National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 3261

Bitter Orange Dietary Supplement Suite

This Standard Reference Material (SRM) consists of two packets each of three bitter orange-related SRMs: SRM 3258 Bitter Orange (Fruit), SRM 3259 Bitter Orange Extract, and SRM 3260 Bitter Orange-Containing Solid Oral Dosage Form. These SRMs are intended primarily for use in validating analytical methods for the determination of citrus alkaloids in bitter orange-containing matrices. These SRMs can also be used for quality assurance when assigning values to in-house control materials. The materials in the suite of bitter orange dietary supplement SRMs have been developed to cover a range of natural matrices and analyte levels. See the Certificate of Analysis for each SRM for additional details; certificates are available at http://www.nist.gov/srm.

The development of SRM 3261 was a collaboration among the National Institute of Standards and Technology (NIST); the National Institutes of Health (NIH), Office of Dietary Supplements (ODS); and the Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER).

Certified Concentration Values: A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account [1]. The certified concentration values of citrus alkaloids and caffeine are provided in Table 1 in bold typeface. Values were derived from the combination of results provided by NIST and collaborating laboratories. The certified values in this material are the equally weighted means of the individual sets of NIST results and the means of the measurements made by collaborating laboratories where available; the associated uncertainties are expanded uncertainties at the 95 % level of confidence [2,3]. Values are reported on a dry-mass basis in mass fraction units [4].

Reference Concentration Values: A NIST reference value is a noncertified value that is the best estimate of the true value based on available data; however, the value does not meet the NIST criteria for certification [1] and is provided with associated uncertainties that may reflect only measurement reproducibility, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods. Reference concentration values for citrus alkaloids are provided in Table 1 in normal typeface.

Information Concentration Values: A NIST information value is a value that may be of interest to the SRM user, but insufficient information is available to assess the uncertainty associated with the value therefore no uncertainty is provided. Information concentration values for selected citrus alkaloids and toxic elements are provided in Tables 1 and 2, respectively.

Expiration of Value Assignment: The value assignment of this SRM is valid until **30 September 2014**, within the measurement uncertainties specified, provided the SRM is handled and stored in accordance with the instructions given in this certificate. Value assignment is nullified if the SRM is damaged, contaminated, or modified.

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

Support for the development of SRM 3261 was provided in part by NIH ODS and FDA CDER. Technical consultation from these agencies was provided by J.M. Betz (NIH ODS) and A. NguyenPho (FDA CDER).

Acquisition and preparation of the material was coordinated by A. NguyenPho of FDA CDER and K.E. Sharpless of the NIST Analytical Chemistry Division.

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Analytical measurements at NIST were performed by M. Bedner, K.E. Murphy, B.C. Nelson, B.J. Porter, K. Putzbach, M.M. Schantz, J.B. Thomas, and L.J. Wood of the NIST Analytical Chemistry Division. Analytical measurements at the FDA National Center for Toxicological Research (NCTR; Jefferson, AR) were made by P.H. Siitonen and R.L. Evans; analytical measurements at ChromaDex (Clearwater, FL) were made by M.C. Roman.

Statistical analysis was provided by J.H. Yen of the NIST Statistical Engineering Division.

The support aspects involved with the certification and issuance of this SRM were coordinated through the NIST Measurement Services Division.

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

NOTICE AND WARNING TO USERS

Storage: The material should be stored at controlled room temperature (20 °C to 25 °C), in an unopened packet, until required for use.

Warning: For laboratory use only. Not for human consumption.

INSTRUCTIONS FOR USE

Prior to removal of a test portion for analysis, the contents of a packet of material should be mixed thoroughly. For certified values to be valid, test portions of the following masses should be used: between 0.05 g and 0.5 g for citrus alkaloid analysis and between 0.06 g and 0.2 g for caffeine analysis. Toxic elements should be measured in test portions greater than or equal to 0.5 g of SRMs 3258 and 3260 and greater than or equal to 0.3 g of SRM 3259. Test portions should be analyzed as received and results converted to a dry-mass basis by determining moisture content (using one of the methods described below) on a separate test portion. The stability of alkaloids and caffeine in previously opened packets has not been investigated.

PREPARATION AND ANALYSIS[1]

Material Acquisition and Preparation: The materials for production of SRMs 3258 and 3259 were obtained from Sinochem Ningbo Ltd. (Ningbo, China) through Modern Nutrition and Biotech (Appleton, WI). While still in China, immature bitter orange fruits were ground to pass through a 250 μm (60 mesh) sieve. Approximately 35 kg of the powder and 12 kg bitter orange extract were shipped to NIST and were packaged as received. SRM 3260 was prepared from several different commercially available products (both tablets and capsules) that were purchased in the marketplace. The tablets and the contents of capsules were ground using a Teflon disc mill at room temperature, and the powdered material was then blended and sieved to 180 μm (80 mesh).

The three materials were transferred to High-Purity Solutions (Charleston, SC) where they were individually blended, aliquotted, and heat-sealed inside nitrogen-flushed 4 mil polyethylene bags, which were then sealed inside nitrogen-flushed aluminized plastic bags along with two packets of silica gel each. Following packaging, the SRMs were irradiated (Neutron Products, Inc.; Dickerson, MD) to an absorbed dose of 7.4 kGy to 9.0 kGy.

Determination of Citrus Alkaloids: Value assignment of the concentrations of the citrus alkaloids in SRM 3261 was based on the combination of measurements from four different analytical methods at NIST and two sets of data provided by collaborating laboratories. NIST provided alkaloid data by using a combination of two extraction techniques (sonication, pressurized-fluid extraction) and four liquid chromatography (LC) methods with different detection (i.e., ultraviolet absorbance [UV], fluorescence [FL], mass spectrometry [MS], and tandem mass spectrometry [MS/MS]). NCTR and ChromaDex generated data by using LC/UV [5,6].

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

NIST Analyses for Citrus Alkaloids: Citrus alkaloids were measured at NIST by using sonication extraction with LC/UV and LC/FL [7] for analysis of all three materials; sonication followed by LC/MS [8] for analysis of SRM 3258; pressurized-fluid extraction with LC/MS [8] for analysis of SRMs 3259 and 3260; and sonication extraction with LC/MS/MS [9] for the analysis of all three materials. Independently prepared calibrants were used for each of the methods. Calibrants were prepared gravimetrically, at levels intended to approximate the levels of the alkaloids in the SRM. A single internal standard solution was used for the calibrants and samples.

Sample Preparation – Sonication Method 1: Two 0.50 g (for LC/UV and LC/FL) test portions from each of six packets of the three SRMs were individually combined with an internal standard solution containing terbutaline and dilute aqueous hydrochloric acid (0.37 % mass fraction), mixed, and placed in an ultrasonicating bath for 60 minutes. The mixture was centrifuged, the supernatant was removed, and the residue was re-extracted into dilute hydrochloric acid using the same procedure. Supernatants were combined, and samples were analyzed by LC/UV and LC/FL, with the two detectors connected in series. Two 0.25 g test portions from each of six packets of SRM 3258 were prepared in the same manner and analyzed by using LC/MS.

Sample Preparation – Pressurized-Fluid Extraction (PFE): Two 0.05 g test portions from each of six packets of SRMs 3259 and 3260 were individually combined with an internal standard solution containing terbutaline and dilute aqueous hydrochloric acid (0.37 % mass fraction), mixed, and extracted by PFE using two extraction cycles. Samples were diluted with water and analyzed by using LC/MS.

Sample Preparation – Sonication Method 2: Two 50 mg test portions from each of six packets of the three SRMs were individually combined with an internal standard solution containing terbutaline and dilute aqueous hydrochloric acid (1 % mass fraction), mixed, and placed in an ultrasonicating bath for 60 minutes. The mixture was centrifuged, the supernatant was removed, and the residue was re-extracted into dilute hydrochloric acid using the same procedure. Supernatants were combined, and samples were analyzed by using LC/MS/MS.

LC/UV and LC/FL: LC/UV and LC/FL were performed with the absorbance and fluorescence detectors connected in series. Analytes were separated on a C_{18} column with a mobile phase of 72 % 10 mmol/L sodium dodecyl sulfate (pH 2.5) and 28 % acetonitrile (volume fractions). Absorbance was monitored at 220 nm. An excitation wavelength of 273 nm and an emission wavelength of 304 nm were used for fluorescence detection.

LC/MS: LC/MS was performed using a pentafluorophenylpropyl column and an isocratic mobile phase of 90 % acetonitrile and 10 % 100 mmol/L ammonium acetate in water (volume fractions). Positive ion electrospray mass spectrometry was used for detection of the alkaloids. The separation was monitored in the selected ion mode at *m/z* 226 (terbutaline), *m/z* 136 (octopamine), *m/z* 168 (synephrine), *m/z* 138 (tyramine), *m/z* 166 (hordenine), and *m/z* 152 (N-methyltyramine).

LC/MS/MS: LC/MS/MS was performed using a pentafluorophenylpropyl column and an isocratic mobile phase of 10 % 10 mmol/L ammonium acetate in water and 90 % 10 mmol/L ammonium acetate in methanol (volume fractions). Multiple reaction monitoring was performed at the following transitions (*m/z*): 138 to 103 (tyramine), 152 to 91 (N-methyltyramine) 154 to 91 (octopamine), 166 to 91 (hordenine), 168 to 135 (synephrine), and 226 to 125 (terbutaline).

Determination of Caffeine: Value assignment of the concentrations of caffeine in SRM 3260 was based on the combination of measurements from sonication extraction followed by two different analytical methods at NIST: LC/UV and LC/MS.

NIST Analyses for Caffeine: Caffeine was measured at NIST by using sonication extraction with LC/UV [10] and sonication extraction with LC/MS. Independently prepared calibrants were used for each of the methods. Calibrants were prepared gravimetrically, at levels intended to approximate the levels of caffeine in the SRM. A single internal standard solution was used for the calibrants and samples.

Sample Preparation – Sonication Method 3: Two 0.12 g to 0.20 g test portions from each of six packets of SRM 3260 were individually combined with an internal standard solution containing β-hydroxyethyltheophylline (internal standard) in methanol and placed in an ultrasonicating bath for 30 minutes. The mixture was centrifuged, and the supernatant was removed, filtered, and analyzed by LC/UV.

Sample Preparation – Sonication Method 4: Two 0.06 g to 0.08 g test portions from each of six packets of SRM 3260 were individually combined with an internal standard solution containing trimethyl–$^{13}C_3$ caffeine

(internal standard) in methanol and placed in an ultrasonicating bath for 60 minutes. The mixture was centrifuge-filtered, and the supernatant was removed, filtered, and analyzed by LC/MS.

LC/UV: LC/UV was performed using a C_{18} column with a mobile phase of 10 % acetonitrile and 90 % water containing 0.5 % acetic acid (volume fractions). Absorbance was monitored at 274 nm.

LC/MS: LC/MS was performed using a C_{18} column and an isocratic mobile phase of 29 % methanol, 69 % water, and 2 % acetic acid (volume fractions). Electrospray ionization mass spectrometry was used for detection of caffeine. The separation was monitored in the selected ion mode at *m/z* 198 for the labeled caffeine and *m/z* 195 for caffeine.

NIST Analyses for Toxic Elements: SRMs 3258, 3259, and 3260 were screened for toxic elements (arsenic, cadmium, and lead) by using inductively coupled plasma mass spectrometry (ICP-MS) following microwave digestion using nitric and hydrofluoric acids. Arsenic mass 75; cadmium masses 111, 112, and 114; indium (internal standard) mass 115; and lead masses 206, 207, and 208 were monitored.

NIST Analyses for Pesticides: SRMs 3258, 3259, and 3260 were screened for pesticide residues by using gas chromatography (GC)/MS following Soxhlet extraction into methylene chloride; a 2 g sample did not contain quantifiable concentrations of hexachlorocyclohexanes (HCHs), chlordanes, nonachlors, dieldrin, mirex, heptachlors, DDT, or metabolites of DDT.

Determination of Moisture: Moisture content of SRM 3261 was determined at NIST (see "Instructions for Use") by (1) freeze-drying to constant mass over 8 days; (2) drying over magnesium perchlorate in a desiccator at room temperature for 35 days (SRMs 3258 and 3260) or 28 days (SRM 3259); and (3) drying for 2 h in a forced-air oven at 80 °C. Unweighted results obtained using all three techniques were averaged to determine a conversion factor of (0.9621 ± 0.0140) gram dry mass per gram as-received mass for SRM 3258, (0.9848 ± 0.0111) gram dry mass per gram as-received mass for SRM 3259, and (0.9693 ± 0.0052) gram dry mass per gram as-received mass for SRM 3260, which were used to convert data from an as-received to a dry-mass basis the uncertainty shown on this value is an expanded uncertainty. An uncertainty component for the conversion factor (0.34 % for SRM 3258, 0.26 % for SRM 3259, and 0.12 % for SRM 3260) obtained from the moisture measurements is incorporated in the uncertainties of the certified and reference values, reported on a dry-mass basis, that are provided in this certificate.

Homogeneity Assessment: The homogeneity of alkaloids was assessed at NIST by using the methods described above. An analysis of variance did not show inhomogeneity for citrus alkaloids in the 0.05 g and 0.5 g test portions of SRMs 3258, 3259, and 3260 analyzed, nor did it show inhomogeneity for caffeine in the 0.06 g to 2.0 g test portions of SRM 3260 analyzed.

Value Assignment: The equally weighted means from appropriate sets of data were used to calculate the assigned values.

Table 1. Certified (Bold), Reference (Normal Typeface), and Information (Normal Typeface, No Uncertainties Provided) Concentration Values for Alkaloids in SRM 3261 Reported on a Dry-Mass Basis [a]

	SRM 3258 Mass Fraction (mg/g)	SRM 3259 Mass Fraction (mg/g)	SRM 3260 Mass Fraction (mg/g)
Synephrine[b,c,d,e,f,g]	**9.10 ± 0.15**	**71.9 ± 2.3**	**18.19 ± 0.49**
Octopamine[c,d,e]	0.124 ± 0.016	0.809 ± 0.051	0.161 ± 0.022
Tyramine[c,d,e]	0.031	**0.800 ± 0.067**	**0.187 ± 0.022**
Hordenine[e]	0.012	0.018	0.0049
N-methyltyramine	**0.178 ± 0.012**[c,d,e]	**5.23 ± 0.66**[b,c,d,e,f]	0.75 ± 0.16 [c,d,e]
Total Citrus Alkaloids[c,d,e]	**9.41 ± 0.17**	**77.5 ± 1.3**	**19.57 ± 0.18**
Caffeine[b,d]			**64.3 ± 1.2**

[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 and NIST Guides [2,3], 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 and NIST Guides [2,3], 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/FL
[d] NIST LC/MS
[e] NIST LC/MS/MS
[f] FDA NCTR LC/UV
[g] ChromaDex LC/UV

Table 2. Information Concentration Values for Toxic Elements in SRM 3261 Reported on a Dry-Mass Basis [a]

	SRM 3258 Mass Fraction (ng/g)	SRM 3259 Mass Fraction (ng/g)	SRM 3260 Mass Fraction (ng/g)
Arsenic	160	350	140
Cadmium	10	14	16
Lead	1500	290	240

[a] Each information concentration value, expressed as a mass fraction, is the mean of two results provided by ICP-MS.

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Users of this SRM should ensure that the certificate in their possession is current. This can be accomplished by contacting the SRM Program at: telephone (301) 975-6776; fax (301) 926-4751; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.