National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 3251

Saw Palmetto (*Serenoa repens*) Extract

This Standard Reference Material (SRM) is intended primarily for use in validating analytical methods for the determination of phytosterols, β-carotene, and γ-tocopherol in extracts of *Serenoa repens* (saw palmetto) and similar matrices. This SRM can also be used for quality assurance when assigning values to in-house control materials. A unit of SRM 3251 consists of five ampoules, each containing approximately 1 mL of saw palmetto extract.

The development of SRM 3251 was a collaboration among the National Institute of Standards and Technology (NIST), the National Institutes of Health, Office of Dietary Supplements (NIH-ODS), and the Food and Drug Administration, Center for Drug Evaluation and Research (FDA CDER).

Certified Mass Fraction 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 mass fraction values for phytosterols, total β-carotene, and γ-tocopherol are provided in Tables 1 and 2. Analyses for value assignment for phytosterols were derived from the combination of results provided by NIST using two independent methods and by an AOAC collaborative study. Analyses for value assignment for β-carotene, and γ-tocopherol were derived from the combination of results provided by NIST using two independent methods. The certified values in this material are the equally weighted means of the individual sets of results; the associated uncertainties are expanded uncertainties at the 95 % level of confidence [2,3]. Values are reported on an as-received basis in mass fraction units [4].

Reference Mass Fraction Values: Reference mass fraction values are non-certified values that are the best estimate of the true values based on available data; however, the values do not meet the NIST criteria for certification [1] and are provided with associated uncertainties that may reflect only measurement reproducibility, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods. Reference mass fraction values for cycloartenol, β-carotene isomers, and δ-tocopherol are provided in Tables 3 and 4. Values are reported on an as-received basis in mass fraction units [4].

Expiration of Value Assignment: The certification of **SRM 3251** is valid, within the measurement uncertainty specified, until **30 November 2029**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (See "Instructions for Storage and Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

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

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

Support for the development of SRM 3251 was provided in part by NIH-ODS. Acquisition of the material was coordinated by A. Nguyenpho of FDA CDER and K.E. Sharpless of the NIST Office of Special Programs.

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Certificate Issue Date: 14 December 2020
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Analytical measurements at NIST were performed by M. Bedner, K. Putzbach, C.A. Rimmer, and J.B. Thomas of the NIST Chemical Sciences Division and M.M. Schantz and T. Yarita, formerly of NIST. The AOAC collaborative study that generated phytosterol data was directed by W. Sorenson (Covance Laboratories; Madison, WI).

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

Support aspects involved with the issuance of this SRM were coordinated through the NIST Office of Reference Materials.

NOTICE AND WARNING TO USERS

SRM 3251 IS INTENDED FOR RESEARCH USE; NOT FOR HUMAN CONSUMPTION.

INSTRUCTIONS FOR STORAGE AND USE

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

Use: Prior to removal of a test portion for analysis, the contents of an ampoule should be mixed by inverting the ampoule several times. For certified values to be valid, test portions of the extract greater than or equal to 125 mg for phytosterol analysis, 100 mg for β-carotene analysis, and 150 mg for tocopherol analysis should be used. The stability of analytes in previously opened ampoules has not been assessed.

PREPARATION AND ANALYSIS[(1)]

Material Acquisition and Preparation: The material for production of SRM 3251 is a carbon dioxide extract of saw palmetto "berries" and was ampouled as received. Two-milliliter amber ampoules were flushed with argon and filled with approximately 1 mL of extract.

Analytical Approach for Determination of Phytosterols: Value assignment of the mass fractions of the phytosterols in SRM 3251 was based on the combination of measurements from two different analytical methods at NIST. In addition to NIST data, phytosterol data from an AOAC collaborative study using gas chromatography-flame ionization detection (GC-FID) were used for value assignment. Ten participating laboratories reported values for campesterol, stigmasterol, and β-sitosterol in duplicate blind analyses using the method reported in reference 5; results were reported in reference 6.

NIST ANALYSES FOR PHYTOSTEROLS

Phytosterols were measured by using GC with FID and by using liquid chromatography (LC) with mass spectrometry (MS) detection. Four independently prepared calibrants were used for each of the methods. Calibrants were prepared gravimetrically at levels intended to approximate the levels of the phytosterols in the SRM. A single internal standard solution was used for the calibrants and samples.

Sample Preparation Method 1: In an adaptation of the method reported in reference 5, single 0.6 g test portions from each of eight ampoules were individually combined with an internal standard solution containing cholesterol and were saponified using an ethanolic potassium hydroxide (KOH) solution. Phytosterols were extracted into toluene, which was then evaporated under nitrogen. Phytosterols were derivatized using hexamethyldisilane and trimethylchlorosilane and analyzed by GC-FID.

Sample Preparation Method 2: Two 0.125 g test portions from each of six ampoules were individually combined with an internal standard solution containing cholesterol, ethanol, and KOH, and the solution was refluxed for 80 min. Analytes were extracted into toluene, which was then evaporated under nitrogen, and the residue reconstituted in isopropanol. Extracts were filtered and analyzed by LC-MS.

GC with Flame Ionization Detection: GC-FID was performed using a 0.25 mm × 30 m fused silica capillary column containing a dimethylpolysiloxane phase. Peak identities were confirmed on a GC x GC time-of-flight mass spectrometer. A typical separation is provided in Appendix A.

[(1)]Certain commercial equipment, instruments, or materials are identified in this Certificate 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.

LC with Mass Spectrometric Detection: LC with atmospheric pressure chemical ionization MS was performed using a 15 cm C18 column. The following ions (*m/z*) were monitored: 369 (cholesterol, internal standard), 383 (campesterol), 395 (stigmasterol), 397 (β-sitosterol), and 409 (cycloartenol). Campesterol and stigmasterol coeluted in the chromatogram, and the peak area for stigmasterol at *m/z* 395 was corrected for a small contribution from campesterol based on the calibrant responses. A typical separation is provided in Appendix A.

ANALYTICAL APPROACH FOR DETERMINATION of β-CAROTENE AND TOCOPHEROLS

Value assignment of the mass fractions of β-carotene and tocopherols in SRM 3251 was based on the combination of measurements from two different analytical methods at NIST, for each analyte.

NIST ANALYSES FOR β-CAROTENE

β-Carotene was measured by using combinations of two LC methods with absorbance detection. Four independently prepared calibrants were used for each of the methods following spectrometric assignment of mass fraction and an LC evaluation of purity. Calibrants were prepared gravimetrically, at levels intended to approximate the levels of the carotenoids in the SRM.

Sample Preparation: Duplicate (for LC with Absorbance Detection, Method 1) or single (for LC with Absorbance Detection, Method 2) 100 mg to 150 mg test portions from each of six ampoules were diluted in ethanol. Samples were mixed until the oil was visibly dissolved in the ethanol and were analyzed by LC with absorbance detection.

LC with Absorbance Detection, Method 1: An isocratic LC method with a methanol/triethylamine/acetonitrile mobile phase and a polymeric C18 column held at room temperature were used for determination of β-carotene. Absorbance was monitored at 452 nm. A typical separation is provided in Appendix B.

LC with Absorbance Detection, Method 2: An isocratic LC method with a mobile phase consisting of 92 % acetone/8 % water (volume fractions) containing 0.0125 mol/L ammonium acetate and a polymeric C30 column held at 10 ºC were used for determination of β-carotene. Absorbance was monitored at 450 nm. A typical separation is provided in Appendix B.

NIST ANALYSES FOR TOCOPHEROLS

Tocopherols were measured by using two LC methods with absorbance or fluorescence detection. Four independently prepared calibrants were used for each of the methods following spectrometric assignment of mass fraction and an LC evaluation of purity. Calibrants were prepared gravimetrically, at levels intended to approximate the levels of the tocopherols in the SRM. For LC with fluorescence detection, a single internal standard solution was used for the calibrants and samples.

Sample Preparation: Two 250 mg test portions (for fluorescence detection) or single 150 mg test portions (for absorbance detection) from each of six ampoules were individually diluted in ethanol containing tocol as an internal standard (fluorescence detection) or ethanol with no internal standard added (absorbance detection). Samples were mixed until the oil was visibly dissolved in the ethanol and were analyzed by LC with fluorescence or absorbance detection.

LC with Fluorescence Detection: An isocratic LC method with 97 % methanol/3 % water mobile phase (volume fractions) and a polymeric C30 column held at 25 ºC were used for determination of δ- and γ-tocopherol. Excitation was at 298 nm; emission was monitored at 325 nm. A typical separation is provided in Appendix B.

LC with Absorbance Detection: An isocratic LC method with a methanol/water mobile phase and a C30 column were used for LC/absorbance determination of γ-tocopherol at 5 ºC. Absorbance detection was at 295 nm. A typical separation is provided in Appendix B.

Homogeneity Assessment: The homogeneity of phytosterols was assessed at NIST by using the LC-MS and GC-FID methods described above. The homogeneity of β-carotene and γ-tocopherol was assessed at NIST by using LC with absorbance detection, Method 1, and LC with fluorescence detection, respectively. An analysis of variance did not show inhomogeneity for the test portions analyzed.

Value Assignment: The equally weighted means from each set of data available from NIST were used to calculate assigned values. For phytosterols, the median values from the AOAC collaborative study, where available, were averaged with the individual NIST mean values to obtain assigned values.

Table 1. Certified Mass Fraction Values for Selected Phytosterols in SRM 3251[a]

	Mass Fraction (mg/g)		
Campesterol	0.533	±	0.031
β-Sitosterol	1.666	±	0.064
Stigmasterol	0.247	±	0.040

[a] Each certified mass fraction value is an equally weighted mean of results provided by GC-FID and LC-MS and the median of the AOAC collaborative study. Values are expressed as $x \pm U_{95\%}(x)$, where x is the certified value and $U_{95\%}(x)$ is the expanded uncertainty of the certified value. The true value of the analyte lies within the interval $x \pm U_{95\%}(x)$ with 95 % confidence. To propagate this uncertainty, the certified value should be treated as a normally distributed random variable with mean x and standard deviation $U_{95\%}(x)/2$ [2,3]. The measurands are the mass fractions of the selected phytosterols listed in Table 1. Metrological traceability is to the measurement unit as realized through the purity determined for the primary chemical standards employed in the NIST methods.

Table 2. Certified Mass Fraction Values for Total β-Carotene and γ-Tocopherol in SRM 3251[a]

	Mass Fraction (μg/g)		
Total β-carotene	46.8	±	4.6
γ-Tocopherol	280	±	13

[a] Each certified mass fraction value is an equally weighted mean of results provided by two LC methods with absorbance and/or fluorescence detection. Values are expressed as $x \pm U_{95\%}(x)$, where x is the certified value and $U_{95\%}(x)$ is the expanded uncertainty of the certified value. The true value of the analyte lies within the interval $x \pm U_{95\%}(x)$ with 95 % confidence. To propagate this uncertainty, the certified value should be treated as a normally distributed random variable with mean x and standard deviation $U_{95\%}(x)/2$ [2,3]. The measurands are the mass fractions of the total β-carotene and γ-tocopherol listed in Table 2. Metrological traceability is to the measurement unit as realized through the purity determined for the primary chemical standards employed in the NIST methods.

Table 3. Reference Mass Fraction Value for Cycloartenol in SRM 3251[a]

	Mass Fraction (mg/g)		
Cycloartenol	0.772	±	0.008

[a] The reference mass fraction value is the mean of results provided by LC-MS. Values are expressed as $x \pm U_{95\%}(x)$, where x is the estimated value and $U_{95\%}(x)$ is the expanded uncertainty of the value. The method-specific value of the analyte lies within the interval $x \pm U_{95\%}(x)$ with 95 % confidence [2,3]. The measurand is the mass fraction of cycloartenol listed in Table 3. Metrological traceability is to the SI derived unit for mass fraction (expressed as milligrams per gram), as realized by the methods used.

Table 4. Reference Mass Fraction Values for β-Carotene Isomers and δ-Tocopherol in SRM 3251[a]

	Mass Fraction (μg/g)		
Trans-β-carotene	36.4	±	5.6
9-*Cis*-β-carotene	10.4	±	1.2
δ-Tocopherol[b]	35.3	±	0.5

[a] Each reference mass fraction value is an equally weighted mean of results provided by two different LC methods with absorbance detection. Values are expressed as $x \pm U_{95\%}(x)$, where x is the estimated value and $U_{95\%}(x)$ is the expanded uncertainty of the value. The method-specific value of the analyte lies within the interval $x \pm U_{95\%}(x)$ with 95 % confidence [2,3]. The measurands are the mass fractions of the β-carotene isomers and δ-tocopherol listed in Table 4. Metrological traceability is to the SI derived unit for mass fraction (expressed as micrograms per gram), as realized by the methods used.

[b] The value for δ-tocopherol was determined using a single LC method with absorbance detection.

REFERENCES

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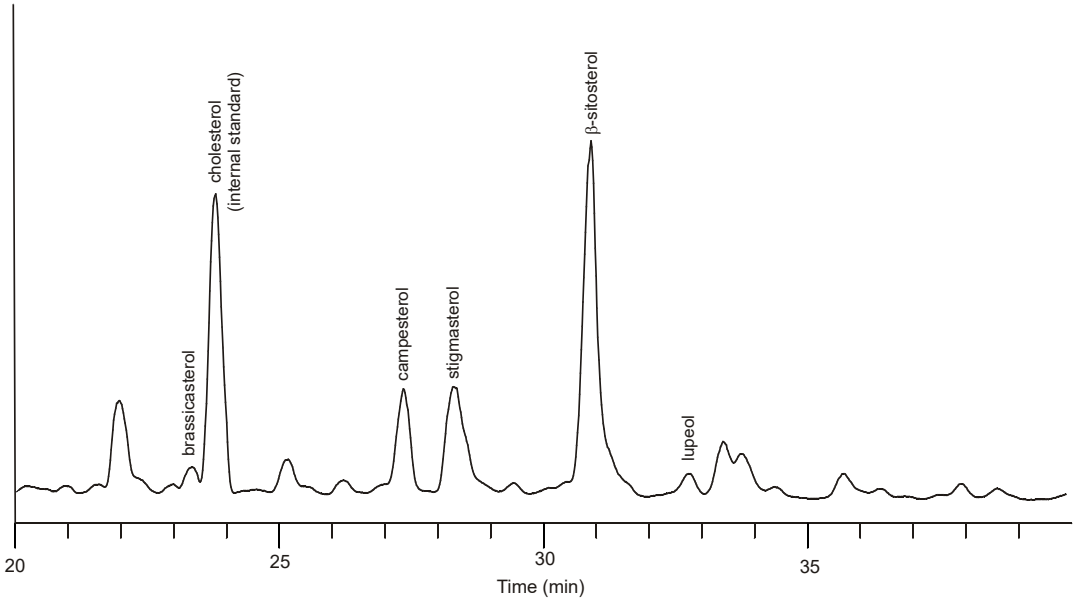
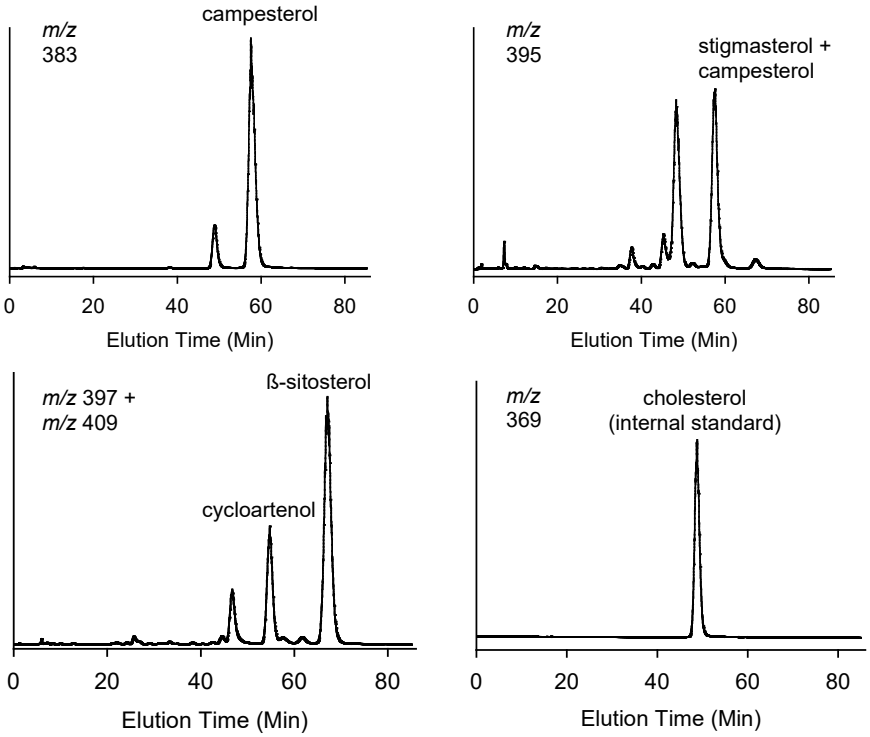
Certificate Revision History: 14 December 2020 (Change of expiration date; removal of certified and reference values for fatty acids due to suspected instability, removal of information values for brassicasterol and lupeol due to NIST's decision to no longer support these values; editorial changes); 12 June 2017 (Editorial changes); 04 April 2014 (Extension of certification period, editorial changes); 13 January 2009 (Original certificate date).

Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; e-mail srminfo@nist.gov; or via the Internet at https://www.nist.gov/srm.

Top: Typical chromatograms obtained by GC-FID with a 30 m dimethylpolysiloxane fused silica capillary column (HP-1MS, Agilent Technologies, Wilmington, DE). The column was held isothermally at 250 ºC for 10 min and then temperature programmed at 4 ºC per min to 280 ºC for 22.5 min. The injection port and FID were maintained at 280 ºC. All injections were done in the split mode (1 µL) with helium as a carrier gas at a constant flow rate of 1.2 mL/min. Bottom: Typical chromatograms for the measurement of phytosterols in SRM 3251 obtained by LC-MS with a 15 cm Ace C18 column (Advanced Chromatography Technologies, Aberdeen, Scotland). The isocratic mobile phase consisted of 10 % water, 90 % methanol (volume fractions) at a flow rate of 0.8 mL/min. MS detection conditions were as follows: nebulizer pressure, 276 kPa (40 psi); fragmentor, 80 V; drying gas temperature, 350 °C; drying gas flow rate, 12 L/min; corona current, 8 µA; capillary voltage, 3000 V; and vaporizer temperature, 400 °C.





APPENDIX B

Typical chromatogram for the measurement of β-carotene and tocopherols in SRM 3251 using LC with absorbance detection. Mobile phase compositions are expressed as volume fractions. First panel: polymeric Vydac C18 201TP54 (The Separations Group; Hesperia, CA), 25 ºC, isocratic mobile phase of 4 % methanol containing 0.05 % triethylamine/96 % acetonitrile at a flow rate of 0.8 mL/min; absorbance detection at 452 nm. Second panel: polymeric YMC C30 column (Waters Corporation, Milford, MA), 10 ºC, isocratic mobile phase of 92 % acetone/8 % water containing 0.0125 mol/L ammonium acetate at a flow rate of 1 mL/min; absorbance detection at 450 nm. Third panel: polymeric YMC C30, 25 ºC, isocratic mobile phase of 97 % methanol/3 % water at a flow rate of 1 mL/min; fluorescence detection with excitation at 298 nm, emission at 325 nm. Fourth panel: Develosil C30 column (Phenomenex, Torrance, CA), 5 ºC, premixed mobile phase of 99 % methanol/1 % water at a flow rate of 1 mL/min; absorbance detection at 295 nm.