRM 3255, mercury was determined by direct combustion atomic absorption (see text)

Figure Captions

Figure 1. Structures of catechins and xanthines in green tea related samples, with CAS designations and relative molecular masses.

Figure 2. Analyses of SRM 3254 by NIST Methods 1 and 2; LC/MS (A, B, C) and LC/abs (D).

Figure 3. Analyses of SRM 3254 - 3256 NIST Method 3, LC/abs with detection at 210 nm.

Figure 4. Analyses of SRM 3255 and SRM 3256 by NIST Method 6; ID-LC/MS

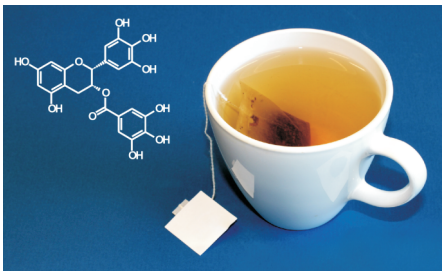
Figure S1. Analyses of SRM 3255 by NIST Methods 1 and 2; LC/MS and LC/abs.

Figure S2. Analyses of SRM 3256 by NIST Methods 1 and 2; LC/MS and LC/abs.

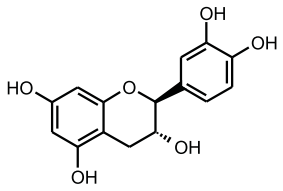
Figure S3. Individual measurements of catechins, xanthines, and other constituents of SRMs 3254, as determined by up to 5 different methods. The solid lines represent the certified values, and the dashed lines the expanded uncertainties. Method 4 data is plotted as a mean +/- the standard deviation.

Figure S4. Individual measurements of catechins, xanthines, and other constituents of SRMs 3255, as determined by up to 5 different methods. The solid lines represent the certified values, and the dashed lines the expanded uncertainties. Method 4 data is plotted as a mean +/- the standard deviation.

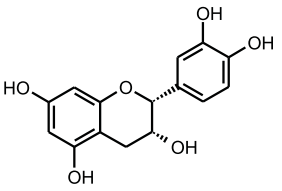
Figure S5. Individual measurements of catechins, xanthines, and other constituents of SRMs 3256, as determined by up to 5 different methods. The solid lines represent the certified values, and the dashed lines the expanded uncertainties. Method 4 data is plotted as a mean +/- the standard deviation.



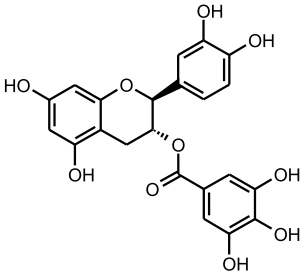
For TOC only



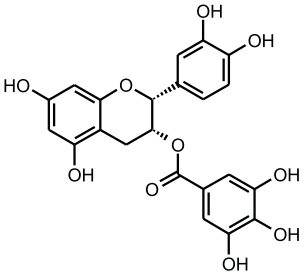
(+)-Catechin, C
$C_{15}H_{14}O_6$ FW 290.27
[18829-70-4]



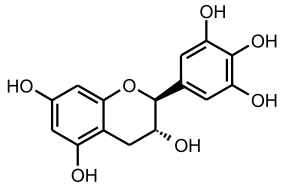
(-)-Epicatechin, EC
$C_{15}H_{14}O_6$ FW 290.27
[490-46-0]



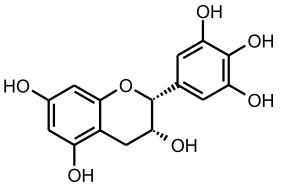
(-)-Catechin 3-gallate, (GCG)
$C_{22}H_{18}O_{10}$ FW 442.37
[130405-40-2]



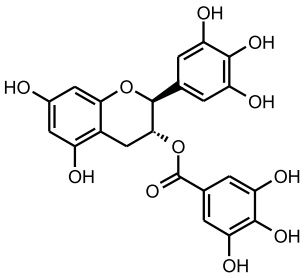
(-)-Epicatechin 3-gallate, (ECG)
$C_{22}H_{18}O_{10}$ FW 442.37
[1257-08-5]



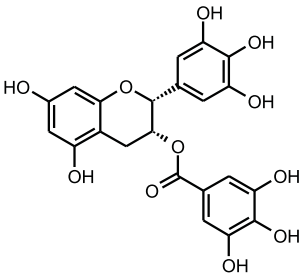
(-)-Gallocatechin, (GC)
$C_{15}H_{14}O_7$ FW 306.27
[3371-27-5]



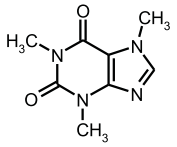
(-)-Epigallocatechin, (EGC)
$C_{15}H_{14}O_7$ FW 306.27
[970-74-1]



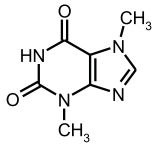
(-)-Gallocatechin 3-gallate, (GCG)
$C_{22}H_{18}O_{11}$ FW 458.37
[4233-96-9]



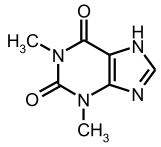
(-)-Epigallocatechin 3-gallate, (EGCG)
$C_{22}H_{18}O_{11}$ FW 458.37
[989-51-5]



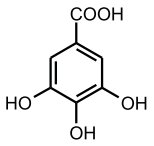
Caffeine, (Caf)
$C_8H_{10}N_4O_2$ FW 194.19
[58-08-2]



Theobromine, (TB)
$C_7H_8N_4O_2$ FW 180.16
[83-67-0]



Theophylline, (TP)
$C_7H_8N_4O_2$ FW 180.16
[58-55-9]



Gallic acid, (GA)
$(HO)_3C_6H_2CO_2H$ FW 170.12
[149-91-7]

Figure 1. Structures of catechins and xanthines in green tea related samples, with CAS designations and relative molecular masses.

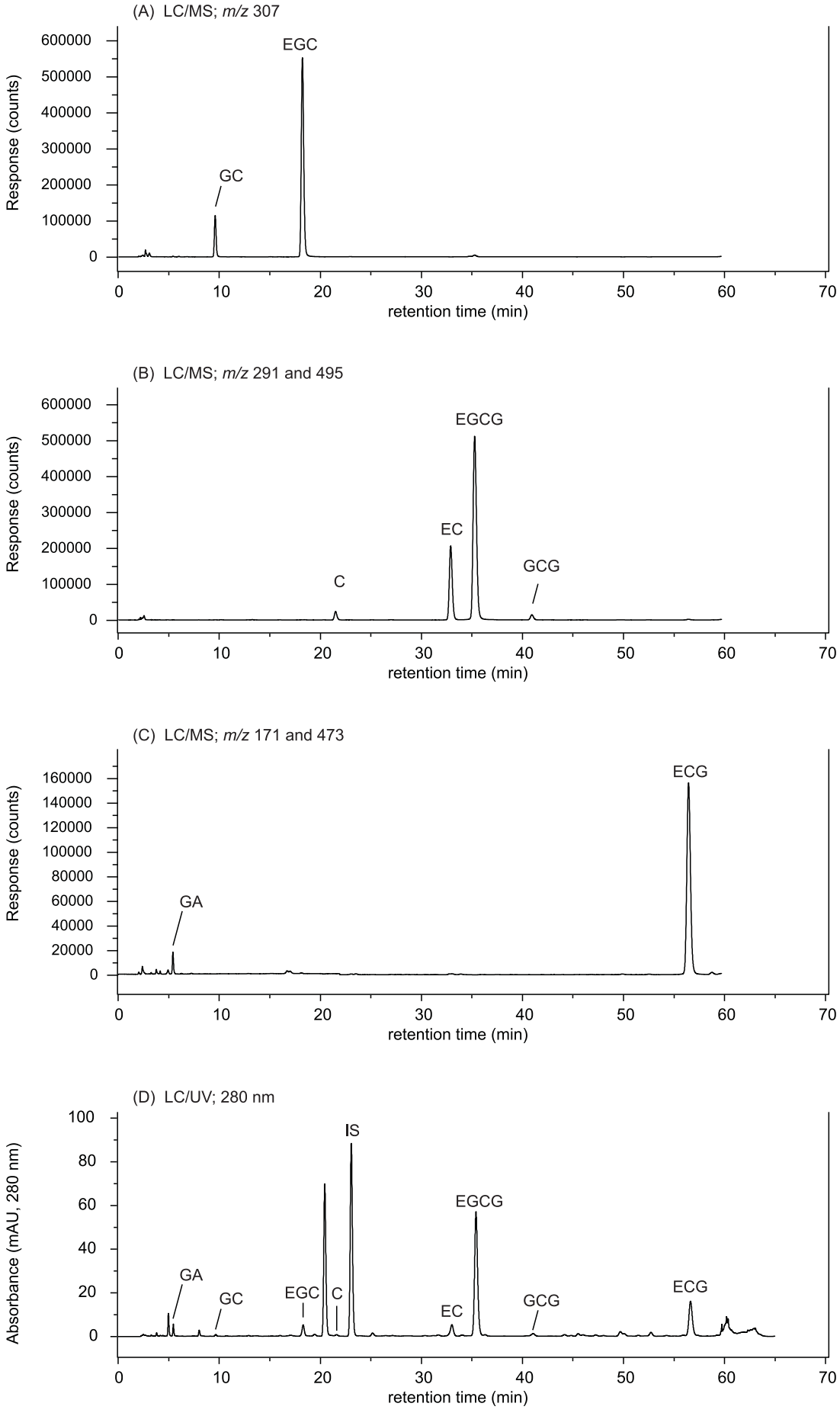


Figure 2. Analyses of SRM 3254 by NIST Methods 1 and 2; LC/MS (A, B, C) and LC/abs (D).

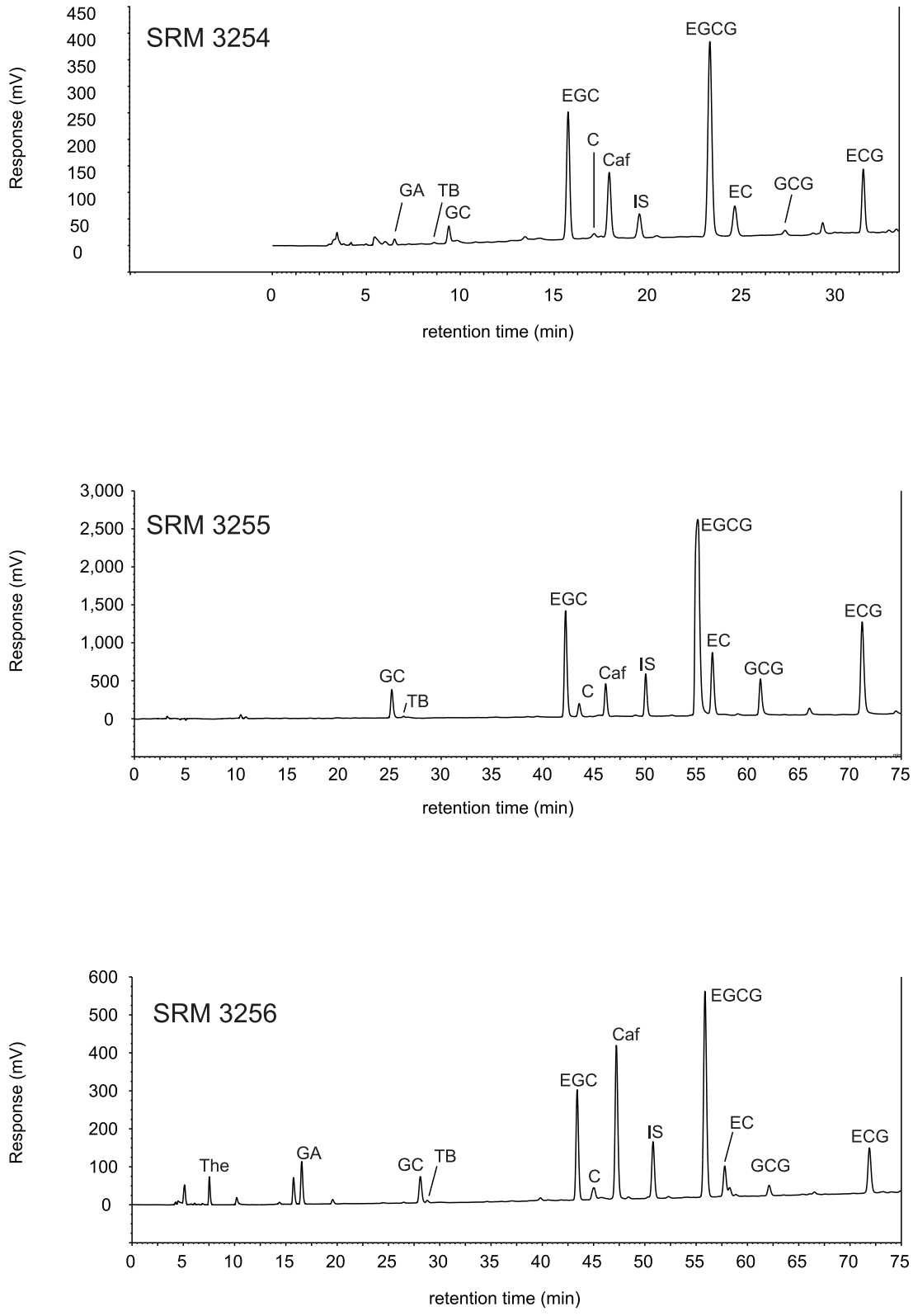


Figure 3. Analyses of SRM 3254 - 3256 NIST Method 3, LC/abs with detection at 210 nm.

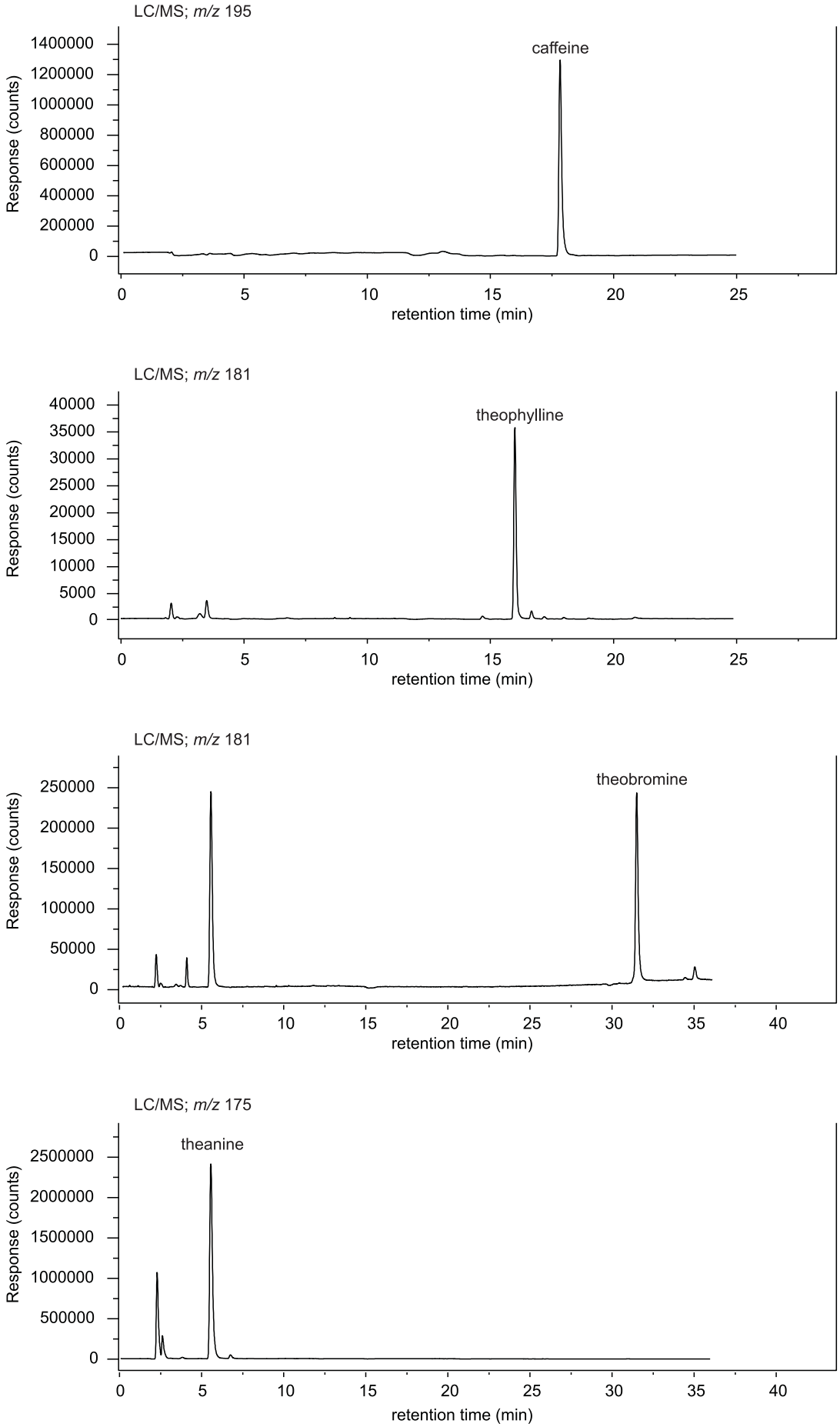


Figure 4. Analyses of SRM 3255 and SRM 3256 by NIST Method 6; ID-LC/MS

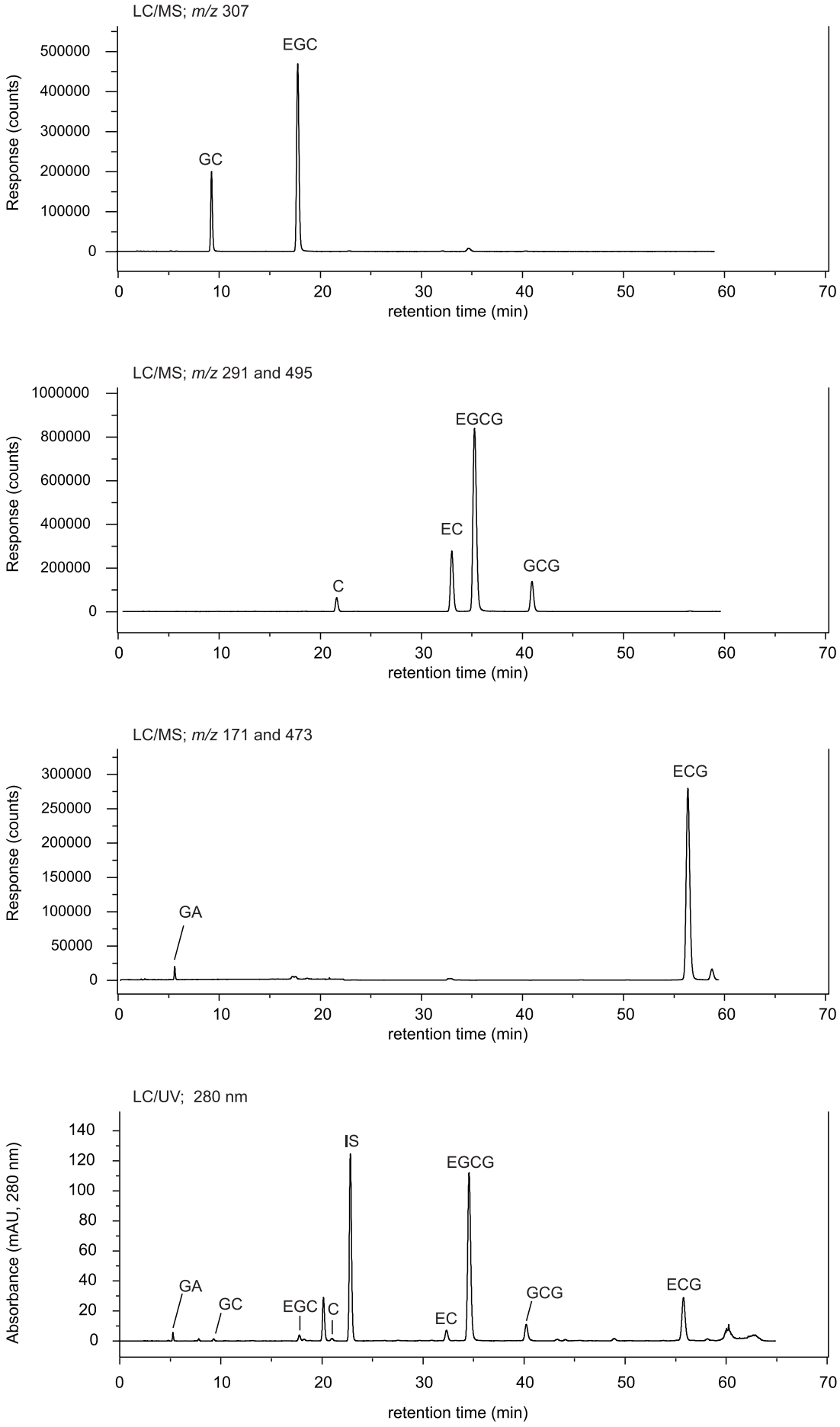


Figure S1. Analyses of SRM 3255 by NIST Methods 1 and 2; LC/MS and LC/abs.

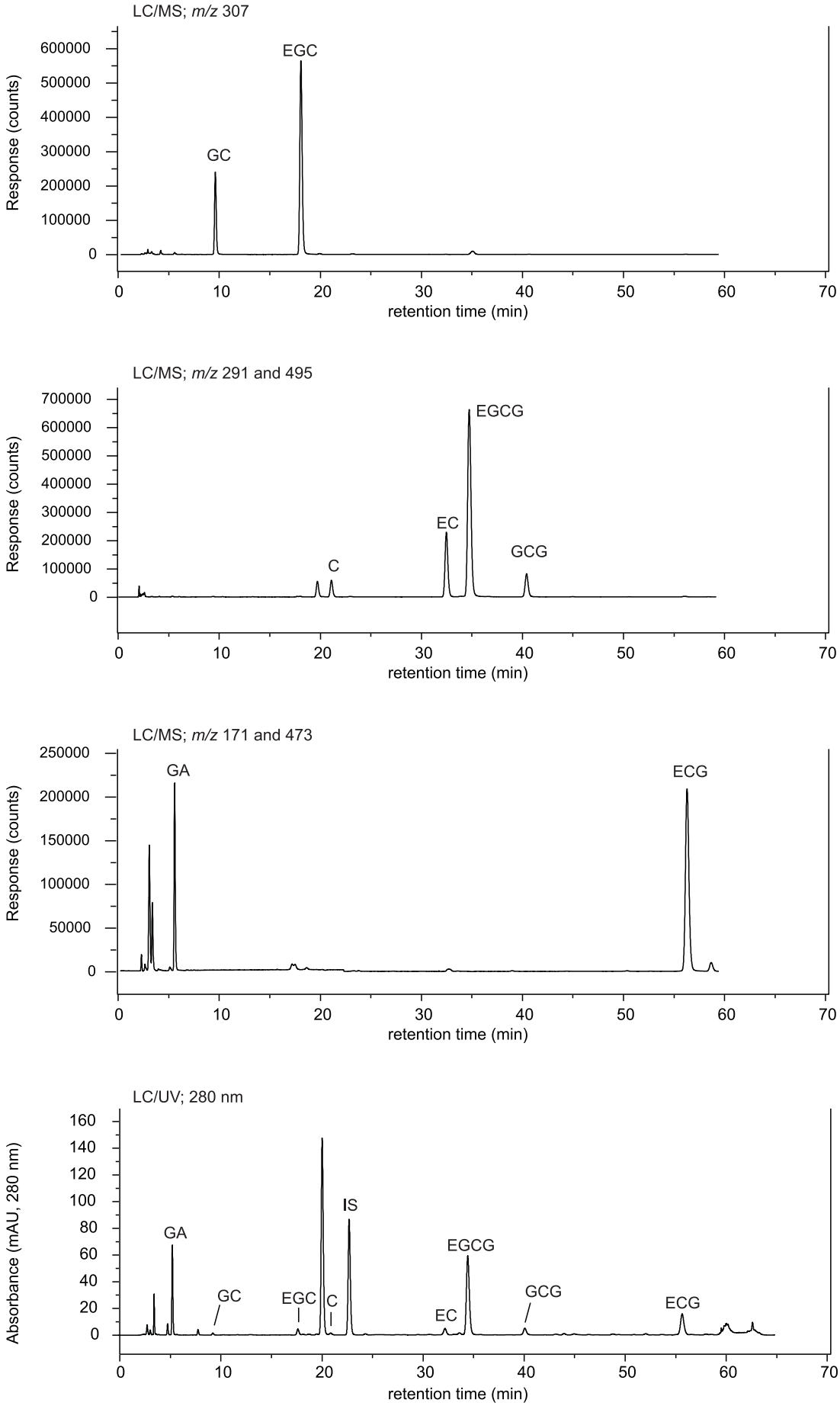


Figure S2. Analyses of SRM 3256 by NIST Methods 1 and 2; LC/MS and LC/abs.

SRM 3254

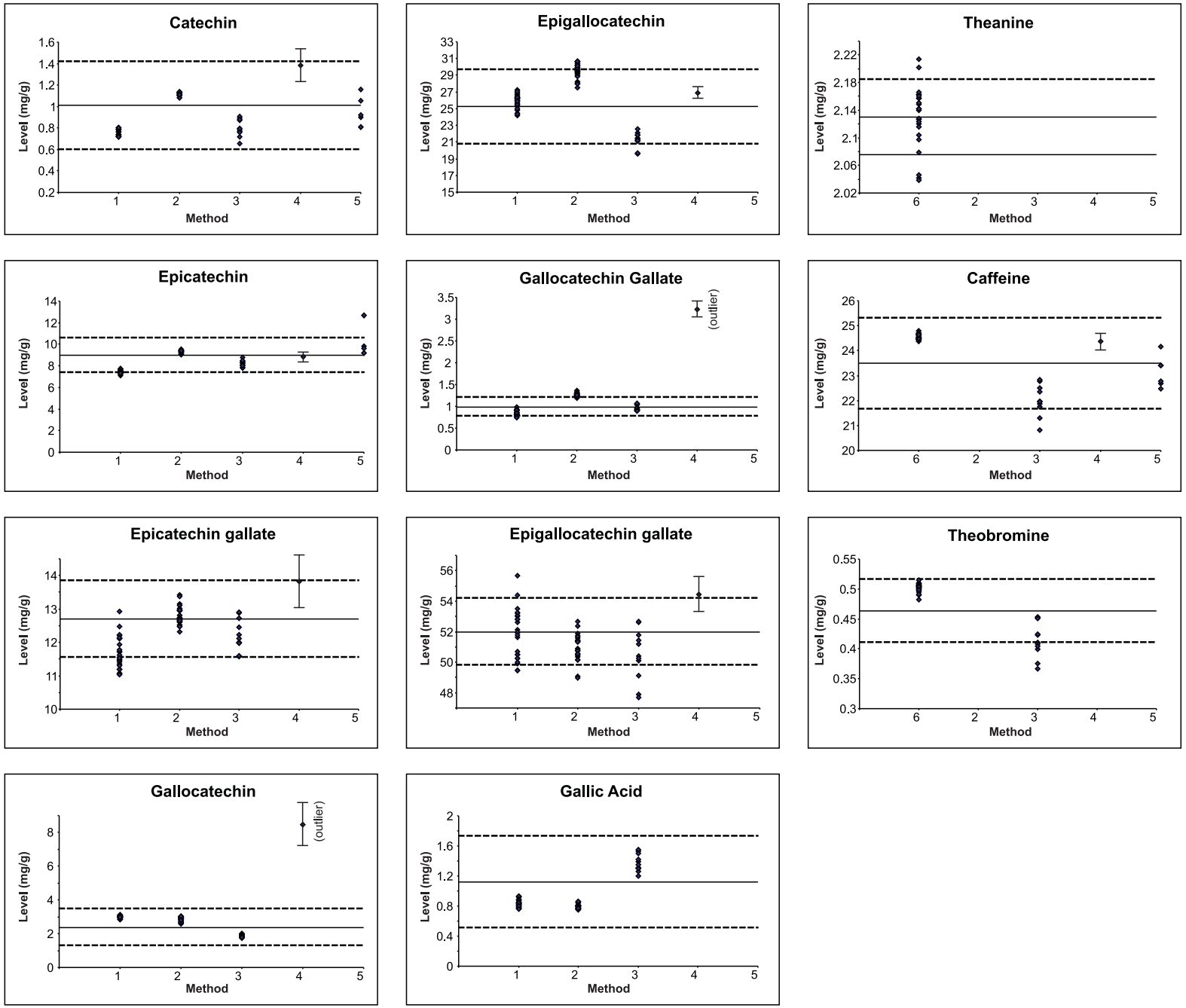


Figure S3. Individual measurements of catechins, xanthines, and other constituents of SRMs 3254, as determined by up to 5 different methods. The solid lines represent the certified values, and the dashed lines the expanded uncertainties. Method 4 data is plotted as a mean +/- the standard deviation.

SRM 3255

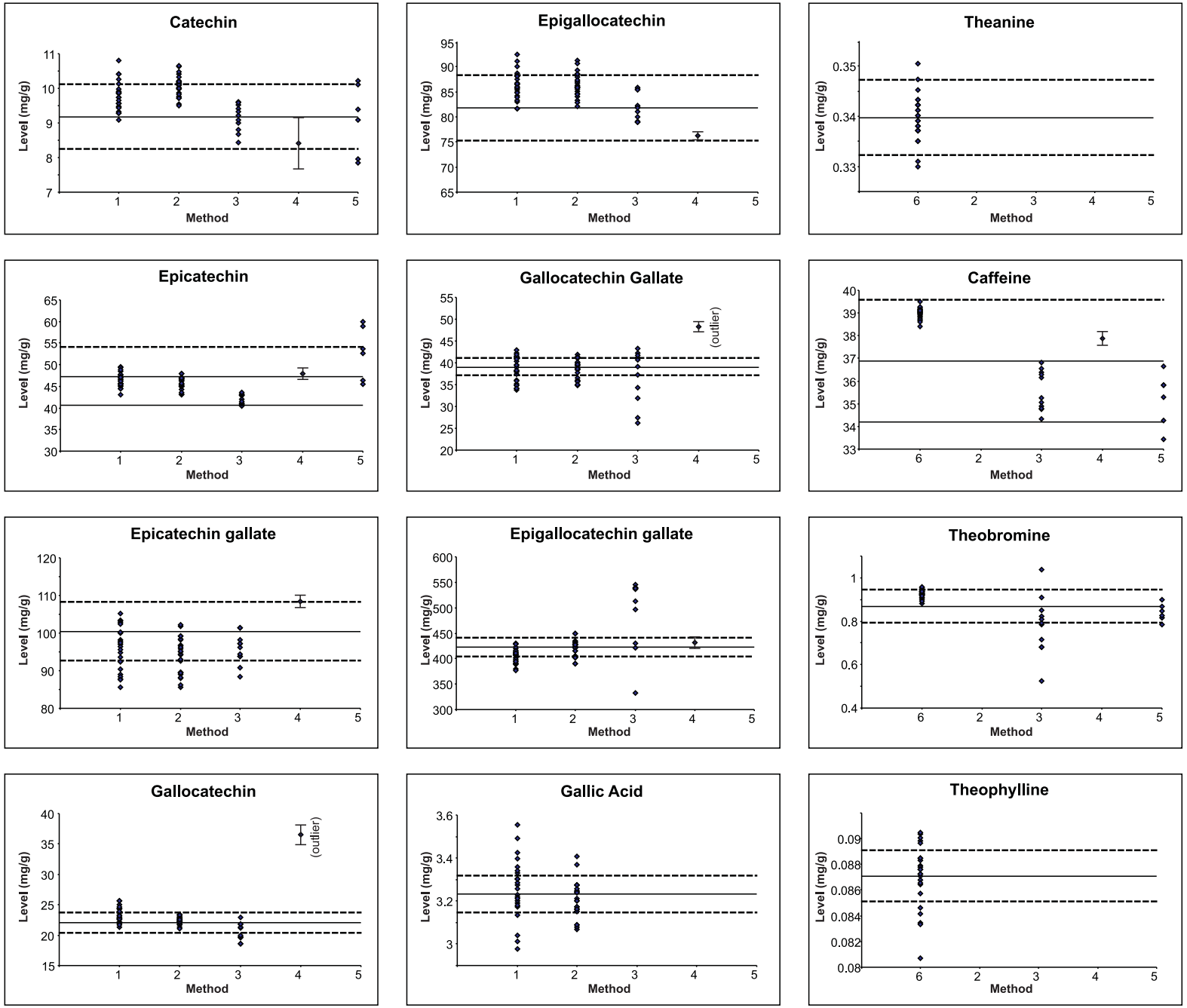


Figure S4. Individual measurements of catechins, xanthines, and other constituents of SRMs 3255, as determined by up to 5 different methods. The solid lines represent the certified values, and the dashed lines the expanded uncertainties. Method 4 data is plotted as a mean +/- the standard deviation.

SRM 3256

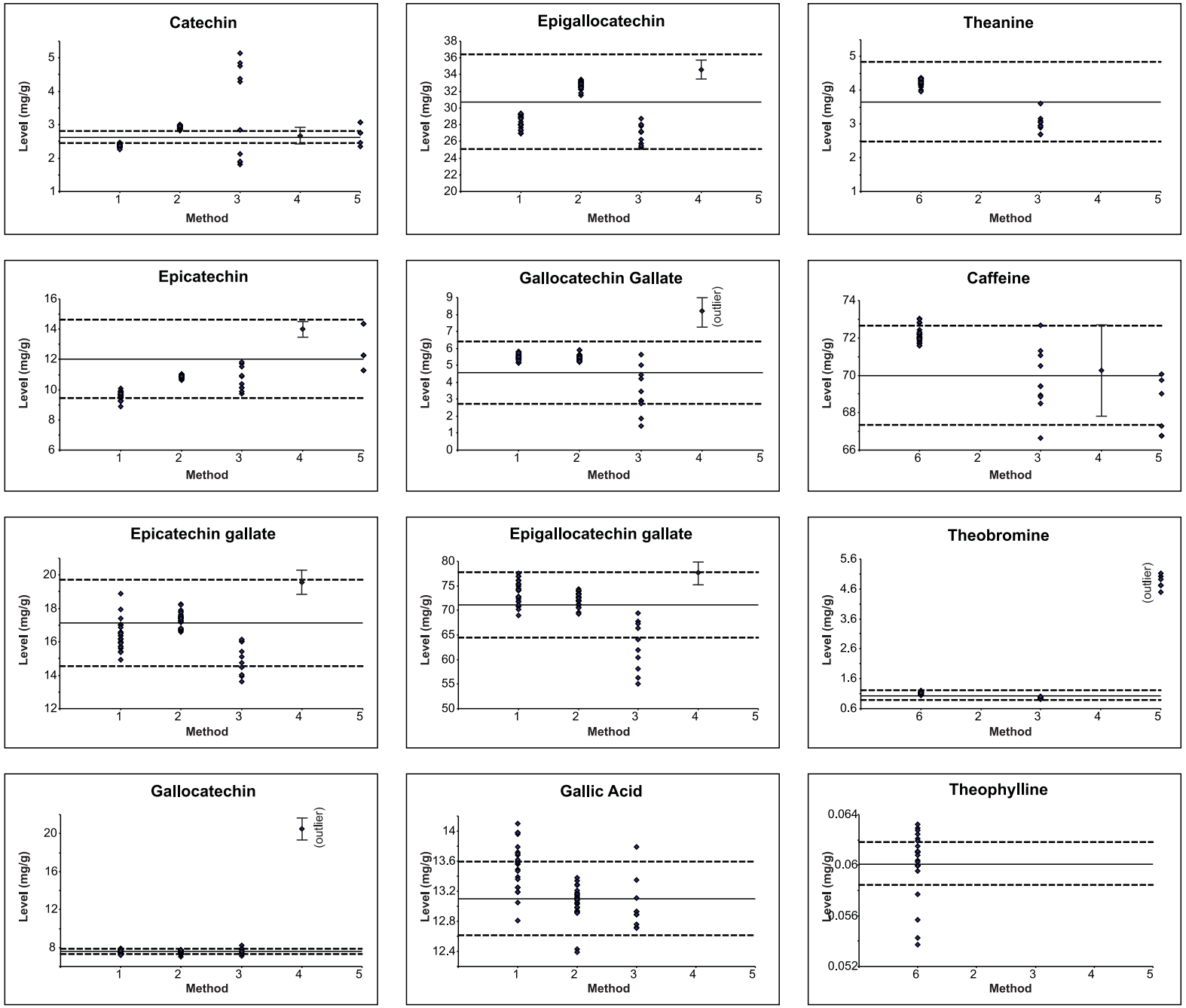


Figure S5. Individual measurements of catechins, xanthines, and other constituents of SRMs 3256, as determined by up to 5 different methods. The solid lines represent the certified values, and the dashed lines the expanded uncertainties. Method 4 data is plotted as a mean +/- the standard deviation.