National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 3246

Ginkgo biloba (Leaves)

This Standard Reference Material (SRM) is intended primarily for use in validating analytical methods for the determination of flavonoids, terpene lactones, and toxic elements in *Ginkgo biloba* and similar matrices. This SRM can be used for quality assurance when assigning values to in-house control materials. This SRM has also been characterized for its DNA sequence. A unit of SRM 3246 consists of five bottles, each containing approximately 3 g of ground leaves.

The development of SRM 3246 was through collaboration among the National Institute of Standards and Technology (NIST); the National Institutes of Health (NIH) Office of Dietary Supplements (ODS); and the Food and Drug Administration (FDA) Center for Drug Evaluation and Research (CDER). Addition of genetic information was accomplished through collaboration among NIST, NIH ODS, the U.S. Department of Agriculture (USDA) Agricultural Research Service (ARS), AuthenTechnologies (Albany, CA), and American Herbal Pharmacopoeia (Scotts Valley, CA).

Certified Properties: The certified species identification is *Ginkgo biloba* and the associated chloroplast DNA sequences from the *psbA-trnH* intergenic spacer and *trnL* intron regions are available in companion FASTA-formatted files [1]. The uncertainty associated with each nucleotide in the sequence, and in turn the uncertainty associated with the DNA sequence as an identifier of species, is expressed in an ordinal scale that represents the strength of the belief in the assigned value (0 = Most Confident, 1 = Very Confident, 2 = Confident, 3 = Ambiguous) (Tables 1 and 2) [2]. In the absence of a fully developed metrology for identity (the current state of affairs), a pragmatic way forward is to consider these DNA sequences as the source of "comparability of identity" for *Ginkgo biloba*. Chloroplast DNA sequences from authenticated *Ginkgo biloba* samples are used to establish inclusivity; chloroplast DNA sequences from close relatives are used to establish exclusivity.

The certified mass fraction values of selected flavonoids, terpene lactones, and elements are provided in Tables 3 and 4. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account [3]. Values were derived from the combination of results provided by NIST and collaborating laboratories. The certified values in this material are the equally weighted means of the individual sets of NIST results and the means of the combined sets of measurements made by collaborating laboratories; the associated uncertainties are expanded uncertainties at the 95 % level of confidence [4,5]. Values are reported on a dry-mass basis in mass fraction units [6].

Reference Values: A NIST reference value is a noncertified value that is the best estimate of the true value based on available data; however, the value does not meet the NIST criteria for certification [3] and is 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. Reference mass fraction values for additional flavonoids and terpene lactones are provided in Table 5.

Expiration of Value Assignment: The certification of **SRM 3246** is valid, within the measurement uncertainty specified, until **31 October 2014** provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Instructions for Handling, Storage, and Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

Coordination of the technical measurements leading to the certification of this SRM was performed by L.C. Sander, K.E. Sharpless, and S.A. Wise of the NIST Chemical Sciences Division.

Carlos A. Gonzalez, Chief
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Gaithersburg, MD 20899
Certificate Issue Date: 23 December 2013
Certificate Revision History on Page 9

Robert L. Watters, Jr., Director
Office of Reference Materials

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

Support for the development of SRM 3246 was provided in part by the NIH ODS and the FDA CDER. Technical consultation from these agencies was provided by J. Betz (NIH ODS) and A. NguyenPho (FDA CDER). Acquisition and preparation of the material was coordinated by A. NguyenPho of FDA CDER and K.E. Sharpless of the NIST Chemical Sciences Division.

Analytical measurements at NIST were performed by S.B. Howerton, S.E. Long, K.E. Murphy, B.J. Porter, K. Putzbach, M.S. Rearick, C.A. Rimmer, L.J. Wood, and R.L. Zeisler of the NIST Chemical Sciences Division and D. Hancock of the NIST Biochemical Science Division. Analyses for value assignment were also performed by C. Scriver and L. Yang of the National Research Council Canada (NRCC, Ottawa, ON); M. Sanders at NSF International (Ann Arbor, MI), C. Nelson at Eurofins (Petaluma, CA), and B. Schaneberg at ChromaDex, Inc. (Boulder, CO). Analyses for toxic elements were also performed by M. Sargent at LGC (Teddington, UK). Data from an AOAC collaborative study for flavonoids in SRM 3246 were also included in value assignment; the directors for this study were D. Gray (Midwest Research Institute, Kansas City, MO), K. LeVanseler and M. Pan (NSF International, Ann Arbor, MI), and E. Waysek (Caravan Products Company, Totawa, NJ). Molecular analyses were coordinated and performed by D.H. Reynaud of AuthenTechnologies (Richmond, CA), with two collaborating laboratories for sequencing analyses: University of California Berkeley DNA Sequencing Facility (Berkeley, CA) and Sequetech Inc. (Mountain View, CA). Technical consultation was provided by J.M. Betz (NIH-ODS), J. Harnly (USDA-ARS), R. Upton (American Herbal Pharmacopoeia), and W. Applequist (Missouri Botanical Garden, St. Louis, MO). Thin layer chromatographic analysis was provided by A. Blatter and E. Reich (CAMAG, Muttenz, Switzerland).

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 AND WARNING TO USERS

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 its unopened bottle, until required for use.

Use: Prior to removal of a test portion for analysis, the contents of a bottle of material should be mixed thoroughly. Test portions used for NIST analyses described below were 1 g for flavonoids, 1 g to 2 g for terpene lactones, 0.1 g for arsenic, 0.5 g for cadmium and lead, and 0.25 g for mercury. These test portions are the minimum amounts recommended for use. Test portions should be analyzed as received and results converted to a dry-mass basis by determining moisture content (described below) on a separate test portion.

PREPARATION AND ANALYSIS[(1)]

Material Acquisition and Preparation: Approximately 20 kg of minced *Ginkgo biloba* leaves were ground at room temperature in a Teflon disk mill containing a concentric Teflon ring and a Teflon puck, and sieved to 180 µm (80 mesh). The sieved material was transferred to ChromaDex, Inc. (Santa Ana, CA) where it was blended and then bottled under nitrogen in amber high-density polyethylene bottles with polypropylene screw caps. After bottling, the material was irradiated by ^{60}Co to an absorbed dose of 12.9 kGy to 15.7 kGy.

MOLECULAR APPROACH FOR SPECIES IDENTITY

Measurement and Analysis: Sanger sequencing was used on two independent chloroplast gene regions, *psbA-trnH* intergenic region [7,8] and *trnL* intron [9], for authentication of SRM 3246. Complete sequencing of both strands of

[(1)] Certain commercial equipment, instruments or materials 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 materials or equipment identified are necessarily the best available for the purpose.

DNA for the two gene regions was performed on replicate samples, and the sequence reads were independently hand-curated and the confidence values were estimated as described below. Certified DNA sequences are available in companion FASTA-formatted files [1].

To certify the species identity, validation studies were performed based on the "Probability of Identification (POI)" model using an Inclusivity and Exclusivity Panel [10]. Multiple samples of authenticated herbarium vouchers, botanical identity reference materials, and published literature were used to comprise the panels from the target and most closely related species (see Appendix A for more information on the reference samples). These samples were analyzed using the same procedures as for SRM 3246 described above. Maximum Likelihood (ML) phylogenetic analyses were performed using the phylogenetic estimation using ML (PhyML) algorithm, with a General Time Reversible (GTR) substitution model, a fixed transition-transversion ratio, and 1,000 bootstrap replicates. ML phylogenies, which can be found in Appendices B and C, were used to determine the species identity of SRM 3246 and to estimate the confidence (as described below). The confidence levels for both the *psbA-trnH* intergenic region and *trnL* intron were "Most Confident" (0).

ANALYTICAL APPROACH FOR DETERMINATION OF FLAVONOIDS

Value assignment of the mass fractions of flavonoids in SRM 3246 was based on the combination of measurements from different analytical methods at NIST, at two collaborating laboratories, and in an interlaboratory comparison using a single analytical method [11]. A total of five sets of measurements was used for the value assignment of the mass fractions of flavonoids. NIST provided measurements by using a combination of two sample extraction procedures and three liquid chromatography (LC) methods with different detection, i.e., ultraviolet absorbance spectrometry (UV) and mass spectrometry (MS) as described below. Results for flavonoids were provided by two collaborating laboratories (NSF International and ChromaDex) and participants in an AOAC collaborative study. All collaborating laboratories' results were based on LC/UV. Two collaborating laboratories analyzed a minimum of six subsamples, one from each of six bottles or two from each of three bottles, and one laboratory analyzed one subsample from three bottles of SRM 3246.

NIST Analyses for Flavonoids

Flavonoid aglycones were measured by using combinations of two sample preparation methods and two LC methods with UV or MS detection. Four independently prepared calibrants were used for each of the methods. Calibrants were prepared gravimetrically, at levels intended to approximate levels of the flavonoid aglycones in the extracts of the SRMs. A single internal standard solution (hesperetin) was used for calibrants and samples. Calculations are based on average response factors for the calibrants (typically duplicate analysis of four calibrant solutions, $n = 8$). The purity of the standards was determined and was used in the calculation of the results. In addition, the water content of quercetin was also corrected since this standard is hydrated in solid form.

Soxhlet Extraction: Twelve 1 g portions of the SRM were weighed into glass-fritted Soxhlet thimbles each containing an approximately 1 cm layer of diatomaceous earth (Hydromatrix, Isco, Lincoln, NE). After stirring the sample, a measured mass of internal standard solution (hesperitin) was transferred to the Soxhlet thimble. The samples were extracted with approximately 200 mL methanol for 24 h. Samples prepared by this approach were hydrolyzed as described below (see "Hydrolysis") and then analyzed by LC/UV.

Pressurized Fluid Extraction: Twelve 1 g portions of the SRM were placed into stainless steel extraction vessels fitted with a cellulose disk to prevent obstruction of the fritted stainless steel disk in the end cap. Each vessel was filled to approximately one half capacity with Hydromatrix. After stirring the sample, a measured mass of internal standard solution (hesperetin) was added, and an additional layer of Hydromatrix material was added to fill the vessel completely. The samples were extracted three times each, with three static holds of 5 min per extraction cycle. A nominal pressure of 13.8 MPa (2000 psi) and a temperature of 100 ºC were maintained. Samples prepared by this approach were hydrolyzed as described below (see "Hydrolysis") and then analyzed by LC/MS.

Hydrolysis: After extraction, 30 g of the extract was refluxed with approximately 10 g of 25 % hydrochloric acid solution (mass fraction) to cleave the sugar residues from the various glycosides to produce the aglycones.

LC/UV Absorbance Detection: A binary gradient LC method with a water/acetonitrile (both containing acetic acid) mobile phase was used for the LC/UV determination. The aglycones were detected at 370 nm and the internal standard was detected at 287 nm. A typical chromatogram is provided in Appendix D.

LC/MS Detection: A C_{18} column was used with an isocratic mobile phase (water/acetonitrile/acetic acid/trifluoroacetic acid) for the LC/MS determination. Positive electrospray mode was used for the determination of the flavonoid aglycones. Quantification of the aglycones was based on selected ion monitoring at *m/z* 303 for

quercetin and hesperetin (internal standard), *m/z* 317 for isorhamnetin, and *m/z* 287 for kaempferol. A typical chromatogram is provided in Appendix D.

ANALYTICAL APPROACH FOR DETERMINATION OF TERPENE LACTONES

Value assignment of the mass fractions of the terpene lactones in SRM 3246 was based on the combination of measurements from two different analytical methods at NIST and at one collaborating laboratory. A total of three sets of measurements were used for the value assignment of the mass fractions of terpene lactones. NIST provided measurements by using two different methods for sample extraction and two different LC methods with MS as described below. Results for terpene lactones were also provided by Eurofins, who analyzed samples using LC with evaporative light scattering detection (ELSD). NIST analyzed single test portions from each of ten bottles or duplicate test portions from each of six bottles, and Eurofins analyzed single test portions from each of five bottles.

NIST Analyses for Terpene Lactones

Terpene lactones were measured by using combinations of two sample preparation methods and two LC/MS methods. Three or six independently prepared calibrants were used for each of the methods. Calibrants were prepared gravimetrically, at levels intended to approximate the levels of the terpene lactones in the extracts of the SRM. A single internal standard solution was used for the calibrants and samples. Calculations are based on average response factors for the calibrants (typically six or three injections of the three or six calibrant solutions, respectively).

Soxhlet Extraction: Ten 2 g portions of the SRM were weighed into glass-fritted Soxhlet thimbles, each containing an approximately 2.5 cm layer of diatomaceous earth (Hydromatrix, Isco). After stirring the sample, a measured mass of internal standard solution (limonin) was transferred to the Soxhlet thimble. The samples were extracted with approximately 200 mL methanol for 22 h. Samples prepared by this approach were analyzed by LC/MS Method 1.

Sonication Extraction: Twelve 2 g portions of the SRM taken from six bottles were placed in 50 mL polyethylene centrifuge tubes, followed by the addition of a measured mass of internal standard solution (hesperitin). Approximately 10 mL of methanol was added to the tubes, and the tubes were capped. The solid matter was suspended by shaking, and the tubes were placed in an ultrasonic bath for 30 min. At the completion of the sonication extraction, the samples were centrifuged, and the methanol was removed and replaced with fresh methanol. The extraction was performed five times, and the five portions of methanol were combined and analyzed by LC/MS Method 2.

LC/MS Method 1: A C_{12} column was used with a mobile phase gradient (water/methanol/acetic acid) for the LC/MS Method 1 determination. Positive ion electrospray mass spectrometry was used for detection of the terpene lactones. Quantification was based on monitoring ions at *m/z* 344 for bilobalide, *m/z* 426 for ginkgolide A, *m/z* 442 for ginkgolides J and B, *m/z* 458 for ginkgolide C, and *m/z* 488 limonin (internal standard). A typical chromatogram is provided in Appendix E.

LC/MS Method 2: A C_{18} column was used with a mobile phase gradient (water/acetonitrile/acetic acid) for the LC/MS Method 2 determination. Positive ion electrospray mass spectrometry was used for detection of the terpene lactones. Quantification was based on monitoring ions at (*m/z*) *m/z* 327 for bilobalide, *m/z* 409 for ginkgolide A, *m/z* 425 for ginkgolides J and B, *m/z* 441 for ginkgolide C, and *m/z* 303 hesperitin (internal standard). A typical chromatogram is provided in Appendix E.

ANALYTICAL APPROACH FOR DETERMINATION OF ELEMENTS

The elements of primary interest for SRM 3246 were the potentially toxic contaminants arsenic, cadmium, lead, and mercury. Value assignment of the mass fractions of toxic elements in SRM 3246 was based on the combination of measurements at NIST using a single analytical method and results from one or two collaborating laboratories (NRCC and LGC) when available. At NIST instrumental neutron activation analysis (INAA) was used for the determination of arsenic, isotope dilution inductively coupled plasma mass spectrometry (ID ICP-MS) was used for determination of cadmium and lead, and cold vapor (CV) ID ICP-MS was used for determination of mercury. For all NIST measurements, botanical-matrix SRMs with certified values for the elements of interest were analyzed concurrently as control samples. NRCC used ID ICP-MS for the determination of cadmium and lead and hydride generation graphite furnace atomic absorption spectrometry (HG GFAAS) for the determination of arsenic. LGC provided results for arsenic using ICP-MS and cadmium and lead using ID ICP-MS. Collaborating laboratories analyzed either three (LGC) or six subsamples (NRCC) of SRM 3246.

NIST Analyses for Elements

For cadmium and lead determinations, a single 0.5 g portion was taken from each of six bottles of the SRM. Isotopically enriched ^{111}Cd and ^{206}Pb were added to the samples prior to digestion in PFA Teflon vessels with nitric and hydrofluoric acids using a high-throughput microwave system. The microwave digests were transferred to PFA Teflon beakers, and heated to evaporate the acids, after which the residue was redissolved in 2 % nitric acid. Measurements were made by using ID ICP-MS [12].

Because of potential interferences at the Cd masses, a matrix separation was performed on a single sample of SRM 3246 to estimate the uncertainty due to interference [13]. The sample was separated using anion exchange chromatography. Samples were evaporated to dryness with concentrated hydrochloric acid to convert residual salts from the nitrate to the chloride form. Samples were redissolved in a mixture of hydrochloric and hydrofluoric acids, separated, evaporated, and redissolved in nitric acid. There was a 1 % difference in determined Cd mass fraction between the separated and unseparated samples of SRM 3246.

For mercury determinations, a single 0.25 g portion was taken from each of six bottles of the SRM. Isotopically enriched ^{201}Hg was added to the samples prior to digestion in quartz vessels with nitric acid in a high-pressure microwave system. Following digestion, samples were diluted and allowed to degas overnight at 4 °C. Measurements were made by using CV mercury generation (using tin (II) chloride reductant) coupled with ICP-MS [14].

Arsenic was measured by using INAA. Individual disks were formed from 100 mg test portions of the SRM using a stainless steel die and hydraulic press. Standards were prepared by transferring a weighed portion of a solution containing a known amount of arsenic onto filter papers. Disks were formed from the dried filter papers. Samples, standards, and controls were packaged individually in clean polyethylene bags, placed together in a polyethylene irradiation container, and exposed to a neutron fluence rate of 1×10^{14} cm^{-2}·s^{-1} for a total of 4 h. Decay times were approximately 4 d to 4.3 d. Gamma rays were collected using an intrinsic germanium detector with a relative efficiency of 35 % and a resolution of 1.75 keV (full-width at half maximum peak height for the 1333 keV line from ^{60}Co). Quantification was based on comparison with standards using the 559-keV and 658-keV lines from ^{76}As. A value for arsenic was not assigned; see "Homogeneity Assignment".

NIST Determination of Moisture

Moisture content of SRM 3246 was determined by (1) freeze-drying to constant mass over 14 d; (2) drying over magnesium perchlorate in a desiccator at room temperature for 12 d; and (3) drying for 24 h in a forced-air oven at 60 °C. Unweighted results obtained using all three techniques were averaged to determine a conversion factor of 0.9518 gram dry mass per gram as-received mass, which was used to convert data from an as-received to a dry-mass basis; NIST arsenic data were moisture-corrected by the analyst using a factor of 0.9474 as determined by drying two 1 g samples over magnesium perchlorate for 20 d. A variability-in-moisture component is included in the uncertainties of both the certified and reference values, reported on a dry-mass basis, that are provided in this certificate.

Homogeneity Assessment

The homogeneity of flavonoids, terpene lactones, and arsenic in SRM 3246 was assessed at NIST by using the methods described above. An analysis of variance did not show inhomogeneity for flavonoids and terpene lactones for the sample sizes employed. Arsenic appeared to be inhomogeneously distributed in 0.1 g samples across the samples tested, ranging from 86 ng/g to 290 ng/g; therefore, a value was not assigned. The homogeneity of the DNA sequences was evaluated by sequencing the DNA from multiple samples from three randomly-selected packets, revealing the degree of homogeneity in DNA sequence or species identity. The DNA data were homogeneous. Other measurands were treated as though they were homogeneously distributed, although homogeneity was not assessed.

Assignment of Values and Properties

The equally weighted means from each set of data were used to calculate the assigned mass fraction values. Each NIST mean was averaged with the grand mean of data provided by collaborating laboratories.

Certified Nucleotide Identity: A set of heuristic, experience-based, rules (see Table 1) were used to establish confidence estimates for the nucleotides comprising the DNA sequences obtained from SRM 3246, and their use in identifying the species. The certified DNA sequences with curated confidence estimates are available in companion FASTA-formatted files [1]. The ML phylogenies used to determine the species identity and their confidence estimates are provided in Appendices B and C. The uncertainty associated with each nucleotide in the sequence in

the FASTA files provided [1], and in turn the uncertainty associated with the DNA sequence as an identifier of species, is expressed in an ordinal scale that represents the strength of the belief in the assigned value (0 = Most Confident, 1 = Very Confident, 2 = Confident, 3 = Ambiguous) [2]. Characteristics of sequence data and phylogenetic data used for species identity associated with the levels of the ordinal scale are described in Tables 1 and 2. The confidence estimates for the *psbA-trnH* intergenic spacer and *trnL* intron region sequences are available in companion FASTA-formatted files [1].

Table 1. Definitions of Heuristic Rules for Confidence Estimates of DNA Nucleotide Identity

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

Table 2. Definitions of Heuristic Rules for Confidence Estimates of Species Identity

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

Certified Mass Fraction Values: Each certified mass fraction value, reported on a dry-mass basis, is an equally weighted mean of results from analytical methods carried out at NIST and at collaborating laboratories. The uncertainty in the certified value, calculated according to the method described in the ISO/JCGM Guide [4,5], is expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory, within-laboratory, and drying components of uncertainty. The uncertainty for cadmium contains an additional component of uncertainty representing the difference in cadmium results for separated and unseparated samples. The coverage factor (k) is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and approximately 95 % confidence for each analyte. For the flavonoids and ginkgolide B in Table 3, the measurand is the total mass fraction listed and the values are metrologically traceable to the SI unit of mass fraction in milligram analyte per gram sample on a dry-mass basis. For the elements in Table 4, the measurand is the total mass fraction

listed and the values listed are metrologically traceable to the SI unit of mass fraction in nanogram analyte per gram sample on a dry-mass basis.

Table 3. Certified Mass Fraction Values for Flavonoids and Ginkgolide B in SRM 3246

	Mass Fraction (mg/g)	*k*
Quercetin[a,b,c,d,e]	2.69 ± 0.31	2.26
Kaempferol[a,b,c,e]	3.02 ± 0.41	2.01
Isorhamnetin[a,b,c,d,e]	0.517 ± 0.099	2.01
Total Aglycones[a,b,c,e]	6.22 ± 0.77	2.01
Ginkgolide B[f,g]	0.470 ± 0.090	2.01

[a] NIST LC/UV
[b] NIST LC/MS
[c] ChromaDex LC/UV
[d] NSF International LC/UV
[e] AOAC collaborative study
[f] Two NIST LC/MS Methods
[g] Eurofins LC/ELSD

Table 4. Certified Mass Fraction Values for Elements in SRM 3246

	Mass Fraction (ng/g)	*k*
Cadmium[a,b]	20.8 ± 1.0	2.36
Lead[a,b]	995 ± 30	2.36
Mercury[c]	23.08 ± 0.17	2.57

[a] NIST ID ICP-MS
[b] NRCC ID ICP-MS
[c] NIST CV ID ICP-MS

Reference Mass Fraction Values: Each reference mass fraction value, reported on a dry-mass basis, is an equally weighted mean of the results from NIST and at collaborating laboratories. The uncertainty in the reference value, calculated according to the method described in the ISO/JCGM Guide [4,5], is expressed as an expanded uncertainty, *U*. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory, within-laboratory, and drying components of uncertainty. The coverage factor (*k*) is determined from the Student's *t*-distribution corresponding to the appropriate associated degrees of freedom and approximately 95 % confidence for each analyte. The measurand is the total mass fraction as determined by the methods listed and values listed are metrologically traceable to the SI unit of mass fraction in milligram analyte per gram sample on a dry-mass basis.

Table 5. Reference Mass Fraction Values for Terpene Lactones in SRM 3246

	Mass Fraction (mg/g)	*k*
Ginkgolide A[a,b,c]	0.57 ± 0.28	2.0
Ginkgolide C[a,b,c]	0.59 ± 0.22	2.0
Ginkgolide J[a,b,c]	0.18 ± 0.10	2.0
Bilobalide[a,b,c]	1.52 ± 0.40	2.0
Total Terpene Lactones[a,b,c]	3.3 ± 1.1	2.0

[a] NIST LC/MS Method 1
[b] NIST LC/MS Method 2
[c] Eurofins LC/ELSD

Supplemental Information

In addition to the analyses of the material described above, further characterization of SRM 3246 was provided using thin layer chromatography (TLC). The experimental procedures and the results are provided in Appendix F. These results are provided only as supplemental information to assist in characterizing SRM 3246 and are not intended for use in identifying *Ginkgo biloba*.

REFERENCES

[1] Certified data can be downloaded at https://www-s.nist.gov/srmors/view_detail.cfm?srm=3246.

— The certified sequence data file for *psbA-trnH* intergenic region is:

SRM3246 psbA-trnH Sequence_v1.FASTA.

— The certified confidence estimates for every base of *psbA-trnH* intergenic region sequence data file is:

SRM3246 psbA-trnH Quality_v1.FASTA.

— The certified sequence data file for *trnL* intron is:

SRM3246 trnL Sequence_v1.FASTA.

— The certified confidence estimates for every base of *trnL* intron sequence data file is:

SRM3246 trnL Quality_v1.FASTA.

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Certificate Revision History: 23 December 2013 (Added DNA sequence certified values and confidence levels; editorial changes); 07 July 2007 (Original certificate date).

Appendix A

Reference Samples and Sequences Used in the Specificity Validation Study for SRM 3246.

Table A1. Inclusivity Panel for *Ginkgo biloba*

Ginkgo biloba Sample Source[a]	Voucher Information or GenBank Accession #	*psbA-trnH*[b], *trnL*[c]
1. AHP	2015	+, +
2. AHP	2947	+, +
3. AHP	812	+, +
4. AHP	2944	+, +
5. AHP	2946	+, +
6. AHP	2945	+, +
7. AHP	2943	+, +
8. AHP	571	+, +
9. AHP	570	+, +
10. AHP	569	+, +
11. AHP	223	+, +
12. AHP	813	+, +
13. AT	050	+, +
14. UC	1717523	+, +
15. UC	1717381	+, +
16. Texas Natural Supply	s.n.	+, +

[a] AHP = American Herbal Pharmacopoeia (AHP-Verified Botanical Identity Reference Material); AT = AuthenTechnologies Herbarium; UC = University Herbarium, University of California, Berkeley.
[b] Intergenic spacer sequence is included when a plus sign (+) is present.
[c] Intron sequence is included when a plus sign (+) is present.

Table A2. Exclusivity Panel for *Ginkgo biloba*

Species	Sample Source[a] or Reference	Voucher Information or GenBank Accession #	*psbA-trnH*[b], *trnL*[c]
Cycas megacarpa	UCBG	2004.0913.506	+, +
Cycas ophiolitica	UCBG	2004.0920.503	+, +
Cycas platyphylla	UCBG	2004.0924.506	+, +
Cycas revoluta	UCBG	50.1816 250B	+, +
Cycas rumphii	UC	1372646	+, +
Bowenia serrulata	[16]	AF531185	-, +
Ceratozamia alvarezii	[15]	GU807395	+, -
Ceratozamia becerrae	[15]	GU807396	+, -
Chigua restrepoi	[15]	GU807394	+, -
Dioon angustifolium	[15]	GU807405	+, -
Dioon mejiae	[15]	GU807410	+, -
Encephalartos ferox	[16]	AF531190	-, +
Epicycas miquelii	[16]	AF531183	-, +
Macrozamia moorei	[16]	AF531188	-, +
Zamia loddigesii	[15]	GU807422	+,-
Zamia pumila	[17]	AY327842	-, +
Zamia vazquezii	[15]	GU807428	+,-

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

Appendix B

Maximum Likelihood *psbA-trnH* Intergenic Spacer Phylogeny of *Ginkgo biloba* and Relatives.

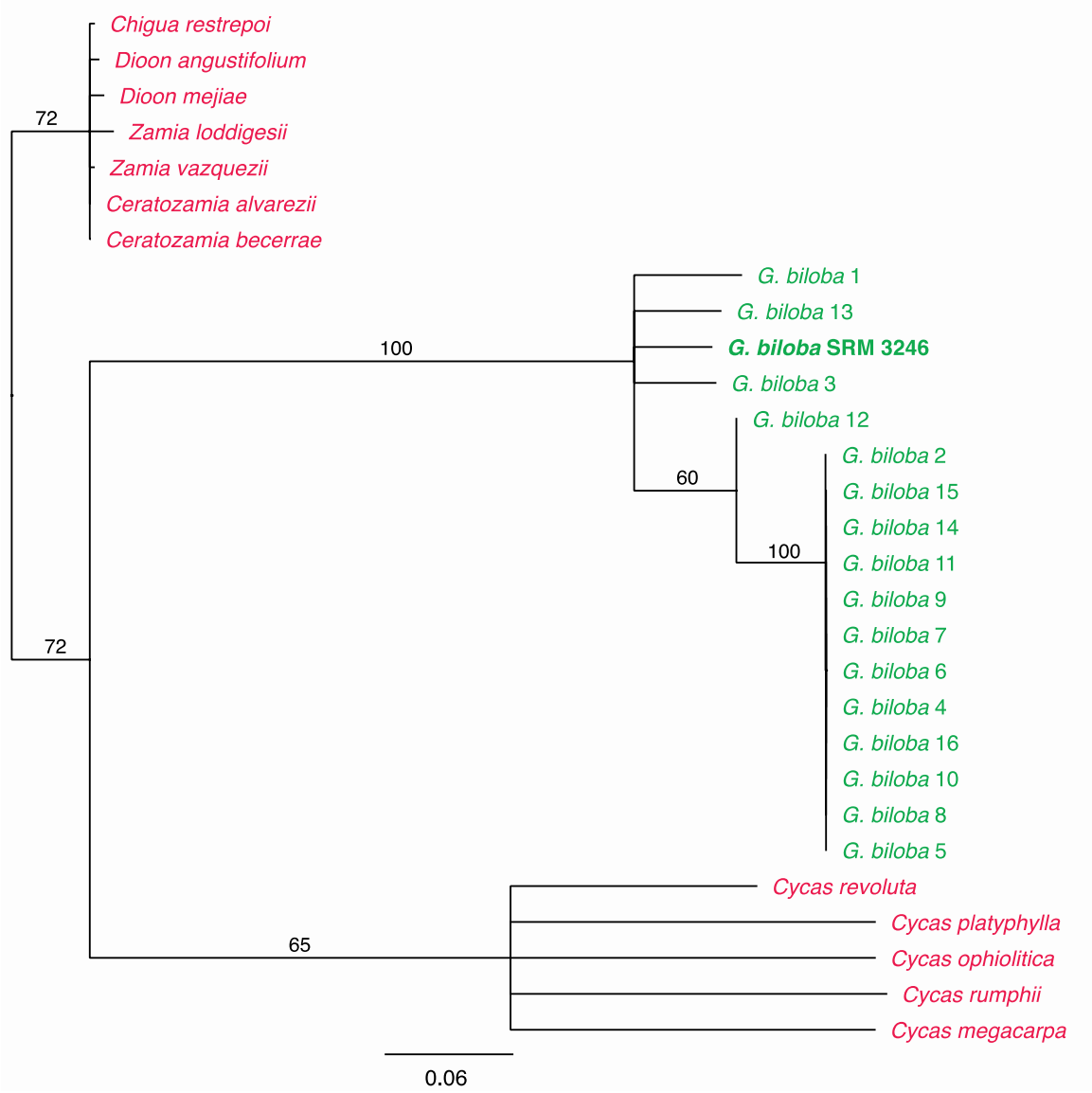

Figure B1. The numbers on the branches represent the bootstrap (BS) [18] support values out of 100 and the branch lengths are proportional to amount of genetic change. The numbers after the taxon names correlate to the numbers in Table A1. The taxon names are shown in red text to indicate the Exclusivity Panel samples while green text indicates the Inclusivity Panel samples. The confidence estimate for the species identification of SRM 3246 as *Ginkgo biloba* is Most Confident (0) and is indicated by the bold text.

Maximum Likelihood *trnL* Intron Phylogeny of *Ginkgo biloba* and Relatives.

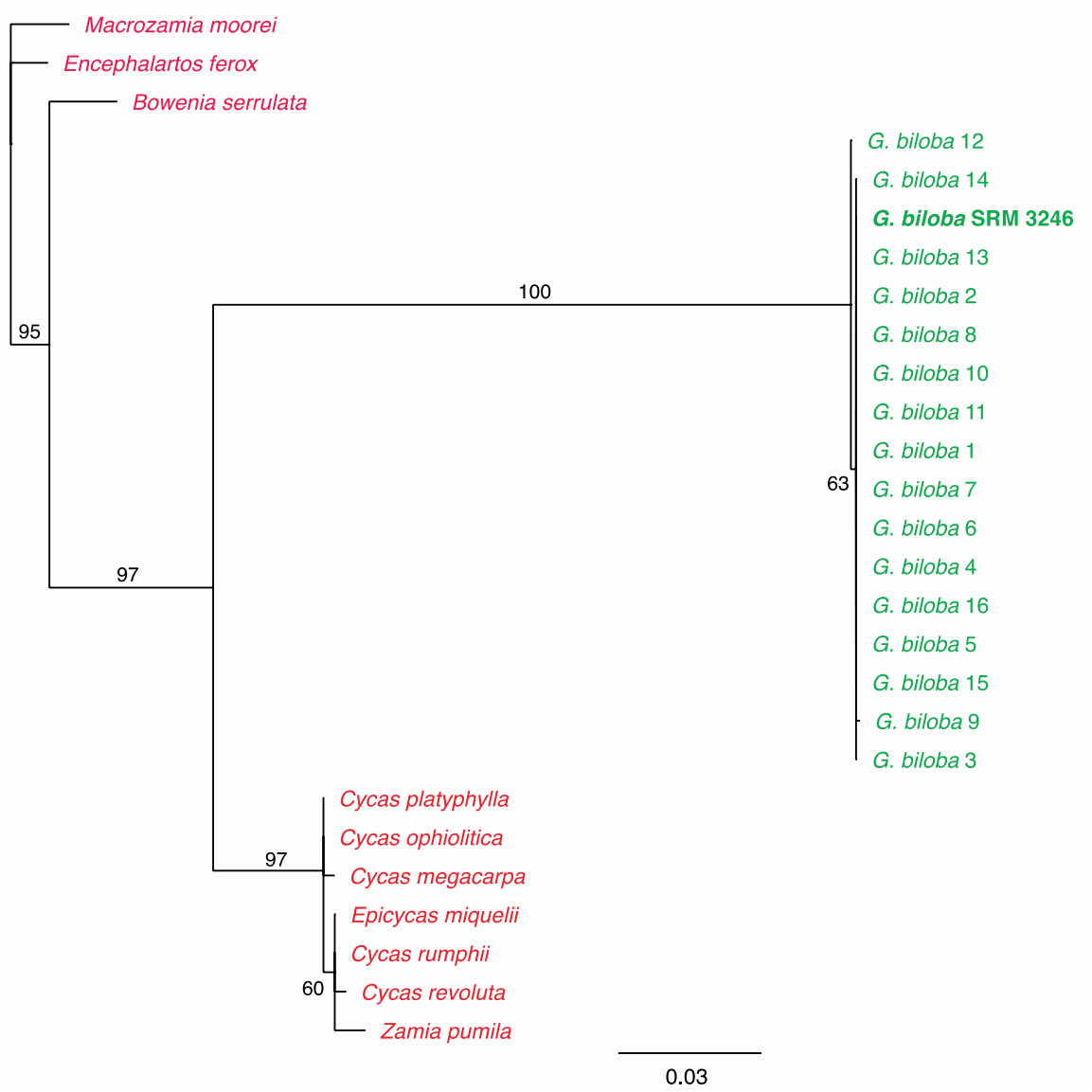

Figure C1. The numbers on the branches represent the bootstrap (BS) [18] support values out of 100 and the branch lengths are proportional to amount of genetic change. The numbers after the taxon names correlate to the numbers in Table A1. The taxon names are shown in red text to indicate the Exclusivity Panel samples while green text indicates the Inclusivity Panel samples. The confidence estimate for the species identification of SRM 3246 as *Ginkgo biloba* is Most Confident (0) and is indicated by the bold text.

Typical chromatograms from the analysis of flavonoids in SRM 3246 by using: (a) LC/UV and (b) LC/MS.

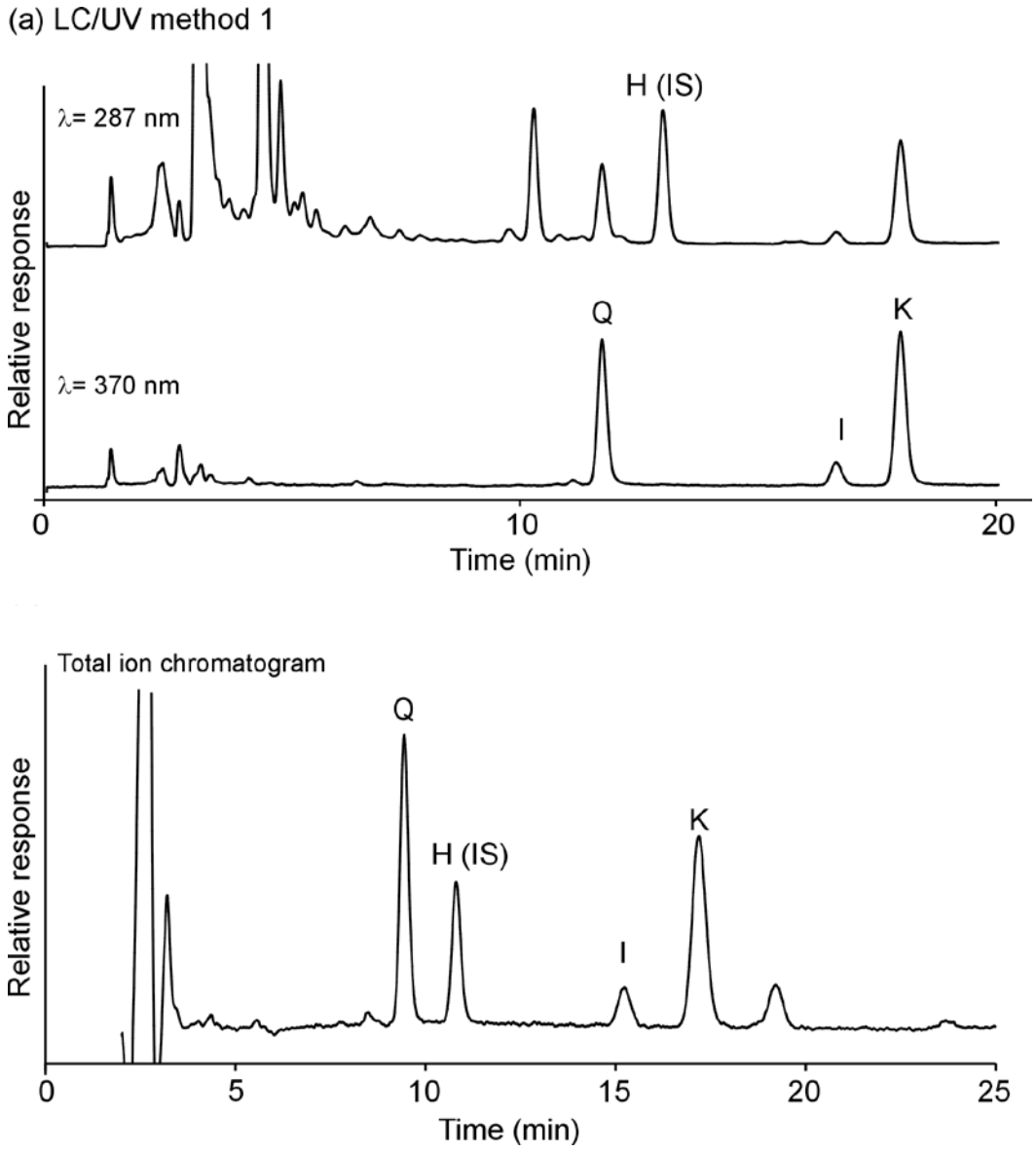

Figure D1. Chromatograms of flavonoids in SRM 3246 by using: (a) LC/UV and (b) LC/MS. For LC/UV, a binary gradient LC method with a water/acetonitrile (both containing acetic acid) mobile phase was used. A 0.46 cm x 25 cm Xterra (Waters, Milford, MA) C_{18} column was used with a SecurityGuard precolumn (C_{18} cartridge) and an inline filter (0.5 μm). A new precolumn and filter were used for each set of measurements. Column temperature was controlled at 25.0 ºC ± 2 ºC with a circulating-water column jacket and water bath. For LC/MS, a 0.46 cm x 25 cm Xterra C_{18} column was used at 25.0 ºC ± 2 ºC with a SecurityGuard precolumn (C_{18} cartridge) and an inline filter with an isocratic mobile phase (water/acetonitrile/acetic acid/trifluoroacetic acid) at 1.0 mL/min. Positive electrospray mode was used for the determination of the flavonoid aglycones. Quantification of the aglycones was based on selected ion monitoring at *m/z* 303 for quercetin and for hesperetin (internal standard), *m/z* 317 for isorhamnetin, and *m/z* 287 for kaempferol. Components are identified as follows: hesperitin (H), quercetin (Q), kaempferol (K), isorhamnetin (I).

Typical chromatograms from the analysis of ginkgolides and bilobalide in SRM 3246 by using: (a) LC/MS Method 1 and (b) LC/MS Method 2.

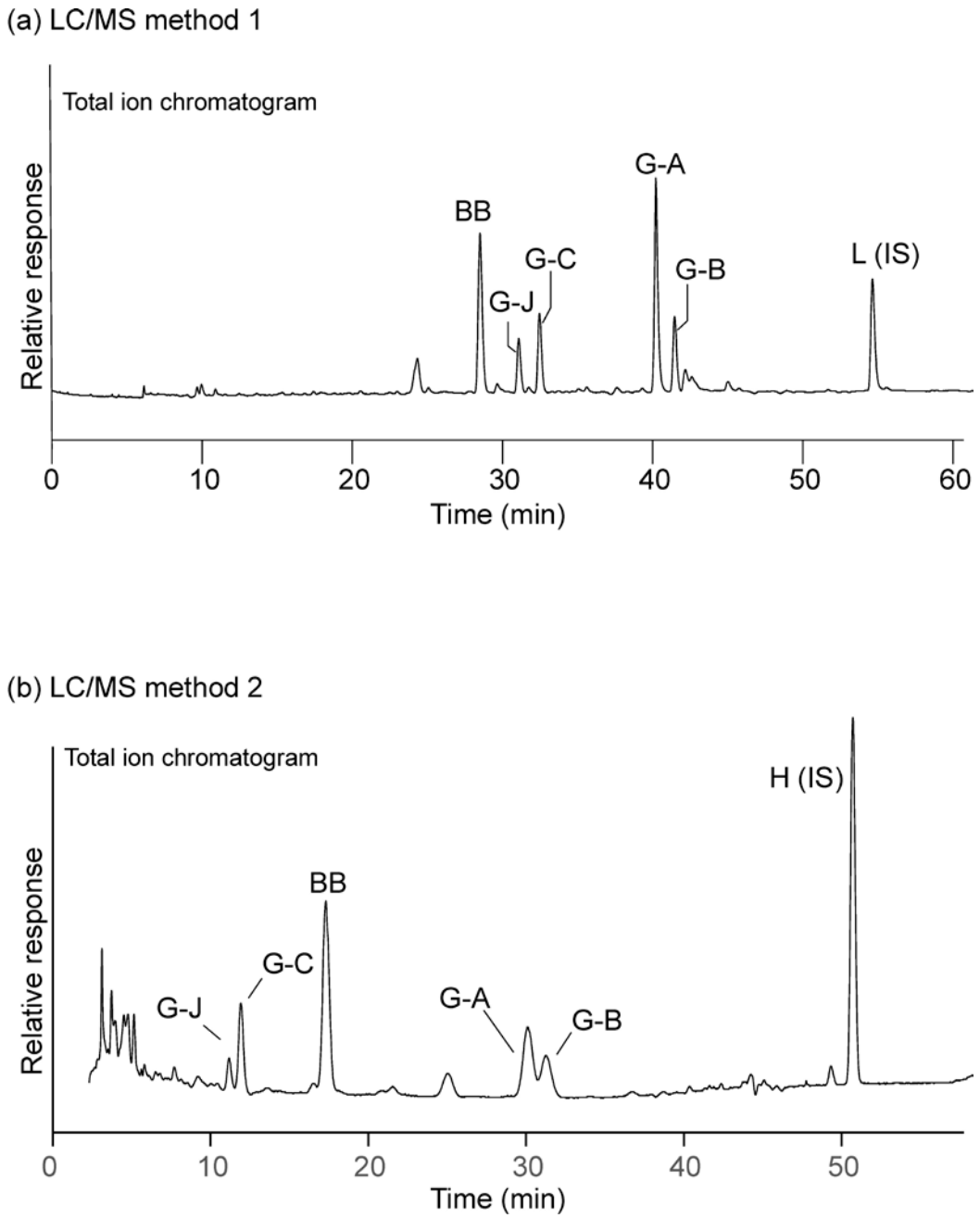

Figure E1. Chromatograms from the analysis of ginkgolides and bilobalide in SRM 3246 by using: (a) LC/MS method 1 and (b) LC/MS method 2. For LC/MS Method 1, a 250 mm x 4.6 mm Synergi-Max RP column (Phenomenex, Madrid, CA) and Synergi-Max RP guard column (Phenomenex) were held at 25 °C ± 1 °C with a column oven. A mobile phase gradient (water/methanol/acetic acid) and a flow rate of 0.75 mL/min were used. Positive ion electrospray mass spectrometry was used for detection of the terpene lactones. Quantification was based on monitoring ions *m/z* 344 for bilobalide, *m/z* 426 for ginkgolide A, *m/z* 442 for ginkgolides J and B, *m/z* 458 for ginkgolide C and *m/z* 488 for limonin (internal standard). For LC/MS Method 2, a 250 mm x 4.6 mm Xterra C_{18} column (Waters) was held at 25 °C ± 1 °C with a column oven. A mobile phase gradient (water/acetonitrile/acetic acid) and a flow rate of 1.0 mL/min were used. Positive ion electrospray mass spectrometry was used for detection of the terpene lactones. Quantification was based on monitoring ions at *m/z* 327 for bilobalide, *m/z* 409 for ginkgolide A, *m/z* 425 for ginkgolides J and B, *m/z* 441 for ginkgolide C and *m/z* 303 for hesperitin (internal standard). Components are identified as follows: bilobalide (BB), ginkgolide-A (G-A), ginkgolide-B (G-B), ginkgolide-C (G-C), ginkgolide-J (G-J), limonin (L), hesperitin (H).

Thin layer chromatography as provided by CAMAG for flavonoids (Figure F1), ginkgolides (Figure F2), and ginkgolic acid (Figure F3).

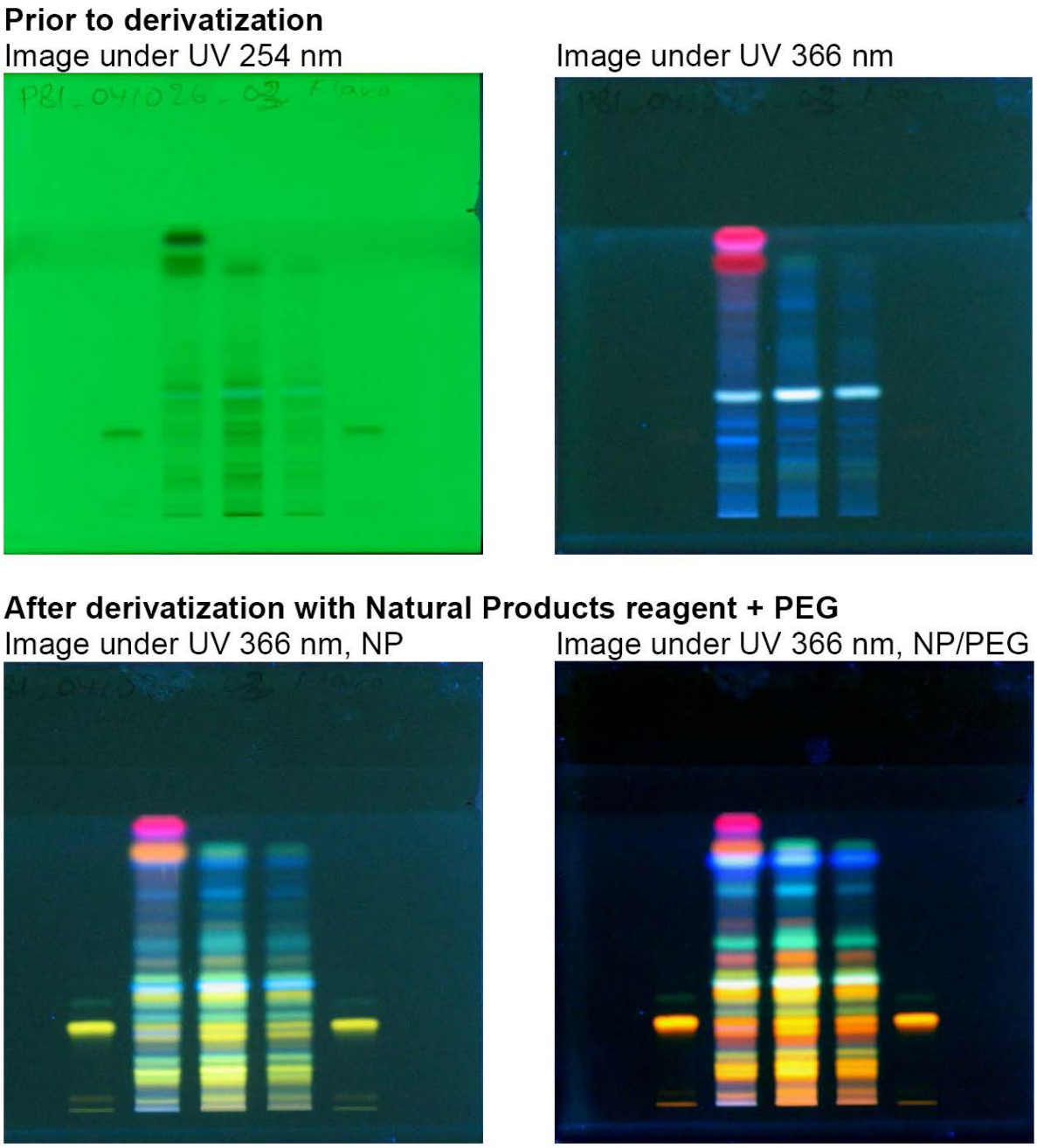

Figure F1. Thin layer chromatography as provided by CAMAG; application note F16B for flavonoids. Stationary phase: HPTLC silica gel 60 F_{254} (Merck KGaA, Darmstadt, Germany); mobile phase: ethyl acetate, acetic acid, formic acid, and water. From left to right, the lanes contain the following:

Lane 1: Rutin (1.5 mg/10 mL), 6 µL
Lane 2: *Ginkgo biloba* (Leaves) NIST SRM 3246 (1 g/10 mL), 5 µL
Lane 3: *Ginkgo biloba* Extract NIST SRM 3247 (100 mg/10 mL), 5 µL
Lane 4: Ginkgo-Containing Tablets NIST SRM 3248 (200 mg/10 mL), 5 µL
Lane 5: Rutin (1.5 mg/10 mL), 6 µL

After derivatization with acetic anhydride

Image under UV 254 nm

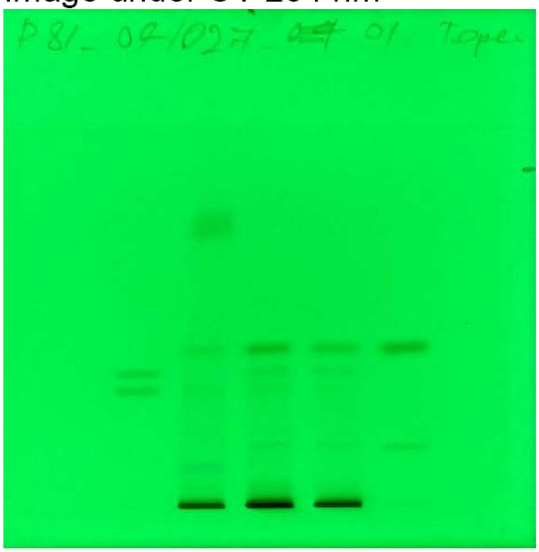

Image under UV 366 nm

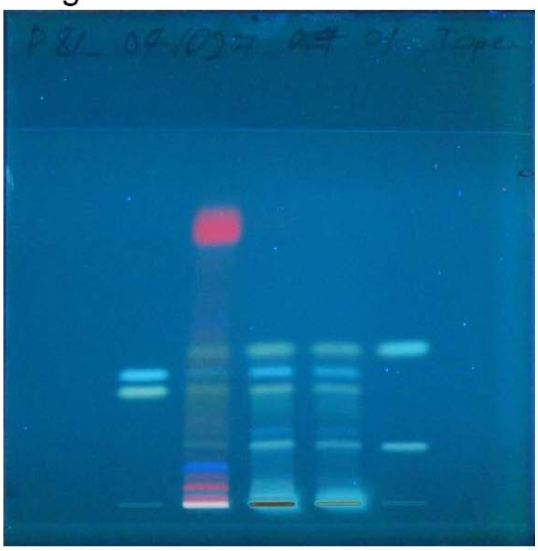

Figure F2. Thin layer chromatography as provided by CAMAG; application note F16A for ginkgolides. Stationary phase: HPTLC silica gel 60 F_{254} (Merck KGaA); mobile phase: ethyl acetate, acetic acid, formic acid, and water. From left to right, the lanes contain the following:

Lane 1: Ginkolide A and B (1 mg/mL), 3 µL each
Lane 2: *Ginkgo biloba* (Leaves) NIST SRM 3246 (1 g/10 mL), 5 µL
Lane 3: *Ginkgo biloba* Extract NIST SRM 3247 (100 mg/10 mL), 15 µL
Lane 4: Ginkgo-Containing Tablets NIST SRM 3248 (200 mg/10 mL), 25 µL
Lane 5: Ginkolide C and bilobalide (1 mg/mL), 3 µL each.

No derivatization

Image under UV 254 nm

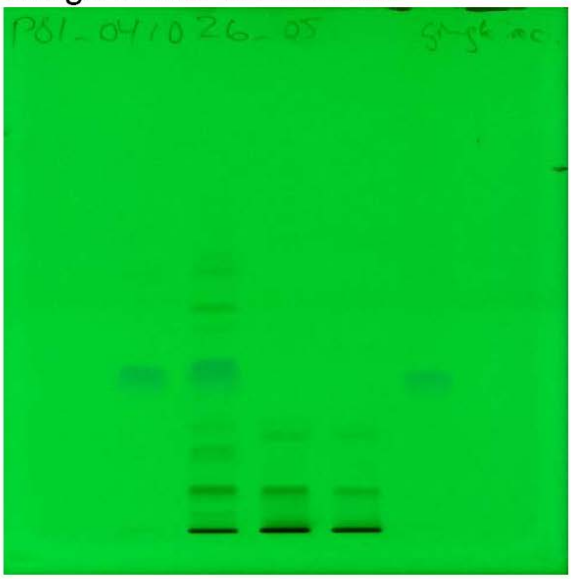

Image under UV 366 nm

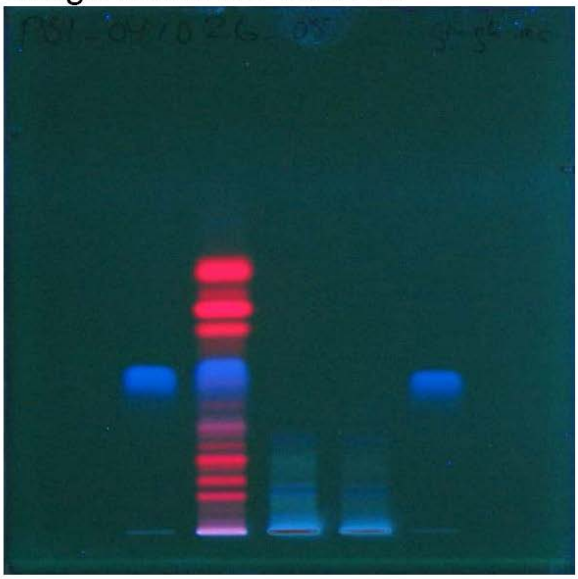

Figure F3. Thin layer chromatography as provided by CAMAG; application note F16C for ginkgolic acid. Stationary phase: HPTLC silica gel 60 F_{254} (Merck KGaA); mobile phase: ethyl acetate, acetic acid, formic acid, and water. From left to right, the lanes contain the following:

Lane 1: Ginkgolic acid (1 mg/10 mL), 10 µL
Lane 2: *Ginkgo biloba* (Leaves) NIST SRM 3246 (1 g/10 mL), 4 µL
Lane 3: *Ginkgo biloba* Extract NIST SRM 3247 (100 mg/10 mL), 10 µL
Lane 4: Ginkgo-Containing Tablets NIST SRM 3248 (200 mg/10 mL), 15 µL
Lane 5: Ginkgolic acid (1 mg/ 10 mL), 10 µL.