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ORIGINAL PAPER

Certification of standard reference materials containing bitter orange

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Abstract A suite of three dietary supplement standard reference materials (SRMs) containing bitter orange has been developed, and the levels of five alkaloids and caffeine have been measured by multiple analytical methods. Synephrine, octopamine, tyramine, N-methyltyramine, hordenine, total alkaloids, and caffeine were determined by as many as six analytical methods, with measurements performed at the National Institute of Standards and Technology and at two collaborating laboratories. The methods offer substantial independence, with two types of extractions, two separation methods, and four detection methods. Excellent agreement was obtained among the measurements, with data reproducibility for most methods and analytes better than 5% relative standard deviation. The bitter-orange-containing dietary supplement SRMs are intended primarily for use as measurement controls and for use in the development and validation of analytical methods.

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Introduction

In December 2003 the Food and Drug Administration ruled that dietary supplements that contain ephedrine alkaloids are adulterated, based on potential health risks associated with their use [1]. This ruling, which became effective in April 2004, prohibits the sale of ephedra-containing dietary supplements in the USA. As a result of this action, many manufacturers of dietary supplements have reformulated weight-loss products with ingredients that are considered to be safer alternatives to ephedra.

Dietary supplements have been developed from extracts derived from the fruits of *Citrus aurantium* L. (Rutaceae). Subspecies or varieties include *C. aurantium* L. var. *amara* (L.) and *C. aurantium* L. var. *sinensis* (L.); however, it is common to refer to mixtures of these plants which are blended to adjust alkaloid content simply as "bitter orange" (see references in [2]). Bitter orange products are formulated with dried powder or extracts of citrus fruits that contain a series of alkaloids that are similar in structure and function to ephedrine alkaloids (Fig. 1) [3]. Synephrine is the most prominent of the alkaloids and can occur naturally in the dried fruit at a level of about 1% (mass fraction); other alkaloids are present at significantly lower levels. Bitter orange extracts used in products that are currently available in the marketplace typically contain 6% (mass fraction) synephrine. Because the bitter orange alkaloids are hydroxylated on the phenyl ring, the compounds are much more polar than ephedrine alkaloids. This alters their pharmacokinetic properties and necessitates the use of alternative analytical methods than are employed for the analysis of ephedra.

Several methods have been developed for the determination of synephrine [2, 4–11]; less effort has been expended towards the measurement of other known bitter orange alkaloids [12–14]. Reversed-phase liquid chromatography (RPLC) methods using C_{18} or phenyl stationary phases provide insufficient retention to easily resolve these compounds, although synephrine alone can be determined in this way. More commonly, methods for the determination of bitter orange alkaloids employ ion-pair chromatography with sodium dodecyl sulfate (SDS) or hexanesulfonic acid to increase retention. Separation of the five bitter orange alkaloids has recently been demonstrated by ion-pair RPLC with fluorescence detection [15]. Unlike the ephedrine alkaloids that do not fluoresce, the bitter orange alkaloids strongly fluoresce at 306 nm with excitation at 273 nm [11]. Ultraviolet (UV) absorbance detection at 210–220 nm can also be used, although this approach is less sensitive and

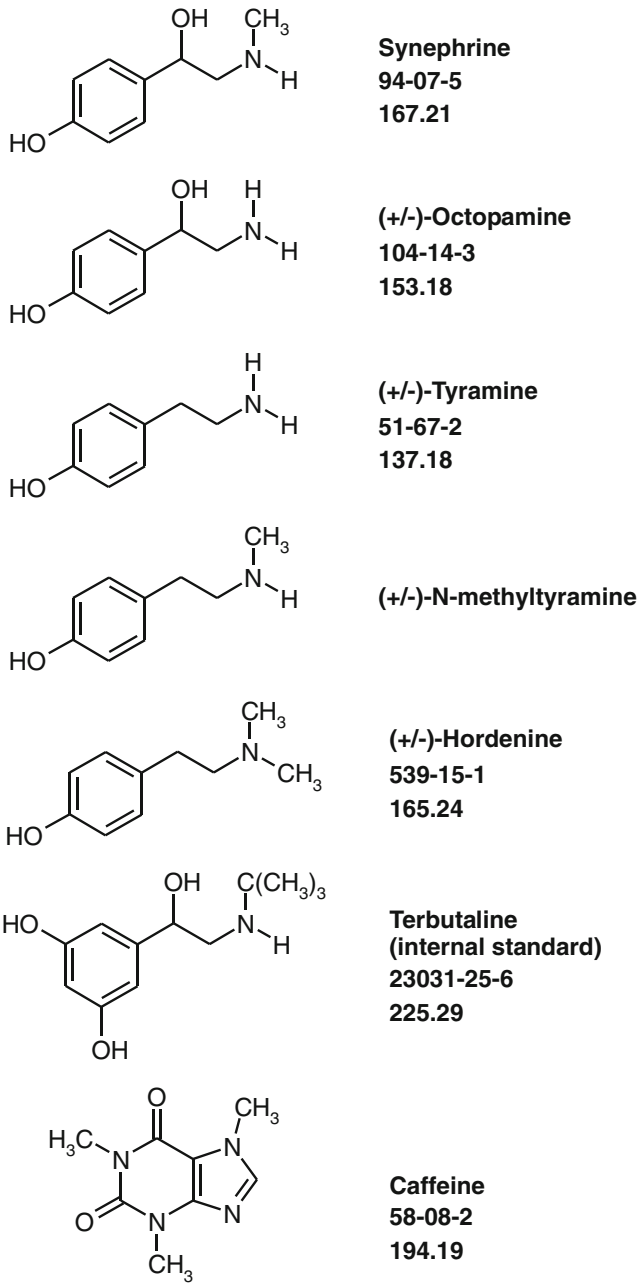

Fig. 1 Structures of bitter orange alkaloids and related compounds, including CAS designations and relative molecular masses

less selective than florescence detection. Owing to the presence of the ion-pair and buffer reagents, mass spectrometry (MS) detection is not possible with this separation method. A different approach using a pentafluorophenyl stationary phase has been reported that does not require nonvolatile mobile-phase additives and is compatible with MS detection [16, 17]. Both single quadrupole (i.e., LC/MS) and tandem MS (i.e., LC/MS/MS) methods have been developed using this novel column [17, 18]. The use of multiple independent methods is an important component in the value assignment of reference materials at the National Institute of Standards and Technology (NIST)

since the comparison of independent data sets provides insight into potential biases among methods and permits realistic assessment of measurement uncertainties [19].

In 2001, NIST and the National Institutes of Health, Office of Dietary Supplements undertook a collaboration to develop a series of Standard Reference Material (SRM) suites based on dietary supplements of botanical origin in support of good manufacturing practices (GMPs) [20] that were being developed by the Food and Drug Administration. These SRM suites have been planned to include a dried and powdered plant material, an extract derived from the plant material, and one or more finished products, i.e., mixtures of commercially available dietary supplements. These SRMs are not to be considered archetypes for product formulation, but they should provide sample matrices that are representative of the analytical challenges that may be encountered by analysts in all segments of the dietary supplement industry. Subparts E and J of the GMPs require manufacturers to establish specifications for identity, purity, strength, and composition; to set limits on contaminants and adulterants for every ingredient in their products; and to analyze both "materials in process" and finished products to determine whether specifications are met [20]. The validity of analytical methods must be verified and then routinely used for monitoring against the manufacturer's specifications. Thus, the SRMs can be used both to demonstrate that a method is appropriate for its intended use and as a quality control material when demonstrating that specifications are met.

Three bitter-orange-containing SRMs have been developed that represent natural, extracted, and processed samples, i.e., SRM 3258 Bitter Orange (Fruit), SRM 3259 Bitter Orange Extract, and SRM 3260 Bitter Orange-Containing Solid Oral Dosage Form. Value assignment of the alkaloid content was approached through the use of multiple analytical methods with measurements at NIST and by collaborating laboratories. In addition to the bitter orange alkaloids, caffeine was certified in SRM 3260 and this material was screened for ephedrine alkaloids. All three materials were screened for pesticide residues and toxic heavy metals. This paper details the analytical approach to the measurement and value assignment of these analytes in the three bitter-orange-containing SRMs.

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

SRM preparation

Immature bitter orange fruit powder used in the preparation of SRM 3258 was procured from Sinochem Ningbo (Ningbo, China) by Modern Nutrition and Biotech (Appleton, WI, USA) and was received by NIST directly from the manufacturer in China. The powder was passed through a 250-μm (60-mesh) sieve by the manufacturer, and was used as received. SRM 3259 was procured from the same source. This material is an alcoholic extract of immature bitter orange fruit that is spray-dried to produce a powder with a nominal synephrine composition of 6% (mass fraction).The extract was passed through a 180-μm (80-mesh) sieve by the manufacturer. SRM 3260 was prepared by grinding, sieving (180 μm, 80 mesh), and blending tablets and the contents of capsules from three commercial dietary supplements. The products used in the formulation of SRM 3260 were processed separately and combined prior to blending. Aliquots of the three SRMs (5 g for SRM 3258, 1.2 g for SRM 3259, and 2.5 g for SRM 3260) were heat-sealed inside nitrogen-flushed 100-μm (4-mil) polyethylene bags, which were then sealed inside nitrogen-flushed aluminized plastic bags along with two packets of silica gel. To prevent mold growth during long-term storage, SRMs 3258, 3259, and 3260 were γ-irradiated in a single lot by Neutron Products (Dickerson, MD, USA) with an absorbed dose of 7.4–9.0 kGy. Prior to irradiation of the SRMs, the potential influence of γ irradiation on chemical composition was evaluated for absorbed doses of 1, 5, and 10 kGy. No discernable differences were apparent in the separations performed on preirradiated and postirradiated samples, and an absorbed dose target range of 7–10 kGy was specified for the SRM production lots. The moisture content of the three SRMs was determined from an average of freeze-drying, oven-drying, and desiccator-drying studies. SRM 3258 was determined to contain 3.79% water (mass fraction), SRM 3259 was determined to contain 1.52% water (mass fraction), and SRM 3260 was determined to contain 3.07% water. Correction factors were applied for the moisture content of the SRMs so that constituent levels could be reported on a dry-mass basis. The uncertainty in the moisture correction factors (0.2–0.6% relative standard deviation, RSD) is included in the expanded uncertainty of each assigned value.

Reagents

Synephrine, octopamine hydrochloride, tyramine, N-methyltyramine, and hordenine sulfate reference standards were obtained from ChromaDex (Santa Ana, CA, USA). Purity of the reference standards was determined from a consensus of measurements which included LC with UV detection at 220 nm and fluorescence detection at 304 nm with excitation at 273 nm, LC with atmospheric pressure

positive ion electrospray MS detection, LC evaporative light scattering detection, differential scanning calorimetry, and manufacturer data. MS and proton nuclear magnetic resonance spectroscopy documentation was supplied by the manufacturer. The internal standards terbutaline hemisulfate, and β-hydroxyethyltheophylline were obtained from Sigma (St. Louis, MO, USA) and caffeine-$^{13}C_3$ was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). SDS was obtained from Polyscience (Warrington, PA, USA). All solvents used for the preparation of mobile phases were high-performance LC grade.

Analytical methods

Method 1: LC/UV (NIST)

Details of the LC/UV method development have been reported separately [15]; however, information relevant to the SRM certification is briefly summarized. Duplicate aliquots (approximately 0.5 g) from each of six SRM units were analyzed for each of the three SRMs. Samples were weighed into 50-mL polyethylene centrifuge tubes, the internal standard terbutaline was added, and a 20-mL aliquot of the extraction solvent (0.37% mass fraction hydrochloric acid in water) was added. Samples were extracted by sonication in an ultrasonic bath. Extraction conditions were studied in detail and the following conditions were employed. For SRM 3258, two 60-min extraction cycles were required for quantitative recovery. SRM 3259 readily dissolved in the extraction solvent, and a single 30-min sonication cycle was used. Two 30-min sonication extraction cycles were used with SRM 3260. Extracts were centrifuged, and an aliquot was filtered prior to analysis using a 0.45-μm polytetrafluoroethylene syringe filter. Analyses were carried out with a reversed-phase ion-pair method. The mobile-phase composition was 72% (volume fraction) of a 10 mmol/L SDS solution adjusted to pH 2.5 using 5% (mass fraction) phosphoric acid and 28% (volume fraction) acetonitrile. An ACE 5 RP C_{18} column (250 mm × 4.6 mm, 5-μm particle size, from Advanced Chromatography Technologies, Aberdeen, UK) was used with a Phenomenex C_{18} Security Guard column (Torrance, CA, USA). The column temperature was maintained at 25 °C using a column jacket and circulating fluid bath. The mobile-phase flow rate was 1 mL/min, and the injection volume was 5 μL. Absorbance detection was at 220 nm.

Method 2: LC/fluorescence detection (NIST)

The details are identical to those of method 1, except that fluorescence detection at 304 nm was used with excitation at 273 nm.

Method 3: LC/MS (NIST)

Details of LC/MS method development have been published separately [17]. Aspects of sample processing are summarized as follows. Duplicate aliquots (approximately 0.25 g for SRM 3258 and SRM 3260; approximately 0.05 g for SRM 3259) from each of six SRM units were analyzed. Terbutaline was used as an internal standard. Pressurized fluid extraction (PFE) and sonication extraction approaches were compared. Quantitative recovery was not achieved using PFE to extract SRM 3258, and therefore a sonication extraction approach similar to method 1 was used. Samples of SRM 3258 were extracted with 20-mL portions of 0.37% (mass fraction) hydrochloric acid in an ultrasonic bath (model 5510, Branson, Danbury, CT, USA) over a period of 60 min. The supernatant solution was decanted, and the process repeated twice. SRMs 3259 and 3260 were extracted by PFE using 0.37% (mass fraction) hydrochloric acid and two extraction cycles. The resulting extracts were further diluted with water such that similar alkaloid levels resulted for the three SRMs. The LC separation method utilized a pentafluorophenyl column (Discovery HSF5, 4.6 mm × 250 mm, 5-μm particle diameter; Supelco, Bellefonte, PA, USA) and an acetonitrile/aqueous ammonium acetate (10 mmol/L) 90:10 (volume fraction) mobile phase at 1 mL/min. The column temperature was maintained at 35 °C, and the injection volume was 5 μL. An Agilent Technologies (Santa Clara, CA, USA) 1100 series liquid chromatograph with an SL series MS detector and an electrospray ionization source was operated in the positive ion mode, and $[M + H]^+$ ions were monitored, i.e., the ions monitored (*m/z*) were 168 synephrine, 138 tyramine, 166 hordenine, and 152 N-methyltyramine; for octopamine the more abundant *m/z* 136 ion was monitored.

Method 4: LC/MS/MS (NIST)

Details of the LC/MS/MS method development are reported separately [18]. Duplicate aliquots (approximately 50 mg) were analyzed from six samples for each of the three SRM materials. Terbutaline was used as an internal standard. Samples were extracted by sonication with 1% (mass fraction) aqueous hydrochloric acid at ambient temperature. SRM 3258 was extracted for 60 min with two extraction cycles, SRM 3259 was extracted for 30 min with one extraction cycle, and SRM 3260 was extracted for 30 min with two extraction cycles. Separations were carried out using a pentafluorophenyl column (Discovery HSF5, 4.6 mm × 150 mm, 5-μm particle diameter) and a methanol/aqueous ammonium acetate (10 mmol/L) 90:10 (volume fraction) mobile phase at 0.5 mL/min. The injection volume was 2 μL. A Q-Trap MS/MS system (Applied Biosystems, Foster City, CA, USA) was operated

in the positive electrospray-ionization mode. Multiple reaction monitoring was performed using $[M + H]^+$ protonated analyte molecules. Product ions were selected on the basis of the formation of tropylium ions (N-methyltyramine, octopamine, and hordenine), the combined loss of water and ammonia (tyramine), or the combined loss of water and a methyl group (synephrine). Samples were diluted by a factor of 10 or more to minimize ion self-suppression effects.

Method 5: LC/UV detection (National Center for Toxicological Research)

Six samples of SRMs 3258, 3259, and 3260 were analyzed using an ion-pair method [21]. A 4.6 mm × 250 mm Phenomenex C_{18} column was used with gradient elution conditions. The initial composition was 17% acetonitrile and 83% 30 mmol/L SDS (volume fraction), pH 2.5 (mobile phase A). The composition was stepped at 2.5 min to 43% acetonitrile and 57% 30 mmol/L SDS (volume fraction), pH 2.5 (mobile phase B) and held to 26 min. The column was re-equilibrated to mobile phase A for 8 min prior to injection. The flow rate was 1.1 mL/min. Detection was by UV absorbance at 222 nm, and calibration was performed by an external standardization approach.

Method 6: LC/UV detection (ChromaDex)

Six samples of SRMs 3258, 3259, and 3260 were analyzed using an ion-pair method that has been validated through a single laboratory validation protocol [22]. Aliquot sizes were 300 mg (SRMs 3258 and 3260) or 100 mg (SRM 3259). Samples were sonicated with 50 mL of 0.1% (volume fraction) phosphoric acid for 60 min (SRM 3258) or 15 min (SRMs 3259 and 3260). Analyses were carried out using a Phenomenex $C_{18}(2)$ (3.0 mm × 150 mm, 5-μm particle diameter) column at 35 °C. Gradient elution conditions were employed: the mobile-phase composition was programmed from 100% solvent A to 100% solvent B over 30 min. Solvent A consisted of 20 mmol/L borate buffer pH 8.2 with 10 mmol/L hexanesulfonic acid; solvent B consisted of 80:20 (volume fraction) 20 mmol/L borate buffer pH 8.2/acetonitrile with 10 mmol/L hexanesulfonic acid. The flow rate was 0.85 mL/min and absorbance detection was at 224 nm.

Method 7: caffeine analysis by LC/MS (NIST)

Caffeine was determined in duplicate aliquots (approximately 60–80 mg) from six samples for SRM 3260 by LC/MS. Samples were extracted by sonication for 60 min using a solution of 60% water and 40% methanol (volume fraction) that contained the internal standard caffeine-$^{13}C_3$. Portions (approximately 3 mL) of the extracts were filtered through a 0.45-μm pore polypropylene centrifuge filter for analysis. An Agilent Technologies 1100 series liquid chromatograph with an SL series MS detector and an electrospray ionization source was used to determine caffeine. A Phenomenex Luna $C_{18}(2)$ column with 5-μm particle size and dimensions of 3.0-mm internal diameter × 25 cm was used under isocratic conditions, and 1-μL injections were made. The mobile phase consisted of 29% methanol, 69% water, and 2% acetic acid (volume fractions) and was delivered at a flow rate of 0.4 mL/min. Between analyses, the column was flushed with 98% methanol and 2% acetic acid to elute extraneous retained compounds. MS detection was carried out using electrospray ionization in positive polarity with selected ion monitoring at $[M + H]^+$ for the caffeine-$^{13}C_3$ (*m/z* 198) and caffeine (*m/z* 195). SRM 3243 Ephedra-Containing Solid Oral Dosage Form was analyzed as a quality control sample.

Method 8: caffeine analysis by LC/UV detection (NIST)

Caffeine was determined by RPLC with absorbance detection [23]. Two test portions (approximately 120–200 mg) from each of six samples of SRM 3260 were weighed and diluted with approximately 10 g of methanol (which contained the internal standard, β-hydroxyethyltheophylline, at about 1 mg/g). Each test portion was sonicated for 30 min, centrifuged, and filtered through a 0.45-μm nylon filter prior to LC analysis. A Zorbax Rx-C_{18} column (4.6 mm × 250 mm, 5-μm particle size, from Agilent Technologies) was used with a mobile-phase composition of 10% acetonitrile/90% water (containing 0.5% acetic acid, volume fractions), a flow rate of 1 mL/min, and a temperature of 25 °C. Absorbance detection was at 274 nm, and the sample injection volume was 10 μL. SRM 3243 was analyzed as a quality control sample.

Method 9: pesticide analysis (NIST)

In addition to the assignment of mass fractions for the alkaloids and caffeine, SRMs 3258, 3259, and 3260 were screened for pesticide residues by gas chromatography/MS (GC/MS). Samples of 1–2 g were Soxhlet-extracted for approximately 16 h using methylene chloride. The samples were analyzed by GC/MS using a 0.25 mm × 60 m DB-5 fused-silica capillary column (Agilent Technologies), 0.25-μm film thickness. The column was held isothermally at 60 °C for 1 min, temperature-programmed at 40 °C/min to 150 °C for 5 min, and then temperature-programmed at 2 °C/min to 280 °C for 30 min. The injection port was maintained in the oven track mode (3 °C above the oven temperature), and the transfer line was maintained at 280 °C.

All injections were on-column (1 μL) with helium as a carrier gas at a constant flow rate of 1.2 mL/min. The mass spectrometer was operated in the scan mode (from 70 to 450 amu).

Method 10: ephedrine alkaloid analysis by LC/MS (NIST)

SRM 3260 was screened for ephedrine alkaloids using the method previously published for the certification of the suite of ephedra-containing SRMs 3240, 3241, 3242, 3243, and 3244 [3]. In brief, a 0.5-g sample of SRM 3260 was extracted by sonication with approximately 30 mL methanol for 90 min, filtered through a 0.45-μm nylon syringe filter, and analyzed without further processing. The LC method used a 4.6 mm × 250 mm, 5-μm particle size YMC phenyl column (Waters) operated at 25 °C. The mobile phase consisted of 96% water, 2% methanol, and 2% glacial acetic acid (mass fractions), and a flow rate of 0.7 mL/min was used. Positive ion electrospray/MS was used for detection of the alkaloids (atmospheric pressure positive ion electrospray). The separation was monitored in the selected ion monitoring mode at the following ions: synephrine *m/z* 150 and 168, (-)-norephedrine and (+)-norpseudoephedrine *m/z* 152, (-)-ephedrine and (+)-pseudoephedrine *m/z* 166, and (-)-methylephedrine *m/z* 180.

Method 11: toxic element screening by inductively coupled plasma MS (NIST)

SRMs 3258, 3259, and 3260 were screened for toxic elements (arsenic, cadmium, mercury, and lead) by using inductively coupled plasma MS (ICP-MS) [24]. Two subsamples of each SRM were weighed by difference into Teflon digestion vessels and digested using nitric and hydrofluoric acids. Digestion was achieved by processing samples in a MarsXpress microwave oven (CEM, Matthews, NC, USA) operated at 1,200 W, with an initial 15-min ramp to a sample temperature of 205 °C, and subsequent hold for 30 min. Following this treatment, the samples appeared as clear, green-blue solutions. Subsample masses were 0.5 g for SRMs 3258 and 3260 and 0.3 g for SRM 3259. Samples were analyzed using a VG PlasmaQuad 3 quadrupole ICP-MS instrument (ThermoElemental; now ThermoElectron, Madison, WI, USA) operated at 1,350 W using standard gas flows. Solution was introduced via a peristaltic pump at a flow rate of 0.1 mL/min into a water-cooled (4 °C) low-volume cyclonic spray chamber. Arsenic mass 75, cadmium masses 111, 112, and 114, indium (internal standard) mass 115, and lead masses 206, 207, and 208 were monitored. Concentrations were calculated using an external calibration curve generated from standards containing 2 and 10 ng/g of each element.

Results and discussion

The development and certification of the bitter-orange-containing SRM suite is similar to that of the ephedra-containing SRMs 3240, 3241, 3242, 3243, and 3244. With the change in dietary supplement weight-loss products to ephedra-free formulations containing bitter orange, new reference materials are required to support analytical measurements. These complex-matrix SRMs are intended for use primarily as control materials and for the development and validation of new analytical methods.

In general, complex-matrix reference materials are not recommended for use in instrument calibration since the analytes may be represented at only a single concentration, and the associated uncertainties are typically larger than for solution calibrants. As with other complex-matrix reference materials produced by NIST, value assignment of the bitter orange SRMs was approached through the use of multiple independent analytical methods with measurements performed at NIST and by collaborating laboratories. A discussion of the "modes" used in the certification of reference materials has been published [19]; in general, more stringent requirements are imposed on certified values compared with reference values or information values. Data from the different methods and sources are evaluated statistically and combined to yield a consensus value and expanded uncertainty interval.

In the current study, as many as six data sets were utilized in value assignment of the bitter orange alkaloids (Fig. 2). The corresponding methods are substantially independent; at NIST at least two extraction approaches, two chromatographic separation methods, and four modes of detection were used. Data were also provided from the National Center for Toxicological Research (NCTR) and ChromaDex. These laboratories used similar methods with sonication extraction of the samples, ion-pair chromatography, and UV absorbance detection. Both methods were based on external standard calibration; all of the methods used by NIST were based on the use of internal standards. Measurements for caffeine were carried out at NIST with two independent methods that utilized internal standards and SRM 3243 control samples.

Extraction studies were carried out to develop approaches for quantitative recovery of the bitter orange alkaloids from the various types of samples. Three extraction methods were studied: sonication extraction, PFE, and Soxhlet extraction. Different solvents and pH conditions were employed and the influence of temperature was investigated. Several trends were apparent from these studies. The three sample types (fruit, extract, and solid oral dosage forms) exhibited significantly different extraction challenges. SRM 3259 was much easier to extract than either the botanical or solid oral dosage form materials, and

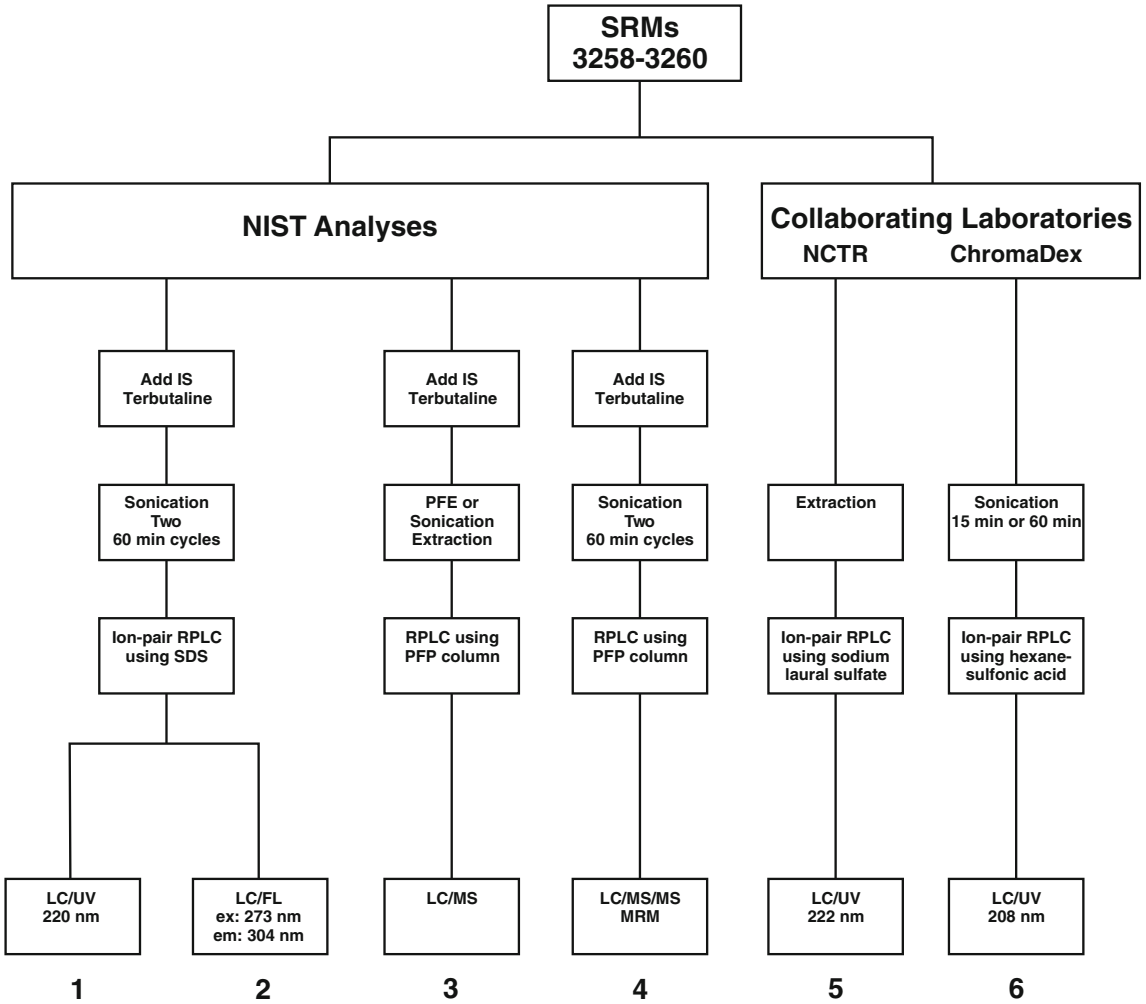

Fig. 2 Analytical approaches used in the determination of bitter orange alkaloids in Standard Reference Materials (SRMs) 3258 Bitter Orange Fruit, 3259 Bitter Orange Extract, and 3260 Bitter Orange-Containing Solid Oral Dosage Form. NIST National Institute of Standards and Technology, NCTR National Center for Toxicological Research, IS internal standard, PFE pressurized fluid extraction, RPLC reversed-phase liquid chromatography, SDS sodium dodecyl sulfate, PFP pentafluorophenyl, LC liquid chromatography, FL fluorescence, MS mass spectrometry, MRM multiple reaction monitoring

in fact SRM 3259 appeared to dissolve fully in methanol and acidic water. In contrast, quantitative recovery of the alkaloids was difficult for the botanical matrix (SRM 3258). Multiple solvent contacts and extended contact times were required to recover all of the alkaloids such that the compounds were not detected in subsequent extraction cycles. It is hypothesized that the cell structure of the plant contributes to the difficulty in extracting the alkaloids. SRM 3259 is a spray-dried residue of a plant extract, and as such dissolution is not impeded by morphological features of the plant. SRM 3260 was prepared from commercial dietary supplements, and the details of manufacture are not known; however, it is common to formulate dietary supplements with spray-dried extracts rather than dried plant material. Extraction of the finished product sample matrix was of intermediate difficulty.

In general, higher levels of the alkaloids were extracted with longer solvent contact times, particularly for SRM 3258. Multiple extraction cycles were also useful in maximizing analyte recovery. Elevated extraction temperature did not always increase the levels of the alkaloids recovered; it is possible that losses may result from degradation of the analytes due to oxidation. Lower levels of octopamine and tyramine (both primary amines) were noted at elevated extraction temperature. The addition of hydrochloric acid appeared to facilitate extraction of analytes from SRM 3258. The acid may help to degrade cellular structures and release contents into solution.

As a result of these studies, sonication extraction with hydrochloric acid was determined to be the preferred extraction approach. Soxhlet extraction worked well for synephrine, but the more aggressive conditions reduced levels of octopamine and tyramine. PFE did not provide quantitative recovery for SRM 3258, although this approach was applicable to the commercial extract and finished product samples. Thus, independent extraction

techniques (sonication extraction and PFE) were utilized for SRMs 3259 and 3260, but only sonication extraction was utilized in the analysis of SRM 3258 since other approaches were shown to yield biased results.

A variety of methods have been published for the determination of synephrine in botanical samples and dietary supplements. In general, these methods preclude the use of MS since nonvolatile mobile-phase additives are required for adequate retention. Ion-pair chromatography with alkyl anions (such as SDS) is one such approach. Potassium phosphate buffers may also provide a measure of retention; however, neither approach can be used with MS. Instead, methods have been developed that use absorbance detection at 210 or 220 nm. This approach is suitable for the determination of synephrine at relatively high levels, but detection of the minor alkaloids at low levels is more challenging owing to the limited sensitivity and selectivity. Niemann and Gay [11] reported the use of fluorescence detection for quantitation of synephrine. Because all of the bitter orange alkaloids exhibit native fluorescence at 304 nm with excitation at 273 nm, fluorescence detection is useful for characterization of extracts of the bitter-orange-containing dietary supplements. A comparison of absorbance and fluorescence detection (methods 1 and 2) is provided in Fig. 3 for extracts of SRM 3258. Synephrine is

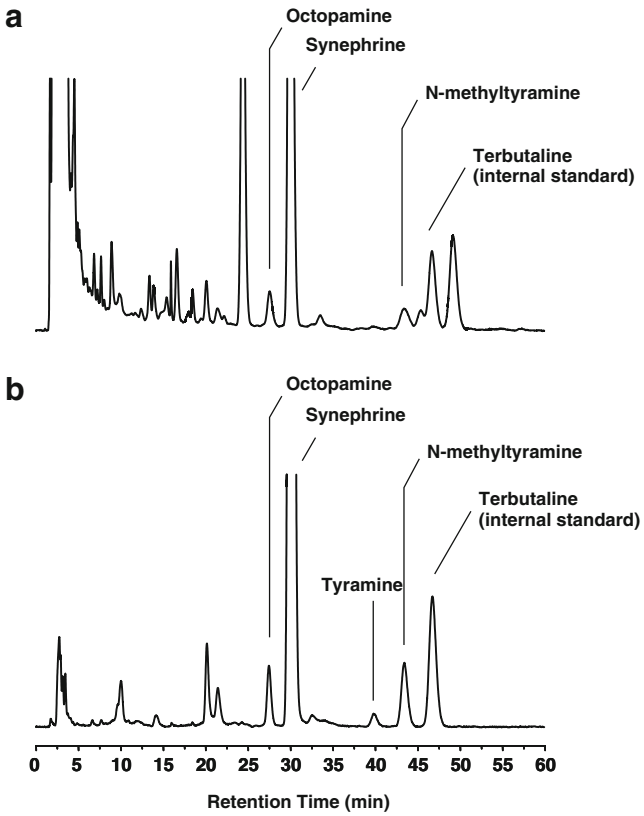

Fig. 3 Separation of SRM 3258 with **a** absorbance detection at 220 nm (method 1) and **b** fluorescence detection (excitation 273 nm, emission 304 nm; method 2)

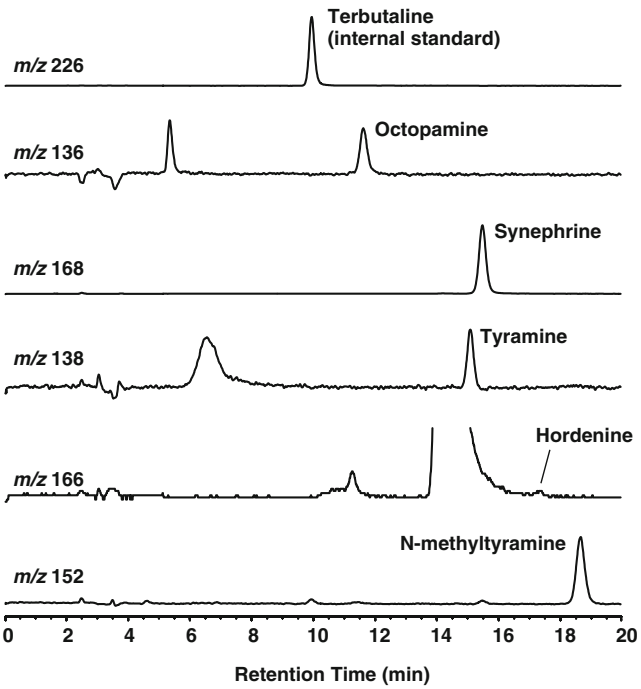

Fig. 4 Liquid chromatography (LC)/mass spectrometry (MS) selected ion chromatograms of SRM 3260

well resolved from matrix constituents for both modes of detection. Other alkaloids, including the internal standard terbutaline, are less well resolved from these interferences with absorbance detection (Fig. 3a). Absorbance detection does not provide sufficient selectivity towards the alkaloids to permit unbiased quantification. By comparison, fluorescence detection (Fig. 3b) is much more selective towards the bitter orange alkaloids. Synephrine, octopamine, tyramine, N-methyltyramine, and terbutaline are baseline-

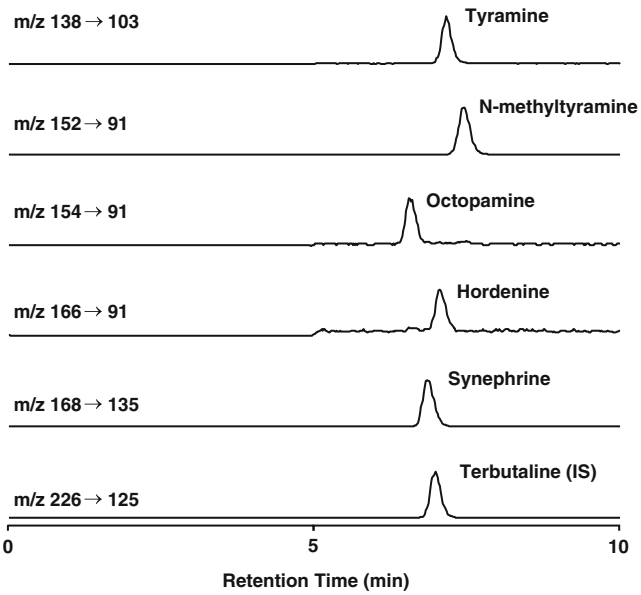

Fig. 5 Representative multiple reaction monitoring chromatograms from the LC/MS/MS analysis of SRM 3259

Table 1 Averages, standard deviations (s), and relative standard deviations (RSD) of N measurements of bitter orange alkaloids (mg/g) in Standard Reference Material (SRM) 3258 Bitter Orange (Fruit), 3259 Bitter Orange Extract, and 3260 Bitter Orange-Containing Solid Oral Dosage Form, as determined by different analytical approaches

	Method 1: NIST LC/UV				Method 2: NIST LC/FL				Method 3: NIST LC/MS				Method 4: NIST LC/MS/MS				Method 5: NCTR LC/UV				Method 6: ChromaDex LC/UV			
	Mean	s	N	RSD (%)	Mean	s	N	RSD (%)	Mean	s	N	RSD (%)	Mean	s	N	RSD (%/	Mean	s	N	RSD (%)	Mean	s	N	RSD (%)
SRM 3258																								
Synephrine	9.24	0.17	12	1.9	9.14	0.07	12	0.7	8.95	0.13	12	1.4	9.13	0.16	11	1.7	9.22	0.25	6	2.7	8.94	0.04	6	0.4
N-Methyltyramine					0.182	0.002	12	1.2	0.169	0.005	12	2.9	0.18	0.02	11	10.0								
Octopamine					0.135	0.002	12	1.1	0.13	0.01	12	6.3	0.11	0.02	11	18.7	0.38	0.01	6	2.8				
Tyramine					0.018	0.001	12	8.1	0.027	0.002	12	7.0	0.049	0.007	11	14.6	0.035	0.002	6	6.5				
Hordenine													0.012	0.002	11	20.4								
Total citrus alkaloids					9.47	0.07	12	0.7	9.27	0.12	12	1.3	9.48	0.16	11	1.7								
SRM 3259																								
Synephrine	74.0	2.1	12	2.8	71.0	0.5	12	0.8	69.4	1.4	12	2.1	72.2	1.0	12	1.3	74.9	1.7	6	2.3	70.1	0.3	6	0.5
N-Methyltyramine	5.88	0.13	12	2.1	5.28	0.05	12	0.9	5.34	0.18	12	3.4	4.39	0.11	12	2.6					5.29	0.03	6	0.6
Octopamine	1.00	0.07	12	7.4	0.85	0.01	12	1.3	0.82	0.03	12	3.9	0.76	0.03	12	3.9	2.19	0.06	6	2.8	1.02	0.03	6	2.8
Tyramine	0.83	0.11	12	13.5	0.76	0.02	12	2.6	0.87	0.05	12	5.4	0.76	0.03	12	3.4	0.90	0.03	6	3.1	0.90	0.02	6	2.5
Hordenine													0.018	0.002	12	8.5								
Total citrus alkaloids	81.7	2.1	12	2.5	77.9	0.6	12	0.7	76.5	1.6	12	2.1	78.2	1.0	12	1.3								
SRM 3260																								
Synephrine	18.4	0.5	12	2.6	18.4	0.1	12	0.6	18.5	0.4	12	2.0	18.6	0.3	12	1.5	17.3	0.2	6	1.0	18.0	0.4	6	2.1
N-Methyltyramine					0.78	0.01	12	0.9	0.84	0.02	12	2.9	0.57	0.01	12	2.0					0.83	0.04	6	4.3
Octopamine					0.17	0.01	12	4.9	0.17	0.01	12	5.0	0.14	0.01	12	3.9	0.61	0.01	6	1.9	0.48	0.01	6	2.4
Tyramine	0.17	0.02	12	13.0%	0.174	0.003	12	1.7	0.21	0.01	12	4.0	0.18	0.01	12	3.2								
Hordenine													0.0049	0.0003	12	6.0								
Total citrus alkaloids					19.5	0.1	12	0.5	19.7	0.4	12	2.0	19.5	0.3	12	1.5								

Values are reported on a dry-mass basis

NIST National Institute of Standards and Technology, LC liquid chromatography, UV ultraviolet, FL fluorescence, MS mass spectrometry, NCTR National Center for Toxicological Research

resolved, and no indications of interferences are apparent in the separations with fluorescence detection. Although hordenine is coeluted with terbutaline, hordenine was not present at a sufficient level to permit detection by either method, and the use of terbutaline as the internal standard was justified.

The development of an LC separation based on the use of a pentafluorophenyl column in combination with volatile mobile-phase components permitted the use of MS detection (method 3). Separation of the five bitter orange alkaloids and the internal standard, terbutaline, was achieved at or near baseline resolution using an acetonitrile/water mobile phase with 10 mmol/L ammonium acetate. Tyramine and synephrine were not fully resolved; however, potential biases were minimized by monitoring appropriate mass fragments. Selected ion LC/MS chromatograms for SRM 3260 are shown in Fig. 4. The five bitter orange alkaloids were detected in the sample; however, hordenine was below the limit of quantitation. No interferences from the sample matrix were apparent in the selected ion chromatograms.

An LC/MS/MS method was also developed using the pentafluorophenyl column (method 4). To achieve an additional measure of independence from method 3, an alternative mobile-phase composition containing methanol/water and 10 mmol/L ammonium acetate was used. Methanol was used instead of acetonitrile to alter the selectivity such that potential interferences might become evident, but no such inteferences were observed. The resolution of the bitter orange alkaloids was degraded slightly by the substitution of methanol for acetonitrile, however, additional specificity was provided by the MS/MS method. An example of multiple reaction monitoring chromatograms for SRM 3259 is provided in Fig. 5.The chromatograms are highly specific for the individual compounds. The MS/MS method also provides significantly enhanced sensitivity, and hordenine was easily detected and quantified in the three bitter-orange-containing SRMs.

Data contributed by NCTR and ChromaDex were also used in the certification of the bitter orange SRM suite. Methods 5 and 6 used by these laboratories were quite similar to each other and to NIST method 1, because all are based on ion-pair chromatography with UV absorbance detection. ChromaDex and NCTR were able to measure synephrine, octopamine, and tyramine in most of the samples. Levels for N-methyltyramine were not reported in several instances, and neither laboratory reported levels for hordenine. A value for "total alkaloids" was not calculated for these data sets.

Caffeine was determined in SRM 3260 by LC/MS (method 7) and LC/UV detection (method 8). Caffeine is often added to dietary supplement weight-loss products from natural sources such as guarana or *Camellia sinensis* (green tea). Samples of SRM 3260 were extracted by two sonication approaches, with different extraction solvents and extraction times. Both methods utilized internal standards. For LC/MS method 7, caffeine-$^{13}C_3$ was used as the internal standard, which coeluted with the measurand. β-Hydroxyethyltheophylline was used as the internal standard for LC/UV method 8. Good agreement was obtained between the two methods. The level of caffeine determined by method 7 was 65.2±0.5 mg/g, and the level determined by method 8 was 63.3±0.4 mg/g (uncertainties represent one standard deviation).

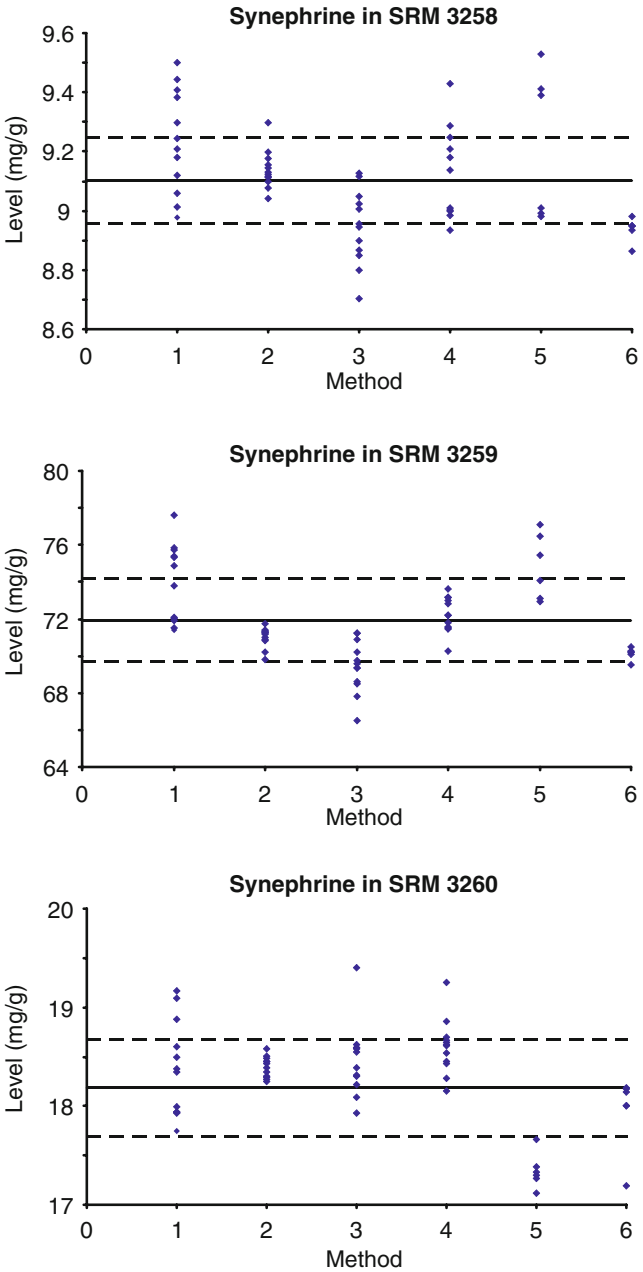

Fig. 6 Individual measurements of synephrine in SRMs 3258, 3259, and 3260, as determined by six different methods. The solid lines represent the certified values; the dashed lines represent the expanded uncertainty of the certified values

Table 2 Certified, reference, and information values for alkaloids in SRMs 3258, 3259, and 3260 (mg/g)

	SRM 3258		SRM 3259		SRM 3260	
Synephrine	9.10[a]±0.15	(2)	71.9[a]±2.3	(3)	18.19[a]±0.49	(3)
N-Methyltyramine	0.178[a]±0.012	(7)	5.23[a]±0.66	(13)	0.75[b]±0.16	(21)
Octopamine	0.124[b]±0.016	(13)	0.809[b]±0.051	(6)	0.161[b]±0.022	(13)
Tyramine	0.031[c]		0.800[a]±0.067	(8)	0.187[a]±0.022	(12)
Hordenine	0.012[c]		0.018[c]		0.0049[c]	
Total citrus alkaloids	9.41[a]±0.17	(2)	77.5[a]±1.3	(2)	19.57[a]±0.18	(1)
Caffeine					64.3[a]±1.2	(2)

[a] Each certified concentration value, expressed as a mass fraction on a dry-mass basis, is an equally weighted mean of the results from two to six analytical methods carried out at NIST and at collaborating laboratories. The uncertainty in the certified value is expressed as an expanded uncertainty (U) about the mean ($\bar{x}$), following the ISO/NIST Guide to the expression of uncertainty in measurement [26]. 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 Student's t distribution corresponding to the appropriate associated degrees of freedom and approximately 95% confidence for each analyte. Values in parentheses represent $U/\bar{x}\%$.

[b] Reference values

[c] Information values, uncertainties are not provided owing to limited data sets

The quantitative results for the alkaloids from methods 1–6 for the three SRMs are summarized in Table 1. Excellent agreement was achieved among the measurements. Measurement reproducibility varied among the samples and methods as expected. Better reproducibility usually resulted for the high-level measurands (i.e., synephrine and total alkaloids). RSDs for synephrine ranged from 0.4 to 2.8% among the different samples and methods. RSDs for low-concentration measurands such as octopamine and tyramine typically were between 5 and 10%. The best overall within-method reproducibility was obtained with method 2 based on LC/fluorescence detection. Measurement reproducibility (RSDs) for method 2 ranged from 0.5 to 1.3% for synephrine, N-methyltyramine, octopamine, and total alkaloids; RSDs for tyramine were somewhat larger. Poorer reproducibility resulted for both MS methods. LC/MS method 3 produced RSDs ranging from about 2 to 7%. Similar within-method reproducibility resulted for LC/MS/MS measurements of SRMs and 3260 (i.e., RSDs ranged from approximately 1.3 to 8.5%). Significantly poorer reproducibility resulted for LC/MS/MS measurements of SRM 3258 (RSDs ranged from 10 to 20% for the low-level alkaloids). No reason for the reduced reproducibility was apparent; however, the alkaloids in SRM 3258 have been shown to be more difficult to extract than those in the other samples, and the imprecision of method 4 may be unrelated to the instrumental analytical approach. Although the MS methods utilized an internal standard (terbutaline), this compound was eluted prior to the analytes of interest. Fluctuations in ionization efficiency are compensated more effectively with internal standards that are coeluted with the analytes, such as with isotopically labeled analyte analogues, but a labeled internal standard was only available for caffeine in this study.

An example of the excellent agreement in the individual data for the three SRMs is illustrated in Fig. 6 for synephrine (similar agreement is evident in the plots for the other analytes; see Electronic Supplementary Material Figs. S1–S6). Evidence of outliers within the data is not apparent, and all data were used in the value assignment of alkaloid levels. Certified, reference, and information values are summarized in Table 2. In the cases for which data exist from five or six methods, the values were calculated as the equally weighted mean of the means from each method. In several instances for which data were provided from two to four methods, the "type B on bias" approach was used that may be more suitable for small data sets [25]. The simple mean of measurements was used for data provided from a single method. The associated expanded uncertainties represent an approximate 95% confidence interval, with the coverage factor as determined with Student's t distribution corresponding to the appropriate associated degrees of freedom, calculated according to the ISO guide [26]. The expanded uncertainties incorporate a component for the moisture measurements. Since no evidence for sample inhomogeneity was evident in any of the SRMs, an inhomogeneity component was not included in calculation of the expanded uncertainties. The expanded uncertainties range from approximately 2 to 4% for the major constituents

Table 3 Information concentration values for toxic elements in SRMs 3258, 3259, and 3260 expressed as a mass fraction (ng/g, dry-mass basis)

	SRM 3258	SRM 3259	SRM 3260
Arsenic	160	350	140
Cadmium	10	14	16
Lead	1,500	290	240

Each information concentration value is the mean of two results provided by inductively coupled plasma mass spectrometry.

(synephrine, caffeine, and total alkaloids) in each of the bitter-orange-containing SRMs.

SRMs 3258, 3259, and 3260 were screened for pesticide residues by GC/MS (method 9). The samples did not contain quantifiable concentrations of the following chlorinated pesticides: hexachlorocyclohexanes, chlordanes, nonachlors, dieldrin, mirex, heptachlors, or DDT and associated metabolites. SRM 3260 was further screened for ephedrine alkaloids using LC/MS method 10. The presence of these compounds is not expected in dietary supplements prepared from bitter orange. However, SRM 3260 is a mixture of commercial dietary supplements and the details of manufacture were not independently verified. Ephedrine alkaloids were not detectable given the following detection limits: norephedrine 0.01 mg/g, norpseudoephedrine 0.012 mg/g, ephedrine 0.067 mg/g, pseudoephedrine 0.017 mg/g, and methylephedrine 0.028 mg/g.

The levels of toxic elements arsenic, cadmium, lead, and mercury were screened by ICP-MS (method 11). The measured results are listed in Table 3 as the average of the two subsample results. The levels of mercury were near the limit of quantitation for the solution-based method employed (10 ng/g) and are not reported. Because the levels of the four toxic elements were considered to be relatively low and of limited significance, additional measurements were not performed and the assigned values are reported as information values.

Conclusions

SRMs 3258, 3259, and 3260 are provided as tools to assist the dietary supplement community and support the chemical metrology of dietary supplements containing bitter orange. The SRMs are intended for use in the development and validation of new analytical methods and as control materials for quality assurance. It is anticipated that the use of such reference materials will improve the reliability of chemical measurements and promote product quality, consistency, and safety.

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References

1. Food and Drug Administration (2004) Final rule declaring dietary supplements containing ephedrine alkaloids adulterated because they present an unreasonable risk. 21 CFR part 119. http://www.fda.gov/OHRMS/DOCKETS/98fr/04–2912.htm
2. Mattoli L, Cangi F, Maidecchi A, Ghiara C, Tubaro M, Traldi P (2005) J Agric Food Chem 53:9860–9866
3. Sander LC, Sharpless KE, Satterfield MB, Ihara T, Phinney KW, Yen JH, Wise SA, Gay ML, Lam JW, McCooeye M, Gardner G, Fraser C, Sturgeon R, Roman M (2005) Anal Chem 77:3101–3112
4. Hashimoto K, Yasuda T, Ohsawa K (1992) J Chromatogr 623:386–389
5. Hurlbut JA, Carr JR, Singleton ER, Faul KC, Madson MR, Storey JM, Thomas TL (1998) J AOAC Int 81:1121–1127
6. Kusu F, Matsumoto K, Arai K, Takamura K (1996) Anal Biochem 235:191–194
7. Marchei E, Pichini S, Pacifici R, Pellegrini M, Zuccaro P (2006) J Pharm Biomed Anal 41:1468–1472
8. Li Q, Huang CZ, Huang YM (2006) Luminescence 21:43–48
9. Gay ML, Niemann RA, Musser SA (2006) J Agric Food Chem 54:285–291
10. Pellati F, Benvenuti S, Melegari M (2005) J Pharm Biomed Anal 37:839–849
11. Niemann RA, Gay ML (2003) J Agric Food Chem 51:5630–5638
12. Avula B, Joshi VC, Weerasooriya A, Khan IA (2005) Chromatographia 62:379–383
13. Avula B, Upparapalli SK, Navarrete A, Khan IA (2005) J AOAC Int 88:1593–1606
14. Pellati F, Benvenuti S, Melegari M (2004) Phytochem Anal 15:220–225
15. Putzbach K, Rimmer CA, Sharpless KE, Sander LC (2007) J Chromatogr A 1156:304–311
16. Bell DS, Cramer HM, Jones AD (2005) J Chromatogr A 1095:113–118
17. Putzbach K, Rimmer CA, Sharpless KE, Wise SA, Sander LC (2007) Anal Bioanal Chem 389:197–205
18. Nelson BC, Putzbach K, Sharpless KE, Sander LC (2007) J Agric Food Chem 55:9769–9775
19. May W, Parris R, Beck C, Fassett J, Greenberg R, Guenther F, Kramer G, Wise S, Gills T, Colbert J, Gettings R, MacDonald B (2000). Definitions of terms and modes used at NIST for value-assignment of reference materials for chemical measurements. NIST special publication 260–136. National Institute of Standards and Technology, US Government Printing Office, Washington
20. Food and Drug Administration (2007) Current good manufacturing practice in manufacturing, packaging, labeling, or holding operations for dietary supplements. Docket no 1996N-0417 (formerly no 96N-0417), 34752–34958. 21 CFR part 111. http://www.fda.gov/OHRMS/DOCKETS/98fr/07–3039.pdf
21. Evans RL, Siitonen PH (2008) J Chromatogr Sci 46:61–67
22. Roman MC, Betz JM, Hildreth J (2007) J AOAC Int 90:68–81
23. Thomas JB, Yen JH, Schantz MM, Porter BJ, Sharpless KE (2004) J Agric Food Chem 52:3259–3263
24. Murphy KE, Long SE, Vocke RD (2007) Anal Bioanal Chem 387:2453–2461
25. Levenson MS, Banks DL, Eberhardt KR, Gill LM, Guthrie WF, Liu HK, Vangel MG, Yen JH, Zhang NF (2000) J Res Natl Inst Stand Technol 105:571–579
26. International Organization for Standardization (1993) Guide to the expression of uncertainty in measurement. 1st edn. ISO, Geneva