Anal Bioanal Chem (2007) 389:179–196
DOI 10.1007/s00216-007-1398-5

ORIGINAL PAPER

Characterization of a suite of ginkgo-containing standard reference materials

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Received: 9 April 2007 / Revised: 15 May 2007 / Accepted: 24 May 2007 / Published online: 7 July 2007

Abstract A suite of three ginkgo-containing dietary supplement Standard Reference Materials (SRMs) has been issued by the National Institute of Standards and Technology (NIST) with certified values for flavonoid aglycones, ginkgolides, bilobalide, and selected toxic trace elements. The materials represent a range of matrices (i.e., plant, extract, and finished product) that provide different analytical challenges. The constituents have been determined by at least two independent analytical methods with measurements performed by

Electronic supplementary material The online version of this article (doi:10.1007/s00216-007-1398-5) contains supplementary material, which is available to authorized users.

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NIST and at least one collaborating laboratory. The methods utilized different extractions, chromatographic separations, modes of detection, and approaches to quantitation. The SRMs are primarily intended for method validation and for use as control materials to support the analysis of dietary supplements and related botanical materials.

Keywords Liquid chromatography · Mass spectrometry · *Ginkgo biloba* · Standard reference material · Dietary supplements

Introduction

In 1994, the Dietary Supplements Health and Education Act (DSHEA) defined a dietary supplement as any product that is intended to supplement the diet that contains one or more of the following: a vitamin, mineral, herb or other botanical, amino acid, or metabolite [1]. Supplements are meant to be ingested in the form of a capsule, powder, soft gel, or gel-cap that is not represented as a conventional food or as a sole item of a meal or the diet. According to DSHEA, dietary supplements are regulated as foods rather than drugs, and the burden of proof for the safety of dietary supplements is placed on the US Food and Drug Administration (FDA).

In a 2001 study, it was estimated that 70% of the US population used a dietary supplement including vitamin supplements [2]. The popularity of dietary supplements has created a nearly US$21 billion industry, and the annual worldwide sales of *Ginkgo biloba* dietary supplements have been estimated at US$1 billion [3].

G. biloba L. (Ginkgoaceae) is also known as the Maidenhair tree. There are at least 12 species of ginkgo found in the fossil record [4]; however, *G. biloba* is the only known remaining species. Many parts of the ginkgo tree have been used in traditional medicine. The fruits/seeds of the female plant are commonly eaten in Japan and have been used for a variety of medicinal purposes in the Far East. The seeds are not commonly consumed in the USA, as they contain 4′-methoxypyridoxine, which acts as an anti-vitamin B_6 and is known to cause seizures in children. Ginkgo leaves and standardized leaf extracts have been used for a number of purposes including treatment of asthma, fatigue, and tinnitus; memory improvement; and Alzheimer's treatment/prevention. At least 33 identified flavonol glycosides, 4 terpene lactones, and bilobalide are associated with the perceived health benefits [3].

The levels of two classes of constituents are commonly determined in *G. biloba*: terpene lactones and flavonol glycosides. The flavonol glycosides are hydrolyzed to three major aglycones that are then measured, usually by liquid chromatography (LC) with ultraviolet/visible absorbance (UV) detection. The aglycone values may be converted to give an estimate of the concentration of individual flavonol glycosides in solution through the following equation:

$$\text{glycoside}\,(\%\,\textit{by mass fraction}) = \frac{(C)(F_v)(D)(F)(100\%)}{(w)}$$

where C is the specific aglycone concentration (mg/ml), F_v is the final volume of the sample solution, D is the dilution factor from the sample preparation (if needed), w is the mass of the sample (mg), and F is a relative molecular mass conversion factor for converting the aglycones to the glycoside. For quercetin, F=(756.7 g/mol)/(302.2 g/mol)= 2.504; kaempferol is (740.7 g/mol)/(286.2 g/mol)=2.588, and isorhamnetin is (770.6 g/mol)/(316.2 g/mol)=2.437 (http://www.nsf.org/business/ina/ginkgo.asp?program=INA). The terpene lactones are unique to *G. biloba* and serve as true marker compounds for the plant. They are extracted and measured, usually by LC with evaporative light scattering detection (ELSD), after the hydrolysis of the flavonol glycosides, as the glycosides may co-elute with the terpene lactones. As is true for many dietary supplements, the tablets are often made from *G. biloba* standardized extract mixed with other botanicals or inert materials; these extracts are marketed as "standardized," and the levels of specific marker compounds are normalized to improve product consistency. *G. biloba* extracts are commonly specified as 6% terpene lactones and 24% flavonol glycosides. The marker compounds are intended to be representative of a specific herbal material, but are not necessarily the active constituents, as the origin of herbal activity may not be known or may result from the synergistic effect of several constituents.

Dietary supplement products are also formulated with *G. biloba* leaves, and label claims may not be provided for these products because the levels of the marker compounds depend on the time of year at which the leaves were harvested and on growing conditions. A series of comprehensive reviews on the methods of determination of terpene lactones and flavonoid aglycones has been written by van Beek [3, 5–7].

The National Institute of Standards and Technology (NIST), in collaboration with the National Institutes of Health, Office of Dietary Supplements (NIH/ODS), and FDA, Center for Drug Evaluation and Research, is working to produce a number of botanical dietary supplement standard reference materials (SRMs) with values assigned for active and/or marker compounds. Botanical materials for consideration have been prioritized based on safety consideration (e.g., ephedra-containing materials [8, 9]) or sales (e.g., ginkgo). NIST has certified the levels of flavonoid aglycones, ginkgolides, and bilobalide in three ginkgo-containing dietary supplements: SRM 3246 *G. biloba* (leaves), SRM 3247 *G. biloba* extract, and SRM 3248 ginkgo-containing tablets. SRM 3249 ginkgo dietary supplement suite contains two bottles of each of the three materials. The structures of

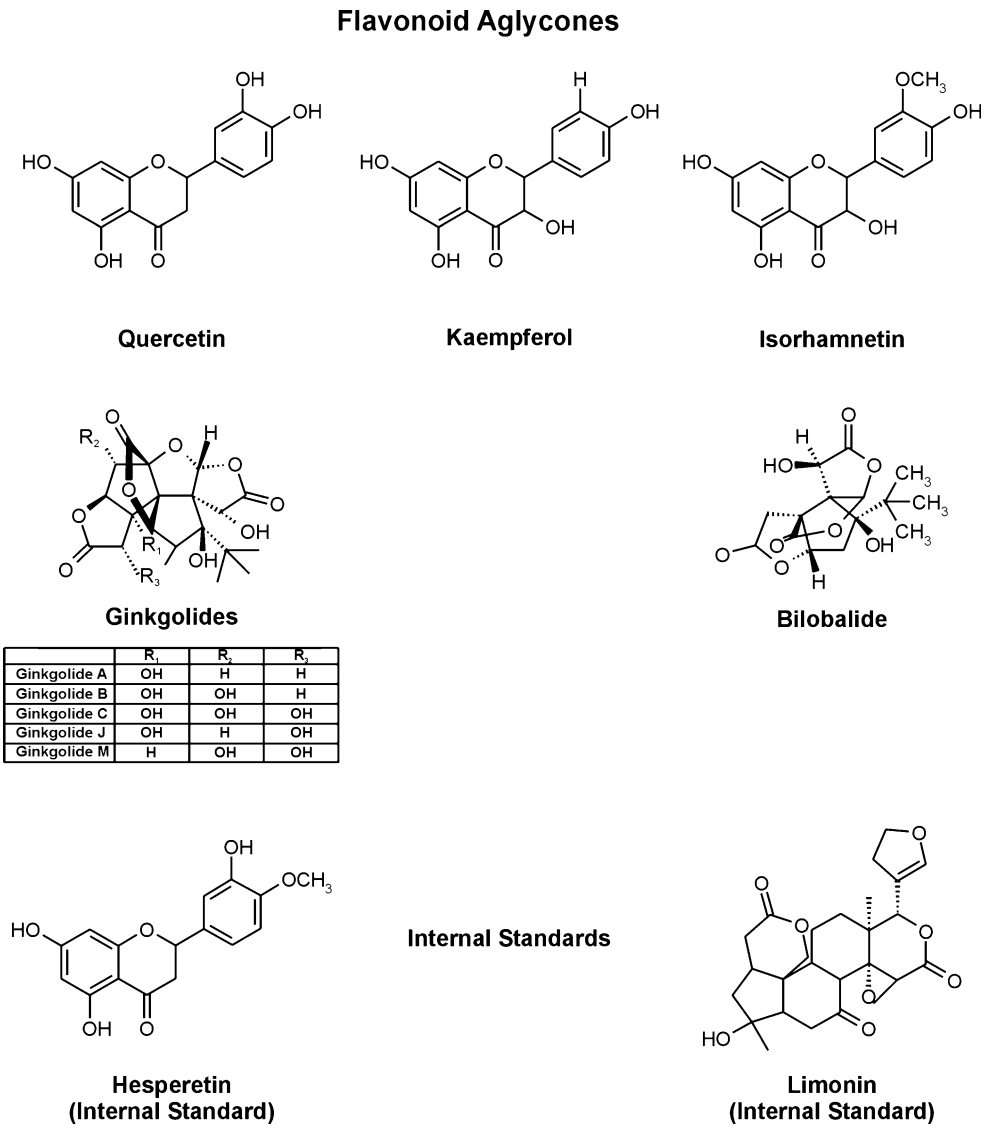
the aglycones and terpene lactones for which values are assigned and the internal standards used by NIST in the value assignment process are shown in Fig. 1. These SRMs are intended for use in method validation and as control materials to support the analysis of similar dietary supplements. Inaccurate labeling, adulteration, contamination (with pesticides, toxic metals, or toxic botanicals), and drug interactions have all been reported for various dietary supplements. The availability of well-characterized, matrix-based reference materials is expected to lead to improved product quality and to reduce public health risks that may be associated with the use of dietary supplements.

The three ginkgo SRMs follow the same model as other botanical-containing dietary supplement SRMs produced by NIST [8, 10] where the matrices are in the form of plant material, extract, and "finished product." Each matrix presents different analytical challenges and represents a matrix type that is encountered in the dietary supplement industry.

Experimental

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 NIST, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

SRM preparation Approximately 20 kg of minced *G. biloba* leaves (SRM 3246) and 7.4 kg of ginkgo tablets (SRM 3248) were obtained from a commercial source. Approximately 7 kg of *G. biloba* extract (SRM 3247), prepared according to the German Pharmacopoeia (non-clinical), was received from the manufacturer. The minced leaves and tablets were each ground (in batches of ≈150 to 200 g) at room temperature in a Teflon disk mill containing a concentric Teflon ring and a Teflon puck, and sieved to



	R_1	R_2	R_3
Ginkgolide A	OH	H	H
Ginkgolide B	OH	OH	H
Ginkgolide C	OH	OH	OH
Ginkgolide J	OH	H	OH
Ginkgolide M	H	OH	OH

Fig. 1 Structures of flavonoid aglycones, ginkgolides (terpene lactones), and the internal standards used in the determination of flavonoid aglycones and ginkgolides in SRMs 3246 through 3248. Note that ginkgolide M is typically found in the roots of the *G. biloba* tree, but not the leaf (therefore it was not measured in the current study)

180 μm (80 mesh). The sieved leaf and tablet materials and the extract material were transferred to ChromaDex (Santa Ana, CA) where each material was blended and then bottled under nitrogen in amber high-density polyethylene bottles with polypropylene screw caps. After bottling, all materials were irradiated by ^{60}Co to an absorbed dose of 12.9 to 15.7 kGy.

Reagents (NIST) Quercetin and isorhamnetin were obtained from Indofine Chemical Company (Hillsborough, NJ). Hesperetin (internal standard) was obtained from Tokyo Chemical Industry (Portland, OR). Reference standards for ginkgolides A, B, C, and J (the terpene lactones), bilobalide, kaempferol, and limonin (internal standard) were obtained from ChromaDex. The purities of the reference standards were determined from a consensus of several methods at NIST, including LC/UV, LC/ELSD, quantitative nuclear magnetic resonance (qNMR), and manufacturer data. The purities of the ginkgolides and bilobalide were found to be better than 97% (mass fraction). The purities of the flavonoid aglycones were found to be better than 99% (mass fraction); however, an additional correction was made for the water of hydration in quercetin as determined by qNMR.

The moisture content of the SRMs was also determined from a consensus of multiple methods including drying in a desiccator over magnesium perchlorate, drying in a forced air oven at 85 °C for 4 h, and lyophilization over the course of 7 or 11 days. Assigned values are reported on a dry-mass basis using the average drying factors for each of the three individual materials. For comparison with the certified and reference values, any of the three drying methods can be employed by the SRM user for conversion of concentrations determined on the material as received to a dry-mass basis.

Analytical approach for determination of flavonoids

Value assignment of the concentrations of flavonoids in the ginkgo SRMs 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. NIST provided measurements by using a combination of two sample extraction procedures, i.e., Soxhlet extraction and pressurized fluid extraction (PFE), and two LC methods with different detection, i.e., ultraviolet absorbance spectrometry (UV) and mass spectrometry (MS) as described below. Results for flavonoids were also 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. Details of sample preparation and analysis are provided in Table 1, and the general approaches employed in value assignment are summarized in Fig. S1. Assigned values were calculated using the individual means of the NIST methods and the mean of the results provided by the collaborating laboratories.

Flavonoid aglycone method 1: LC/UV (NIST) In brief, samples of SRM 3246 *G. biloba* (leaves) and SRM 3248 ginkgo-containing tablets were extracted by Soxhlet extraction. Because SRM 3247 *G. biloba* extract dissolved completely in methanol at room temperature, these samples were not extracted but instead were dissolved by sonication (to assure dissolution). Hesperitin was added for use as an internal standard. After extraction or dissolution, the solutions were heated under reflux with hydrochloric acid to hydrolyze the glycosides to produce the aglycones. Solutions originating from SRM 3247 and 3248 underwent a color change from yellow to orange and SRM 3246 from a dark green to a dark red. Color change has not been correlated to completion of the hydrolysis and as such should not be used as a visual indicator for completion. The aglycones were determined using a gradient LC/UV method with UV/visible absorbance detection at 287 and 370 nm simultaneously (see Fig. 2 for typical chromatograms).

Flavonoid aglycone method 2: LC/MS (NIST) Samples of SRM 3246 and 3248 were extracted by PFE with methanol; samples of SRM 3247 were dissolved as with method 1; see Table 1. Samples were weighed into stainless steel extraction vessels (22 ml) and mixed with sufficient Hydromatrix to fill the vessel. The internal standard solution (hesperetin, 0.5 mg/g in methanol, mass fraction) was added using a gas-tight syringe. The extraction vessels were extracted three times each, with three static holds of 5 min per extraction cycle. The pressure was maintained at a nominal pressure of 13.8 MPa (2,000 psi) and a temperature of 100 °C. Following hydrolysis (see method 1), PFE-extracted samples were centrifuged and analyzed by LC/MS in positive ion electrospray mode using selected-ion monitoring. Representative total ion chromatograms of the three materials are provided in Fig. 3.

Flavonoid aglycone method 3: LC/UV (ChromaDex) Samples were processed and analyzed as summarized in Table 1. A simultaneous extraction and hydrolysis approach was developed for the measurements. The samples and calibrants were analyzed by LC/UV using isocratic elution and absorbance detection at 370 nm.

Flavonoid aglycone method 4: LC/UV (NSF International) Samples were processed and analyzed as summarized in Table 1. As with method 3, a simultaneous extraction and hydrolysis approach was used; however, the conditions differed significantly from those of method 3. An isocratic LC/UV method was used to determine levels of the flavonoid aglycones; a different column and different mobile phase

Table 1 Flavonoid methods of analysis

Method identification		Extraction type	Extraction solvent	Extraction conditions	Mass of sample	Internal standard	Hydrolysis	Chromatographic conditions	Detection conditions
Flavonoid aglycone method 1	LC/UV NIST	Soxhlet (SRM 3246, 3248); dissolution by sonication (SRM 3247)	Methanol	22–24 h (extraction), 5 min (dissolution)	≈1 g SRM 3246, ≈50 mg SRM 3247, ≈1 g SRM 3248	Hesperetin, ≈1 ml; 0.5 mg/g in methanol	≈10 g, 2.4 mol/l HCl added to ≈30 g extract solution, heated to reflux for 3 h	Xterra C18 column (4.6×250 mm; 5 μm particle size, Waters, Milford, MA, USA), water/acetonitrile gradient; 0.5% acetic acid (mass fraction) additive; 1.0 ml/min and 25 °C gradient conditions: 0 min, 65:35 H_2O/ACN; 10 min, 65:35 H_2O/ACN; 30 min, 50:50 H_2O/ACN	UV/visible absorbance detection at 287 nm (IS) and 370 nm (aglycones)
Flavonoid aglycone method 2	LC/MS NIST	PFE (SRM 3246, 3248); dissolution by sonication (SRM 3247)	Methanol	3 extractions in succession; PFE program, 3 cycles at 13 MPa (2,000 psi), 5-min static hold/cycle; 100 °C,5 min (dissolution)	≈1 g SRM 3246, ≈50 mg SRM 3247, ≈1 g SRM 3248	Hesperetin, ≈1 ml, 0.5 mg/g in methanol	≈10 g 2.4 mol/l HCl added to ≈30 g extract solution, heated to reflux for 3 h	Xterra C18 column (4.6×150 mm; 5-μm particle size), isocratic acetonitrile–water (40:60% volume fraction; 0.5% acetic acid and 0.1% trifluoroacetic acid (mass fraction) additive at 1.0 ml/min and 25 °C	Positive ion electrospray mode (fragmentor voltage, 140 V; capillary voltage, 3,000 V; drying gas, 350 °C and 12.0 l/min, using single-ion monitoring (m/z quercetin and hesperetin, 303; isorhamnetin, 317; and kaempferol, 287)
Flavonoid aglycone method 3	LC/UV ChromaDex	Reflux	15 ml 15% HCl solution followed by methanol	≈1 h	500 mg (all)	External standard	Extraction and hydrolysis simultaneous	Luna C18 column (4.6×250 mm, 5-μm particle size, Phenomenex, Torrance, CA, USA), isocratic, 45:55 methanol/water (containing 0.1% phosphoric acid) at 1.5 ml/min and 30 °C. Column was rinsed with methanol between runs	UV/visible absorbance detection at 370 nm
Flavonoid aglycone method 4	LC/UV NSF International	Ultrasonic bath	Ethanol/water/hydrochloric acid (50:20:8)	5 to 60 min	Equivalent of 3.6 mg of flavonol glycosides	External standard	Heated in a sealed container for 60 min at 90 °C	Prodigy ODS 3 C18 column (4.6×250 mm, 5-μm particle size, Phenomenex, Torrance, CA, USA), isocratic, 50:50 acetonitrile/water (containing 0.85% phosphoric acid) at 1 ml/min and 35 °C	UV/visible absorbance detection at 370 nm
Flavonoid aglycone method 5	LC/UV AOAC collaborative study	Ultrasonic bath	Ethanol/water/hydrochloric acid (50:20:8)	5 min sonication	≈200 mg SRM 3246, ≈15 mg SRM 3247, ≈100 mg SRM 3248	External standard	Heated in a sealed container for 60 min at 90 °C	Prodigy ODS 3 C18 column (4.6×250 mm, 5-μm particle size), isocratic 50:50 acetonitrile/water (containing 0.85% phosphoric acid) at 1 ml/min and 35 °C	UV/visible absorbance detection at 370 nm

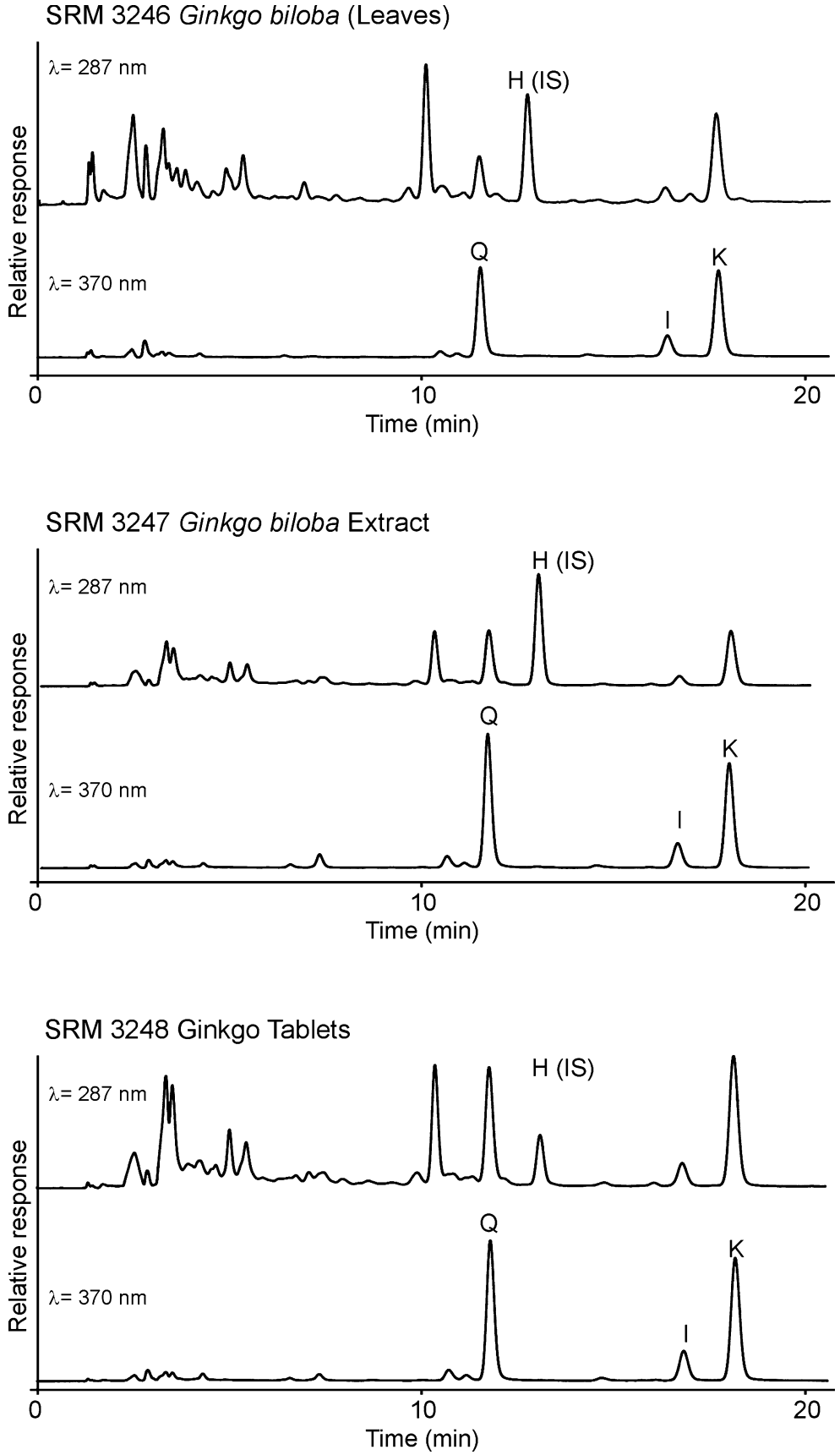


Fig. 2 LC/UV separation of flavonoid aglycones in SRMs 3246 through 3248. Two wavelengths were utilized: 287 nm for hesperetin (*H*) and 370 nm for quercetin (*Q*), isorhamnetin (*I*), and kaempferol (*K*)

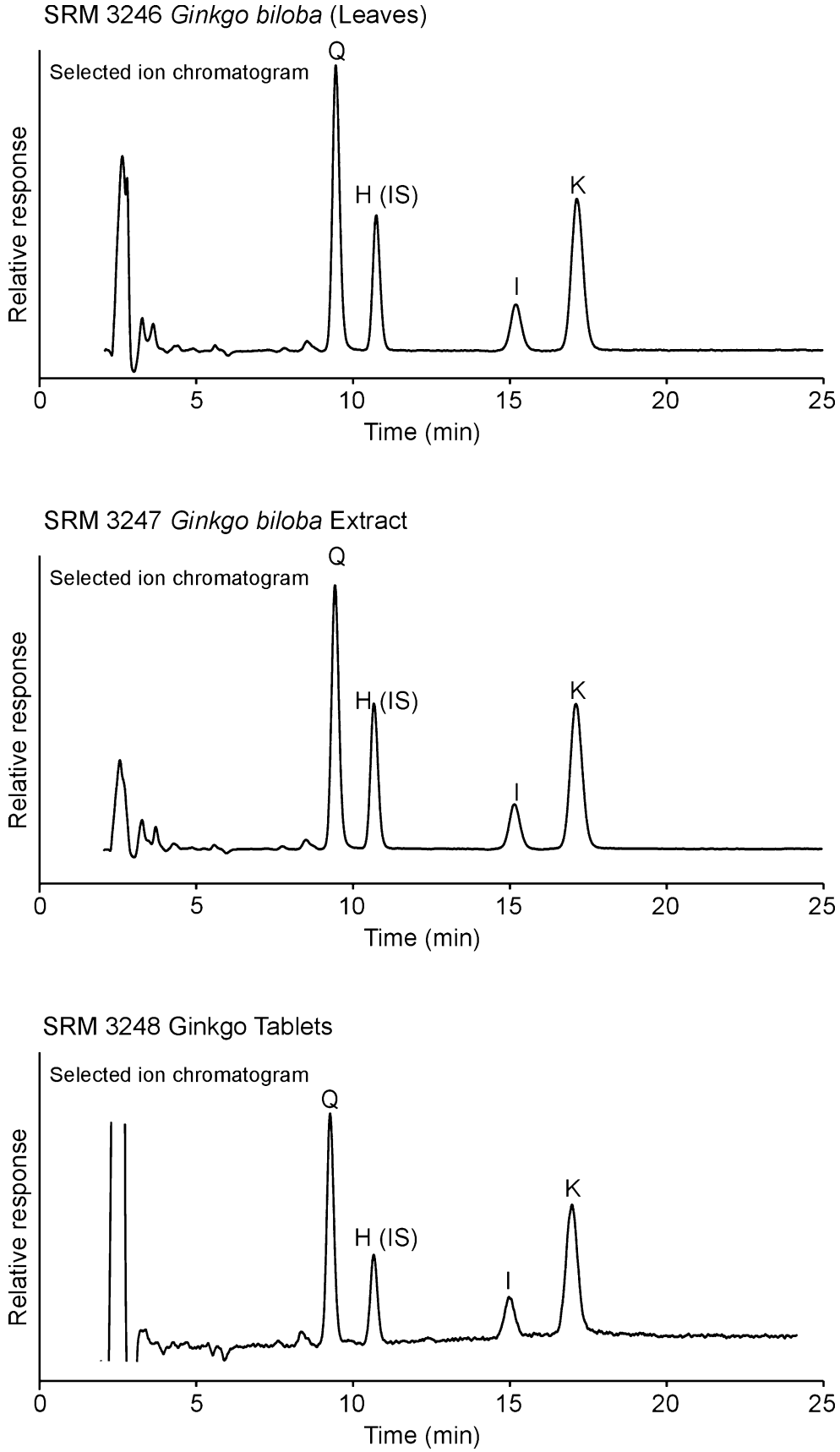


Fig. 3 LC/ESI–MS selected ion chromatograms of flavonoid aglycones in SRMs 3246 through 3248. Ions at *m*/*z* 287, 303, 317. Peak identities are hesperetin (*H*), quercetin (*Q*), isorhamnetin (*I*), and kaempferol (*K*)

conditions were used compared with methods 1, 2, and 3. Absorbance detection was at 370 nm.

Flavonoid aglycone method 5: LC/UV (AOAC collaborative study) The sample preparation, chromatographic conditions, and detection were the same as those used in flavonoid aglycone method 4; however, in this case, the method was applied by 11 laboratories rather than one (in an AOAC collaborative study, participating laboratories use a single method for the analysis of a number of samples to demonstrate the applicability of a method and its precision in the hands of a number of analysts). Total flavonol glycoside concentrations were then calculated using individual conversion factors for the three aglycones.

Analytical approach for determination of terpene lactones

Value assignment of the concentrations of the terpene lactones was based on the combination of measurements from two different analytical methods (three methods for SRM 3248) at NIST and measurements from one collaborating laboratory. Assigned values were calculated as the means of the means from the individual data sets. Details of sample preparation and analysis are provided in Table 2, and the general approaches employed in value assignment are summarized in Fig. S2.

Terpene lactone method 1: LC/MS (NIST) Samples were processed and analyzed as summarized in Table 2. Before extraction, the SRM samples were spiked with aliquots of the internal standard limonin. The samples analyzed by this method were the same samples that were prepared for the determination of the flavonoid aglycones method 1; however, small aliquots were removed from the extracts before the hydrolysis step (hydrolysis was not required for the determination of the lactones). Typical selected ion chromatograms from the three SRM materials are provided in Fig. 4.

Terpene lactone method 2: LC/MS (NIST) Details of sample processing and analysis are provided in Table 2. Different extraction approaches were used depending on the sample type. Samples of SRM 3246 were extracted with methanol in an ultrasonic bath, samples of SRM 3247 were dissolved in methanol with assistance by sonication, and samples of SRM 3248 were extracted by SPE using the approach of flavonoid aglycone method 2. A multistep gradient elution method was developed for simultaneous determination of flavonoid aglycones and ginkgolides. Ultimately, it was determined that resolution of the flavonoid aglycones was not sufficient to permit determination of flavonoids by this method; however, the separation was used to determine ginkgolides because it provided additional method independence. Ginkgolides and bilobalide were determined by positive ion electrospray ionization (ESI)–MS similar to terpene lactone method 1; however, different ions were monitored. Selected ion chromatograms from the analysis of the three materials are shown in Fig. 5.

Terpene lactone method 3: LC/MS (NIST) SRM 3248 ginkgo-containing tablets was Soxhlet extracted as outlined in Table 2. The same chromatographic method used for terpene lactone method 2 was used, except in the case of method 3, negative-ion atmospheric pressure chemical ionization mass spectrometry (NI–APCI–MS) was used for the detection of the ginkgolides and bilobalide. Representative selected ion chromatograms are shown in Fig. 6.

Terpene lactone method 4: LC (Eurofins) The sample preparation, liquid chromatography, and detection methods used are proprietary.

Analytical approach for determination of elements

The elements of primary interest for the ginkgo SRMs were the potentially toxic contaminants arsenic, cadmium, lead, and mercury. Value assignment of the concentrations of toxic elements was based on the combination of measurements at NIST using a single analytical method and results from one or two collaborating laboratories [National Research Council Canada (NRCC) and Laboratory of the Government Chemist (LGC)] when available. NIST analyses indicated apparent inhomogeneity of mercury distribution in SRM 3247 and low levels of cadmium in SRMs 3247 and 3248; therefore, NRCC was asked to measure cadmium and mercury in the leaves but not in the extract or in the tablets. Quantitation of arsenic and lead in all three samples was also requested. Levels of mercury in the leaves were below NRCC's limits of detection using cold vapor atomic absorption spectrometry; therefore, results were not reported. Methods and methodological details are provided in Table 3.

At low cadmium concentrations, spectral interference can affect the accuracy of cadmium measurement by inductively coupled plasma (ICP)-MS [11]. Certain interference such as the isobaric interference from ^{112}Sn and ^{114}Sn at cadmium masses 112 and 114 can be measured and a correction applied, but other matrix-related interference can be difficult to identify and correct. To estimate the uncertainty due to interference from the leaves, extract, and tablet matrices, one digested sample of each material was subjected to matrix separation using anion exchange chromatography. The percent difference in the cadmium concentration (corrected for isobaric interference from tin) obtained from direct introduction of the digested samples and the result obtained after matrix separation of a single sample was 1, 4, and 3% for the leaves, extract, and tablets, respectively. For each material, the

Table 2 Terpene lactone methods of analysis

Method identification		Extraction type	Extraction solvent	Extraction conditions	Mass of sample	Internal standard	Chromatographic conditions	Detection Conditions
Terpene lactone method 1	NIST LC/MS 1	Soxhlet (SRM 3246, 3248); dissolution by sonication (SRM 3247)	Methanol	22 h (SRM 3246, 3248); 30 min dissolution by sonication (SRM 3247)	≈1 g SRM 3246, ≈1 g SRM 3248, ≈50 mg SRM 3247	Limonin	Synergi-Max RP C12 column (4.6×250 mm; 4-μm particle size, Phenomenex, Torrance, CA, USA), water/methanol gradient; 0.1% acetic acid (mass fraction) additive; 0.75 ml/min and 25 °C gradient conditions: 0 min, 90:10 H_2O::methanol, 90 min, 0:100 H_2O::methanol	Positive ion electrospray mode (fragmentor voltage, 80 V; capillary voltage, 4,000 V; drying gas, 300 °C and 12.0 l/min; nebulizer pressure, 50 psi) using single-ion monitoring (m/z (BB) 344, (G–A) 426, (G–J) and (G–B) 442, (G–C) 458, (L) 488)
Terpene lactone method 2	NIST LC/MS 2	Ultrasonic bath, 5 successive extractions (SRM 3246) dissolution by sonication (SRM 3247) PFE (SRM 3248)	Methanol	5 extractions in succession, 30 min sonication (SRM 3246), 30 min dissolution by sonication (SRM 3247), 3 extractions in succession PFE program 3 cycles at 100 °C, 13 MPa (2,000 psi), 5 min static hold/cycle (SRM 3248)	≈1 g SRM 3246, ≈200 mg SRM 3247, ≈1 g SRM 3248	Hesperetin	Xterra C18 column (4.6×250 mm; 5-μm particle size), water/acetonitrile gradient; 0.5% acetic acid (mass fraction) additive; 1.0 ml/min and 25 °C gradient conditions: 0 min, 80:20 H_2O/ACN; 30 min, 80:20 H_2O/ACN; 45 min, 65:35 H_2O/CAN; 53 min, 60:40 H_2O:CAN; 60 min, 10:90 H_2O/ACN	Positive ion electrospray mode (fragmentor voltage: 80 V, capillary voltage: 4000 V, drying gas: 300°C and 12.0 L/min, nebulizer pressure 50 psi) using single-ion monitoring (m/z (BB) 327, (G–A) 409, (G–J) and (G–B) 425, (G–C) 441, (H) 303).
Terpene lactone method 3	NIST LC/MS 3	Soxhlet (SRM 3248 only)	Methanol	22–24 h	≈1 g SRM 3248	Hesperetin	Xterra C18 column (4.6×250 mm; 5-μm particle size), water/acetonitrile gradient; 0.5% acetic acid (mass fraction) additive; 1.0 ml/min and 25 °C gradient conditions: 0 min, 80:20 H_2O/CAN; 30 min, 80:20 H_2O/CAN; 45 min, 65:35 H_2O/CAN; 53 min, 60:40 H_2O/CAN; 60 min, 10:90 H_2O/ACN	Negative ion APCI mode (fragmentor voltage, 70 V; capillary voltage, −1,750 V; drying gas, 350 °C and 4.0 l/min, nebulizer pressure 55 psi, vaporizer temperature 400 °C) using single-ion monitoring [*m/z* (BB) 325, (G–A) 467, (G–J) and (G–B) 423, (G–C) 439, (H) 301, (G–J) 483]
Terpene Lactone Method 4	Eurofins	Proprietary	Proprietary	Proprietary	Proprietary	Proprietary	Proprietary	Proprietary

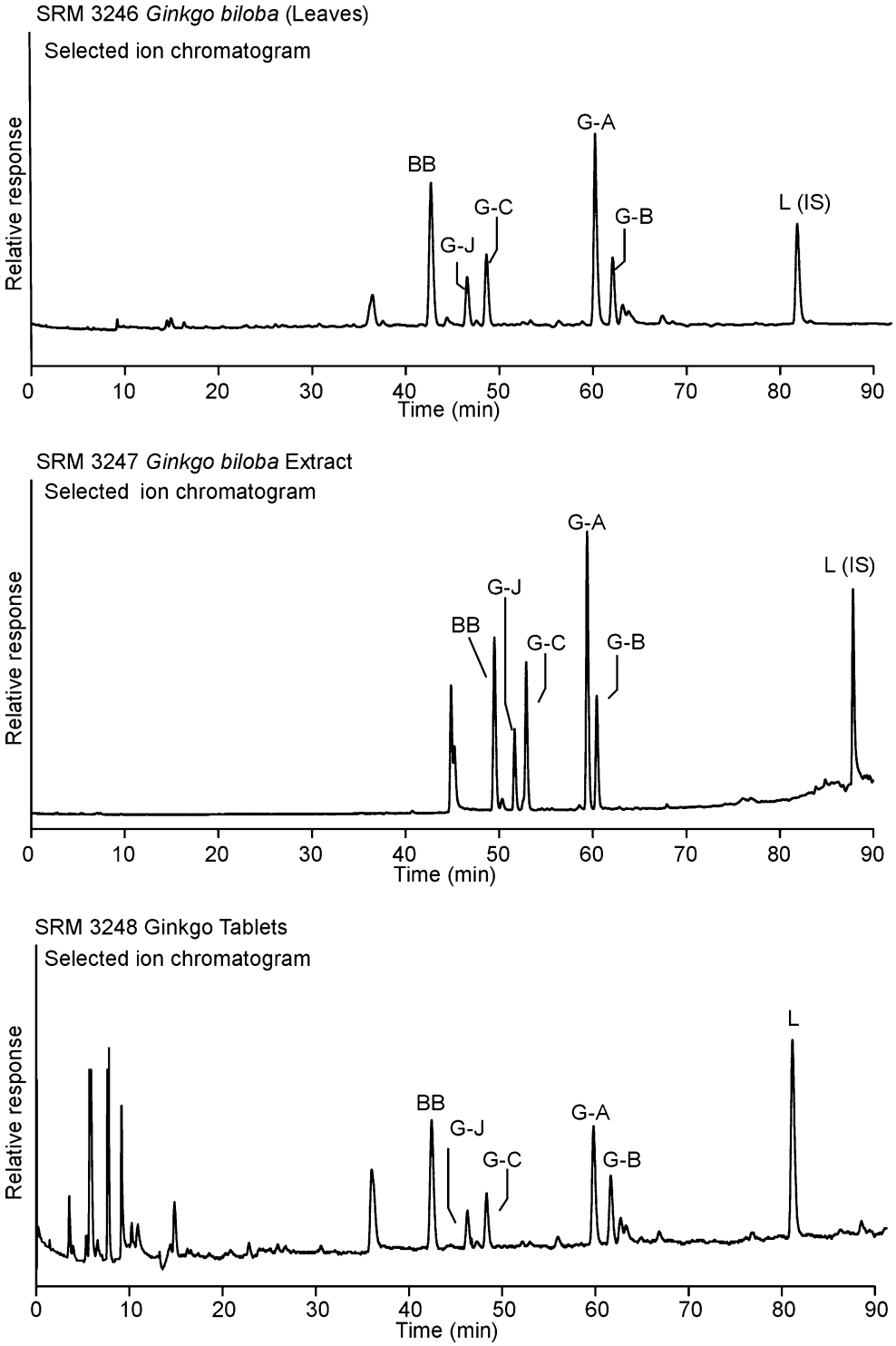


Fig. 4 LC/ESI–MS selected ion chromatograms of ginkgolides in SRMs 3246 through 3248 (method 1). Ions at *m/z* 344, 426, 442, 458, and 488. Peak identities are bilobalide (*BB*), ginkgolide J (*G–J*), ginkgolide C (*G–C*), ginkgolide A (*G–A*), ginkgolide B (*G–B*), and limonin (*L*)

cadmium concentration result obtained after matrix separation was lower than the concentration results generated by direct analysis of the digested sample, presumably indicating some interference at the cadmium 112 and cadmium 114 masses in the directly analyzed samples. Accordingly, an uncertainty component equal to the magnitude of the differences obtained was included in the NIST cadmium concentration uncertainty budget for each material.

Results and discussion

The process of assigning values for chemical composition of reference materials at NIST has been described in detail [12]. Three types of assigned values may be reported in SRMs: certified values, reference values, and information values. Certified values are values upon which NIST places the highest level of confidence in that known or suspected

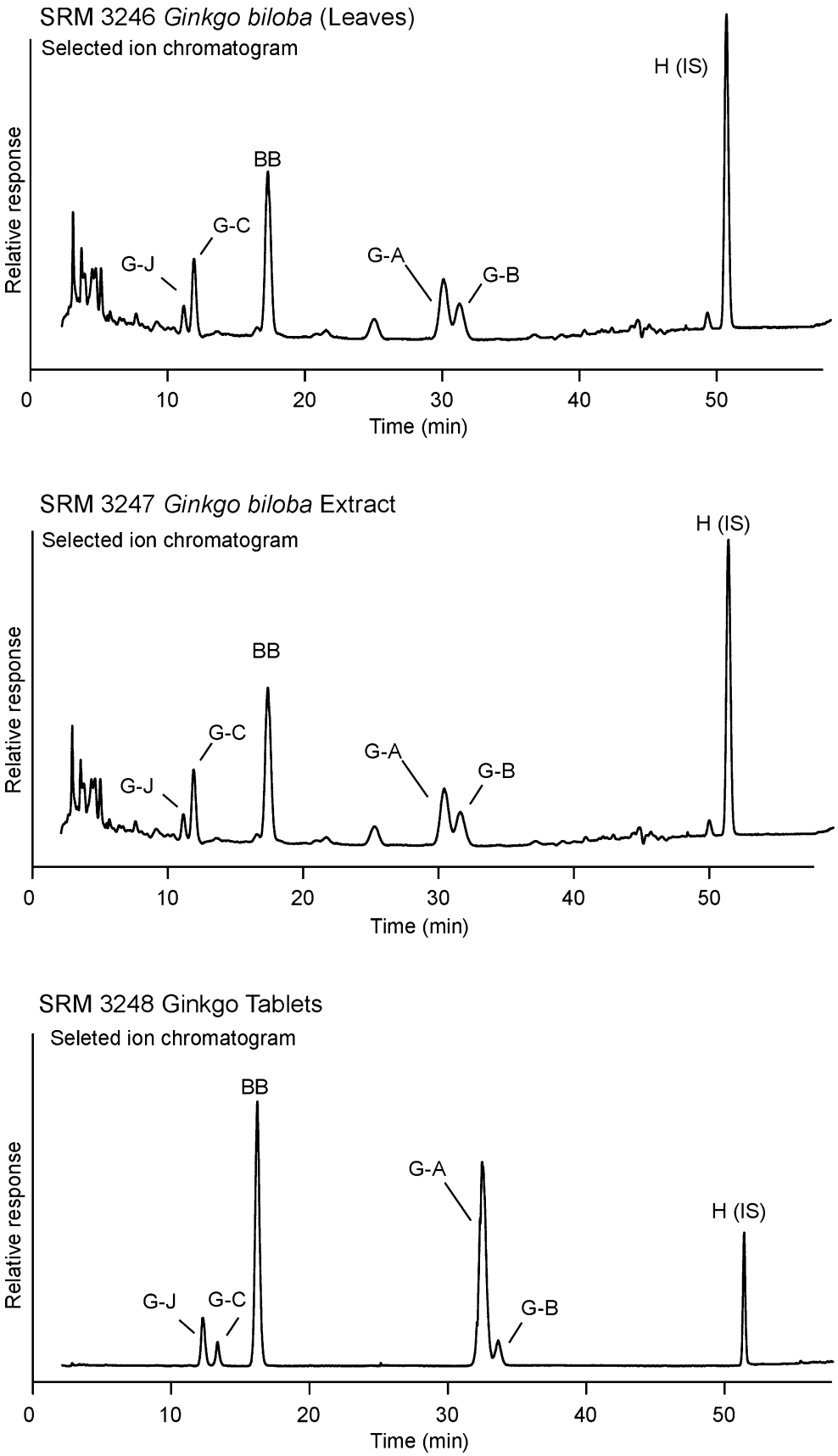


Fig. 5 LC/ESI–MS selected ion chromatograms of ginkgolides in SRMs 3246 through 3248 (method 2). Ions at *m/z* 303, 327, 409, 425, and 441. Peak identities are bilobalide (*BB*), ginkgolide J (*G–J*), ginkgolide C (*G–C*), ginkgolide A (*G–A*), ginkgolide B (*G–B*), and hesperetin (*H*)

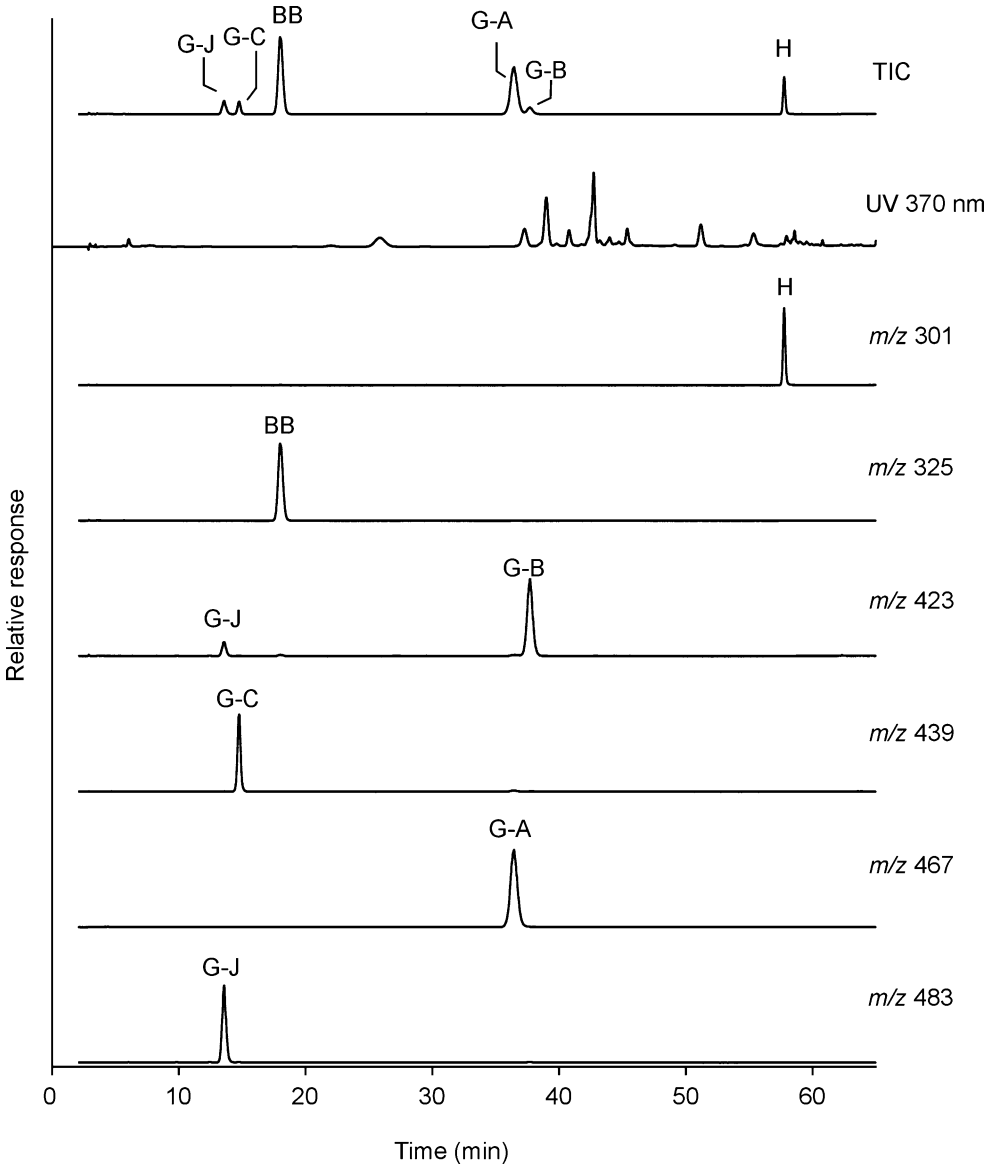


Fig. 6 UV/Vis chromatogram and extracted ion chromatograms from LC/APCI–MS separation of ginkgolides in SRMs 3246 through 3248. Peak identities are bilobalide (*BB*), ginkgolide J (*G–J*), ginkgolide C (*G–C*), ginkgolide A (*G–A*), ginkgolide B (*G–B*), and hesperetin (*H*)

sources of bias have been investigated or accounted for. The most common approach at NIST for certification of chemical composition is measurement by two or more independent analytical methods. If results obtained using independent methods (including but not limited to extraction, cleanup, separation and detection, and approach to quantification, i.e., internal vs external standard approach) are in agreement, there is greater confidence in the accuracy of the assigned value; method biases would result in disagreement among methods. Values that do not meet the criteria required for certification are reported as reference values or information values.

All measurements performed at NIST were based on an internal standard approach to quantitation. A series of aglycones that have not been reported in *G. biloba* were screened for potential use as internal standards. Hesperetin was selected based on similarity to the three aglycones found in the ginkgo SRMs and because it is well resolved chromatographically from the aglycones of interest and from matrix constituents. In addition, it is stable under the extraction and hydrolysis conditions, and it provides adequate detector response. As isotopically labeled standards were not available, hesperetin was also used as the internal standard for the LC/MS method. For additional method independence, a different internal standard, limonin, was used for the determination of ginkgolides. Limonin was selected primarily for its retention characteristics; unfortunately, more closely related ginkgolide analogs were not available. Measurements performed by collaborating laboratories utilized external standard calibration approaches.

Value assignment of the concentration of the flavonoid aglycones was based on the combination of five methods

Table 3 Toxic metal methods of analysis

Analyte	Method identification	Sample mass	Sample preparation	Method	Reference
Arsenic	NIST	100 mg	Individual sample disks formed, exposed to neutron flux of $1\times1{,}014$ cm–$2s^{-1}$ for 4 h. Decay times 4 to 4.3 days	Instrumental neutron activation analysis (INAA); germanium detector, 599 and 568 keV lines of 76As	
Arsenic	NRCC	250 mg	Microwave digestion with nitric acid and hydrogen peroxide; Ir, internal standard	Hydride generation graphite furnace atomic absorption spectrometry (HG GFAAS)	[47]
Arsenic	LGC	500 mg (leaves)	Microwave digestion using nitric acid and hydrogen peroxide	Inductively coupled plasma mass spectrometry (ICP–MS) in helium gas mode	
Cadmium	NIST	250 mg extract, 500 mg leaves, 750 mg tablets	Microwave digestion with nitric and hydrofluoric acid	Isotope dilution (ID) ICP–MS using 112Cd/111Cd and 114Cd/111Cd	[48]
Cadmium	NRCC	250 mg (leaves)	Microwave digestion with nitric acid and hydrogen peroxide	ID ICP–MS using 113Cd/111Cd	
Cadmium	LGC	500 mg (leaves)	Microwave digestion using nitric acid and hydrogen peroxide	ID ICP–MS using 111Cd /106Cd	
Lead	NIST	250 mg extract, 500 mg leaves, 750 mg tablets	Microwave digestion with nitric and hydrofluoric acid	ID ICP–MS using 208Pb/206Pb	[48]
Lead	NRCC	250 mg	Microwave digestion with nitric acid and hydrogen peroxide	ID ICP–MS using 208Pb/207Pb	
Lead	LGC	500 mg (leaves)	Microwave digestion using nitric acid and hydrogen peroxide	ID ICP–MS 208Pb/206Pb	
Mercury	NIST	250 mg	Microwave digestion with nitric acid	Cold vapor (CV) ID ICP–MS with 201Hg	[49]
Mercury	NRCC			Below limit of detection for CV-atomic absorption spectrometric method	

(two from NIST, two from outside laboratories, and one from an AOAC collaborative study involving 11 laboratories). Laboratories reported either flavonoid aglycones or flavonol glycosides; all results were converted to flavonoid aglycones because these were the compounds that were directly determined. It is important to note that most manufacturers report a percentage flavonol glycoside, although flavonoid aglycones are measured. The conversion is imperfect, as there can be multiple glycosolated versions of each aglycone and the conversion factor is at best an average.

Flavonoid aglycones

The quantitative determination of the individual flavonol glycosides in *G. biloba* is problematic due to a lack of pure reference standards. An alternate approach to characterizing flavonol composition is to convert the glycosides to aglycones, for which reference standards are readily available. The extraction of flavonol glycosides and the conversion to flavonoid aglycones has been described in the literature [3, 5, 13–19]. At NIST, 24-h Soxhlet extractions in methanol were shown to be equivalent to

three PFEs at 100 °C and 10.34 MPa (1,500 psi) with three 5-min static holds per extraction [15].

A number of chromatographic methods have been reported in the literature for the separation of the flavonoid aglycones. Most of the separations have utilized reversed-phase LC with C_{18} stationary phases [16, 17, 19–22]. The separations are similar; however, the elution order of isorhamnetin and kaempferol changes depending on the C_{18} stationary phase used. Even on phases that are well end-capped and on inert silica, the addition of acid to the mobile phases is necessary to prevent peak broadening and tailing. Due to improved resolution, methods in which isorhamnetin eluted before kaempferol were preferable for quantitation.

Typical LC/UV chromatograms for the analysis of the three ginkgo-containing SRMs using NIST flavonol aglycone method 1 are provided in Fig. 2. Two UV absorbance wavelengths are shown for each material; 287 nm was used for the detection of hesperetin, which did not have significant absorbance at 370 nm. Quercetin, isorhamnetin, and kaempferol were detected at 370 nm for improved sensitivity.

From Fig. 2, it is evident that SRM 3246 *G. biloba* (leaves) was a more complex sample than SRM 3247 *G. biloba* extract or SRM 3248 ginkgo-containing tablets. This was not unexpected as SRMs 3247 and 3248 were standardized products that are made through the extraction, concentration, and cleanup of *G. biloba* leaves. This is further demonstrated by the thin-layer chromatography that was performed on the three samples (see Figs. S3 to S5), indicating that the ginkgolic acid, for example, was present in the leaves but not in the extract or tablets; see also certificates of analysis for SRMs 3246 through 3248 (http://www.nist.gov/srm) and CAMAG Laboratory Services application notes F-16A, F-16B, and F-16C [23–25]. The quality of the separation is similar to those found in the literature. In the current approach, several modifications were made to optimize the methods for quantitation. New method features resulted as a consequence of extraction and hydrolysis studies, and an internal standard was utilized to reduce losses introduced during sample handling. In published methods, it is common for aglycones and terpene lactones to be determined together in the same method. The lack of a chromophore with the ginkgolides necessitates the use of refractive index, light scattering, or UV detection at approximately 210 nm. It appears that it is preferable to decouple the aglycone and terpene lactone methods to permit more sensitive and selective detection of the aglycones. Sample processing for the terpene lactones is reduced, as it is not necessary to hydrolyze the samples for the determination of the ginkgolides (although, if a universal detector is used

Table 4 Averages and standard deviations (1s) of measurements of aglycones (mg/g) in SRMs 3246, 3247, and 3248 as determined by different analytical approaches and laboratories

Method	1		2		3		4		5		*n*
	NIST		NIST		Chromadex		NSF		AOAC		
	LC/UV		LC/MS		LC/UV		LC/UV		LC/UV		
	Mean	1s	Mean	1s	Mean	1s	Mean	1s	Mean	1s	
SRM 3246											
Quercetin	2.6	0.29	2.98	0.12	2.57	0.05	2.58	0.03	2.26	0.03	10
Kaempferol	3.2	0.08	3.27	0.1	2.48	0.07	2.58	0.02	2.7	0.05	10
Isorhamnetin	0.56	0.04	0.58	0.02	0.4	0.02	0.4	0.01	0.41	0.01	10
Total	6.36	0.37	6.83	0.2	5.45	0.12	5.57	0.05	5.37	0.09	9
SRM 3247											
Quercetin	47.52	0.89	44.87	1.03	45.36	0.28	43.53	0.42	37.43	1.43	11
Kaempferol	42.68	0.78	41.23	1.02	37.58	0.35	38.34	0.31	38.28	1.7	11
Isorhamnetin	11.5	0.16	10.76	0.28	10.97	0.15	9.64	0.04	8.92	3.5	9
Total	101.7	1.73	98.88	2.25	93.91	0.77	91.51	0.75	84.62	0	9
SRM 3248											
Quercetin	7.82	0.23	7.5	0.21	7.79	0.12	7.39	0.01	6.6	0.11	10
Kaempferol	7.77	0.2	7.2	0.28	6.45	0.16	6.55	0.01	6.84	0.11	9
Isorhamnetin	2.09	0.03	1.86	0.09	1.86	0.06	1.64	0.01	1.63	0.08	7
Total	17.68	0.44	16.56	0.44	16.11	0.33	15.58	0.01	15.08	0.22	7

For NIST LC/UV analyses of SRMs 3246 and 3247, $n=12$; for analysis of SRM 3248, $n=7$. For NIST LC/MS analyses of SRMs 3246 and 3247, $n=7$; for analysis of SRM 3248, $n=12$. For Chromadex analyses of all three materials, $n=4$. For NSF analyses of all three materials, $n=3$. For the AOAC collaborative study, n is provided in the table.

for the determination of the ginkgolides, sample hydrolysis may be required because the glycosolated flavonoids coelute with the ginkgolides).

Typical LC/MS selected-ion chromatograms for the three ginkgo-containing SRMs are shown in Fig. 3. The addition of the trifluoroacetic acid (TFA) was found to stabilize the signal of the aglycones in the samples and calibrants leading to consistent response factors throughout the course of the run (several days). Individual laboratory results for quercetin, kaempferol, isorhamnetin, and total aglycones in addition to the number of samples tested by each laboratory are provided in Table 4; individual results for ginkgolides (see discussion below) are provided in Table 5. The certified values for quercetin, kaempferol, isorhamnetin, and total aglycones are provided in Table 6. These data, in addition to the data from duplicate samples prepared from the same bottle, were examined with respect to LC run order and to bottle fill order. Examination of the data indicate that there were no significant sources of sample inhomogeneity.

A detailed comparison of the data from each of the methods indicates some inconsistency among the data sets. The data reported from method 5 for aglycones and total aglycones are consistently lower than values from other methods. Plots of the means and standard deviations for each of the methods are presented in Fig. S6. A lack of consistency is not uncommon for large intercomparison exercises; however, the apparent bias in method 5 data may be indicative of a methodological problem, such as incomplete sample extraction and/or incomplete sample hydrolysis. The extraction and hydrolysis steps used with method 5 are less extensive than those used with method 1 or 2. For example, the number of extraction cycles, the temperature of extraction or hydrolysis, and the concentration of the acid used in hydrolysis are lower for method 5 than with method 1 or 2. Interestingly, the data provided by method 4 was similar to the data from method 5, and the two methods were nominally the same. Somewhat better agreement was obtained for method 4 data for quercetin, although the method 4 data were still slightly lower than the values from other methods.

Terpene lactones

During method development, the extraction of ginkgolides and bilobalide from the leaf and tablet materials was investigated at NIST. Soxhlet extraction was shown to be the best method for the removal of the ginkgolides and bilobalide from the leaf and tablet matrices. Successive extractions in an ultrasonic bath were also used for the value assignment of SRM 3246 [15]. SRM 3247 *G. biloba*

Table 5 Averages and standard deviations (1s) of measurements of ginkgolides (mg/g) in SRMs 3246, 3247, and 3248 as determined by different analytical approaches and laboratories

Method	1		2		3		4	
	NIST		NIST		NIST		Eurofins	
	LC/MS 1		LC/MS 2		LC/MS 3			
	Mean	1s	Mean	1s	Mean	1s	Mean	1s
SRM 3246								
Bilobalide	1.98	0.05	1.29	0.15			1.28	0.12
J	0.27	0.01	0.17	0.02			0.09	0.03
C	0.79	0.02	0.59	0.072			0.4	0.05
A	0.84	0.03	0.54	0.068			0.34	0.06
B	0.57	0.01	0.42	0.056			0.42	0.03
Total	4.5	0.11	3	0.339			2.54	0.25
SRM 3247								
Bilobalide	29.4	1.3294	26.5	1.9175			29.63	0.74
J	5.16	0.1001	3.82	0.2157			2.66	0.34
C	13.7	0.4494	12.2	0.6183			11.43	0.59
A	13.4	0.3631	10.3	0.342			11.21	0.4
B	6.3	0.1773	5.57	0.1857			5.88	0.34
Total	67.96	2.3078	58.39	3.0806			60.86	1.62
SRM 3248								
Bilobalide	5.7	0.34	5.03	0.17	6.5	0.92	5.43	0.05
J	0.96	0.02	0.76	0.02	1	0.07	0.44	0.04
C	2.81	0.08	2.34	0.04	4.74	0.82	2.02	0.05
A	2.77	0.08	1.86	0.04	2.82	0.18	2.07	0.1
B	1.31	0.04	0.98	0.12	1.38	0.17	1.07	0.06
Total	13.55	0.48	10.97	0.23	16.44	1.8	11.03	0.61

For NIST LC/MS1 analyses of all three materials, $n=10$. For NIST LC/MS2 analyses of all three materials, $n=12$. For NIST LC/MS3 analyses of SRM 3248, $n=6$. For Eurofins analyses, $n=5$ for SRM 3246, $n=12$ for SRM 3247, and $n=4$ for SRM 3248.

Table 6 Certified (bold) and reference (normal typeface) concentration values for flavonoid aglycones and terpene lactones in SRM 3246, SRM 3247, and SRM 3248[a]

	SRM 3246 Mass fraction (mg/g)	SRM 3247 Mass fraction (mg/g)	SRM 3248 Mass fraction (mg/g)
Quercetin[b,c,d,e,f]	**2.69±0.31**	**45.1±4.6**	**7.56±0.40**
Kaempferol[b,c,d,f]	**3.02±0.41**	**40.8±3.0**	**7.19±0.70**
Isorhamnetin[b,c,d,e,f,]	**0.517±0.099**	**10.8±1.3**	**1.90±0.22**
Total aglycones[b,c,d,f]	**6.22±0.77**	**96.8±8.3**	**16.6±1.2**
Ginkgolide A	0.57±0.28[g,h]	**11.6±1.7**[g,h]	2.42±0.63[h,i]
Ginkgolide B[g,h]	**0.470±0.090**	**5.92±0.45**	**1.12±0.20**
Ginkgolide C[g,h]	0.59±0.22	**12.4±1.4**	**2.36±0.42**
Ginkgolide J	0.18±0.10[g,h]	**3.9±1.5**[g,h]	0.81±0.36[h,i]
Bilobalide	1.52±0.40[g,h]	**28.5±2.1**[g,h]	5.7±1.2[h,i]
Total terpene lactones[g,h]	3.3±1.1	**62.4±5.7**	**11.8±1.4**[j]

[a] Each certified concentration value, expressed as a mass fraction 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 guide [50–52], 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. Each reference concentration value, expressed as a mass fraction on a dry-mass basis, is an equally weighted mean of the results from NIST and collaborating laboratories. The uncertainty in the reference value, calculated according to the method described in the ISO guide [50–52], 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.

[b] NIST LC/UV

[c] NIST LC/MS

[d] ChromaDex LC/UV

[e] NSF International LC/UV

[f] AOAC collaborative study

[g] Two NIST LC/MS Methods

[h] Eurofins LC/ELSD

[i] Three NIST LC/MS Methods

[j] Because two or three methods were used to determine individual terpene lactones, the value for total terpene lactones does not equal the sum of the individual values.

extract was readily soluble in solvent at room temperature and did not require further extraction for determination of the ginkgolides.

The detection of ginkgolides is especially difficult, as there are no chromophores and the ginkgolides are difficult to separate from many of the flavonol glycosides. Researchers have avoided this problem by first hydrolyzing the samples and then utilizing either the refractive index or ELSD [5–7, 19, 26–30]. The terpene lactones are stable under acid-catalyzed hydrolysis conditions; however, the lactones are subject to ring-opening reactions in alkaline solutions. Other methods include the derivatization of the sample followed by the subsequent gas chromatographic separation with flame ionization or MS detection [31–33] and electrophoretic separations [34–36]. Recently, LC/MS has become a common method for the determination of terpene lactones in *G. biloba* [5, 7, 37–45]. To avoid performing sample hydrolysis after extraction, two different LC/MS methods were utilized for the determination of the ginkgolides. The LC/MS analyses of the three SRMs using positive ion electrospray ionization are shown in Figs. 4 and 5, and methods 1 and 2, respectively. The LC separations were performed on different stationary phase columns (a C_{12} column in Fig. 4 and a C_{18} column in Fig. 5), with different

Table 7 Certified (bold) and reference (normal typeface) concentration values for toxic elements in SRMs 3246 through 3248[a]

	SRM 3246 Mass fraction (ng/g)	SRM 3247 Mass fraction (ng/g)	SRM 3248 Mass fraction (ng/g)
Arsenic[b]		314±12[c,d]	56.5±4.3[c]
Cadmium	**20.8±1.1**[e,f]	7.53±0.77[e]	1.56±0.19[e]
Lead	**995±30**[e,f]	**4273±31**[e,f]	**775.3±8.9**[e,f]
Mercury [g]	**23.08±0.17**	–[h]	0.271±0.034

[a] Each certified concentration value, expressed as a mass fraction on a dry-mass basis, is an equally weighted mean of the results from NIST and NRCC. The uncertainty in the certified value, calculated according to the method described in the ISO guide [50–52], 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. Each reference concentration value, expressed as a mass fraction on a dry-mass basis, is an equally weighted mean of the results from NIST and NRCC (where available). The uncertainty in the reference value, calculated according to the method described in the ISO guide [50–52], 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.

[b] Arsenic in SRM 3246 ranged from 86 to 290 ng/g when tested at NIST using INAA. Because of this apparent inhomogeneity, a value was not assigned.

[c] NIST INAA

[d] NRCC HG–GFAAS

[e] NIST ID ICP–MS

[f] NRCC ID ICP–MS

[h] Mercury in SRM 3247 ranged from 0.29 to 4.5 ng/g when tested at NIST using CV ID ICP–MS. Because of this apparent inhomogeneity, a value was not assigned.

[g] NIST CV ID ICP–MS

chromatographic selectivity (note the change in elution order for G–J and G–C compared to BB), thereby, providing independence between the two methods. The change in elution order may result as a consequence of polar endcapping with the C_{12} phase. Other changes in elution order for C_{18} phases have been attributed to the use of different mobile phase modifiers, such as tetrahydrofuran [46]. The separation of the ginkgolides in SRM 3248 by negative ion LC/APCI–MS is shown in Fig. 6. In this case, the individual extracted ion chromatograms are shown, indicating the increased selectivity that is gained with MS detection.

The levels of the ginkgolides and bilobalide determined by each of the different methods and laboratories are summarized in Table 5. Plots of the individual means and standard deviations are provided in Fig. S7. As with the data for the flavonoid aglycones, some inconsistency in results is apparent among the methods. Overall, NIST method 1 consistently provided higher levels for most of the analytes than the other methods. This could be the result of more complete extraction resulting from the Soxhlet extraction approach (in which case method 1 would provide a better estimate of the true value), or a bias resulting from a problem in the methodology.

Determination of certified and reference values

The results from the different methods for the flavonoid aglycones and ginkgolides (Tables 4 and 5) were combined to assign the certified and reference values in the three SRMs as shown in Table 6. Certified values were assigned for the three flavonoid aglycones and for total aglycones in all three SRMs, with relative uncertainties ranging from 5.3 to 19%. Certified or reference values were assigned for the ginkgolides and total terpene lactones in the three SRMs depending on the agreement of the methods. The relative uncertainties of the certified values for ginkgolides ranged from 7.5 to 18%. The expanded uncertainty in the certified and reference values reflects the combined effect of between-laboratory, within-laboratory, and drying components of uncertainty at the level of approximately 95% confidence for each analyte. No indications of significant inhomogeneity in any of the three ginkgo-containing materials were observed.

Toxic elements

Assigned values for toxic elements are provided in Table 7, and the data used for value assignment are provided in Fig. S8. Mercury was not homogeneously distributed in SRM 3247 *G. biloba* extract; therefore, no value is reported. The range of toxic element concentrations in the three ginkgo materials is somewhat different from the range in a series of ephedra-containing SRMs, the first suite in NIST's dietary supplements SRMs [8]. A match of both concentration and matrix is best when choosing a control material. In some cases, the ephedra-containing materials contain the highest or lowest concentrations and, in others, the ginkgo-containing materials do (Fig. 7), making the two suites complementary for the analysis of toxic elements in materials of similar matrices.

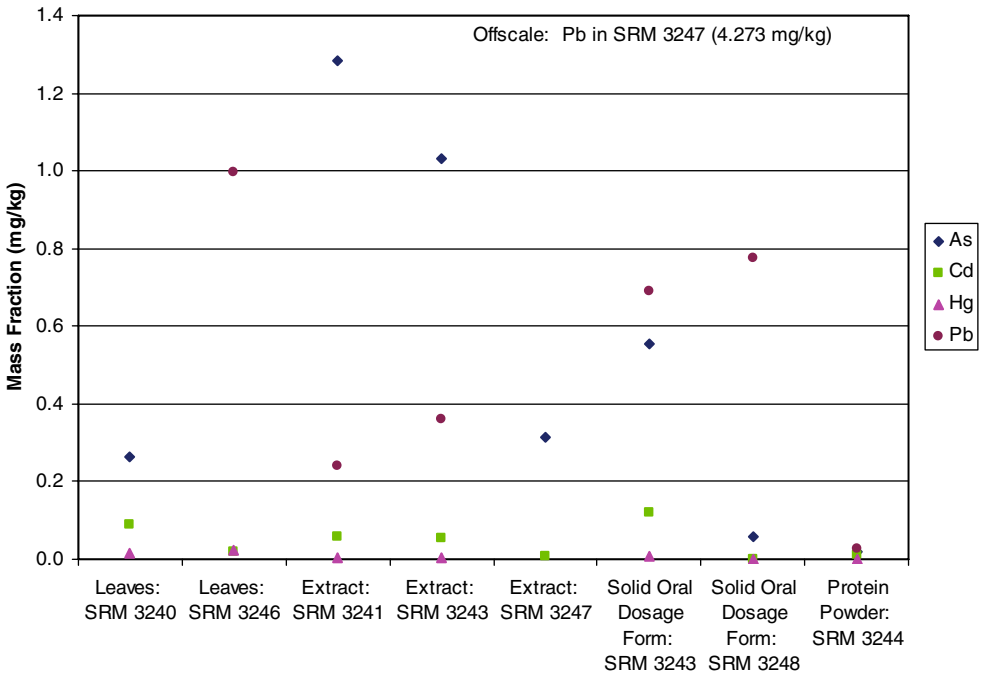


Fig. 7 Comparison of toxic elements in botanical dietary supplement SRMs, grouped by matrix type

Conclusions

SRMs 3246 through 3248 represent an addition to the series of dietary supplement SRMs offered by NIST with certified and reference values for organic constituents and trace elements. Other materials in preparation include suites of materials for green tea, bitter orange, cranberries, blueberries, and bilberries. In addition, SRMs are being prepared for omega-3- and omega-6-containing fatty acids, tocopherols in vegetable oil, and a multivitamin/multielement tablet. These materials are intended for use in method development and as control materials to support analytical methods for the determination of these constituents. Dietary supplements now available include five ephedra materials, cod liver oil, and a carrot extract material.

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