



National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material[®] 3250

Serenoa repens (Fruit)

This Standard Reference Material (SRM) is intended primarily for use in validating analytical methods for the determination of phytosterols and fatty acids in the fruit 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 3250 consists of five packets, each containing approximately 6 g of ground saw palmetto fruit.

The development of SRM 3250 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 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 selected phytosterols and fatty acids are provided in Tables 1 and 2, respectively. Values 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 results of the individual sets of data; 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: Reference values are noncertified 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 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 [1]. Reference concentration values for additional total fatty acids (as triglycerides) as well as free fatty acids are provided in Tables 3 and 4, respectively.

Expiration of Certification: The certification of **SRM 3250** is valid, within the measurement uncertainty specified, until **30 June 2019**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Instructions for Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

Maintenance of SRM Certification: 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.

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

Analytical measurements at NIST were performed by M. Bedner, K.E. Murphy, B.J. Porter, M.M. Schantz, and L.J. Wood of the NIST Chemical Sciences Division.

Acquisition of the material was coordinated by A. NguyenPho of FDA CDER and K.E. Sharpless.

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Gaithersburg, MD 20899
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Certificate Revision History on Page 7

Support for the development of SRM 3250 was provided in part by NIH ODS and FDA CDER. Technical consultation was provided by J.M. Betz (NIH ODS) and A. NguyenPho (FDA CDER).

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

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

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 powder equal to or greater than 0.5 g for fatty acid analysis and 0.5 g for phytosterol analysis should be used. When the contents of an opened packet have been transferred to a clean amber screw-cap bottle and stored in a desiccator, phytosterols have been found to be stable for 5 days. The stability of fatty acids in opened packets has not been investigated. Test portions should be analyzed as received and results converted to a dry-mass basis by determining moisture content (described below) on a separate test portion.

PREPARATION AND ANALYSIS⁽¹⁾

Material Acquisition and Preparation: The material (ground fruit) for production of SRM 3250 was packaged as received. The ground fruit of *S. repens* was heat-sealed inside a nitrogen-flushed 4 mil polyethylene bag, which was then sealed inside a nitrogen-flushed aluminized plastic bag along with two packets of silica gel. Following packaging, SRM 3250 was irradiated (Neutron Products, Inc.; Dickerson, MD) at an absorbed dose of 3.6 kGy to 4.5 kGy.

Analytical Approach for Determination of Phytosterols and Fatty Acids: Value assignment of the concentrations of the phytosterols and fatty acids in SRM 3250 was based on the combination of results from two different analytical methods at NIST. NIST provided total fatty acid (including fatty acids occurring both as free fatty acids and triglycerides) measurements by using a combination of two gas chromatography (GC) methods with different detection (i.e., flame ionization detection [FID] and mass spectrometry [MS]). Free fatty acids (i.e., those that were not naturally present as triglycerides) were measured by GC-FID. NIST made phytosterol measurements by using a combination of GC-FID and liquid chromatography (LC)/MS.

NIST ANALYSES FOR FATTY ACIDS

Fatty acids were measured by using combinations of two GC methods with FID or 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 fatty acids in the SRM. A single internal standard solution was used for the calibrants and samples.

Sample Preparation Method 1: Two 0.5 g test portions from each of eight packets were combined with diatomaceous earth (Hydromatrix, Isco, Lincoln, NE) in individual extraction cells. An internal standard solution containing myristic-*d*₂₇ acid, stearic-*d*₃₅ acid, and arachidic-*d*₃₉ acid was added to the top of the contents in the cells, and the contents were extracted using a pressurized-fluid extractor with hexane:acetone (4+1 volume fraction) as the extraction solvent. A two-step process involving methanolic sodium hydroxide and boron trifluoride was used to convert the fatty acids to their methyl esters. Methyl esters were extracted into hexane three times, and the combined hexane portions were concentrated to approximately 2 mL. Samples were analyzed by using GC-FID.

Sample Preparation Method 2: Two 0.5 g test portions from each of eight packets were combined with diatomaceous earth (Hydromatrix) in individual extraction thimbles. An internal standard solution containing

⁽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.

myristic-*d*₂₇ acid, stearic-*d*₃₅ acid, and arachidic-*d*₃₉ acid was added to the top of the contents in the extraction thimbles, and the contents were Soxhlet-extracted for approximately 42 h using methylene chloride as the extraction solvent. The solvent was exchanged to benzene, and approximately 0.25 mL of the extract was combined with an equal volume of MethPrep II (0.1 mol/L methanolic (*m*-trifluoromethylphenyl)trimethyl ammonium hydroxide, Alltech Associates, Deerfield, IL). Samples were mixed for 1 min, and allowed to sit for at least 1 h prior to analysis by using GC/MS.

For analysis of free fatty acids, single 1.5 g test portions from each of eight packets were combined with diatomaceous earth (Hydromatrix) in individual extraction thimbles. An internal standard solution containing myristic-*d*₂₇ acid, stearic-*d*₃₅ acid, and arachidic-*d*₃₉ acid was added to the top of the contents in the extraction thimbles, and the contents were Soxhlet-extracted for approximately 40 h using hexane:acetone (4+1 volume fraction) as the extraction solvent. Approximately 0.1 mL of the extract was combined with 0.5 mL of MethPrep I (0.1 mol/L aqueous (*m*-trifluoromethylphenyl)trimethyl ammonium hydroxide, Alltech Associates; Deerfield, IL). Samples were mixed for 1 min and analyzed by using GC-FID.

GC with Flame Ionization Detection: GC-FID was performed using a 0.25 mm × 100 m fused silica capillary column containing a nonbonded biscyanopropyl polysiloxane phase. A typical separation is provided in Appendix A.

GC with Mass Spectrometric Detection: GC/MS was performed using a 0.25 mm × 60 m fused silica capillary column containing a 50 % cyanopropyl + 50 % phenylpolysiloxane (mole fraction) phase. The MS was operated in the scan mode (70 amu to 450 amu).

NIST ANALYSES FOR PHYTOSTEROLS

Phytosterols were measured by using GC with FID and LC with 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: Single 3 g test portions from each of eight packets 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 using GC-FID.

Sample Preparation Method 2: Two 0.5 g test portions from each of six packets were individually combined with an internal standard solution containing cholesterol, ethanol, and hydrochloric acid. After heating for 30 min, KOH was added, and the solution was refluxed for 45 min. Analytes were then extracted into toluene, which was then evaporated under nitrogen, and the residue reconstituted in isopropanol. Extracts were filtered and analyzed by using 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 by using a GC x GC time-of-flight mass spectrometry system.

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), and 397 (β-sitosterol). A typical separation is provided in Appendix B. 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.

NIST ANALYSES FOR TOXIC ELEMENTS

Two 0.5 g test portions from a single packet of SRM 3250 were screened for arsenic, cadmium, and lead using inductively coupled plasma with mass spectrometric detection (ICP-MS). Three 0.05 g test portions from a single packet were screened for mercury using a mercury analyzer. The material contains approximately 20 ng/g arsenic, 10 ng/g cadmium, and 3 mg/kg to 5 mg/kg lead; lead is not homogeneously distributed in this material. The material contains <5 ng/g mercury.

Determination of Moisture: Moisture content of SRM 3250 was determined at NIST (see “Instructions for Use”) by (1) freeze-drying to constant mass over 7 days; (2) drying over magnesium perchlorate in a desiccator at room temperature for 38 days; and (3) drying for 4 h in a forced-air oven at 100 °C. The results from all three techniques

were averaged to determine a conversion factor of (0.9358 ± 0.0097) gram dry mass per gram as-received mass, which was 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 (0.24 %) 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 phytosterols and fatty acids, respectively, was assessed at NIST by using the LC/MS and GC/MS methods described above. An analysis of variance did not show inhomogeneity for the test portions analyzed (0.5 g).

Value Assignment: The equally weighted means from each set of data available were used to calculate the assigned values.

Table 1. Certified Concentration Values for Selected Phytosterols in SRM 3250^(a)

Phytosterols	Mass Fraction (mg/g, dry-mass basis)		
Campesterol	0.1175	±	0.0025
β-Sitosterol	0.454	±	0.018
Stigmasterol	0.0477	±	0.0020

^(a) Each certified concentration value, expressed as a mass fraction, is an equally weighted mean of results provided by GC-FID and LC/MS. The uncertainty in the certified value, calculated according to the method described in the ISO/JCGM Guide [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.

Table 2. Certified Concentration Values for Selected Fatty Acids (as Triglycerides) in SRM 3250^(a)

Fatty Acids	Mass Fraction (%, dry-mass basis)		
Octanoic Acid (C8:0) (Caprylic Acid)	0.1072	±	0.0027
Decanoic Acid (C10:0) (Capric Acid)	0.1175	±	0.0055
Dodecanoic Acid (C12:0) (Lauric Acid)	2.962	±	0.062
Tetradecanoic Acid (C14:0) (Myristic Acid)	1.103	±	0.065
Hexadecanoic Acid (C16:0) (Palmitic Acid)	0.869	±	0.027
(Z)-9-Hexadecenoic Acid (C16:1 n-7) (Palmitoleic Acid)	0.0158	±	0.0010
Octadecanoic Acid (C18:0) (Stearic Acid)	0.1791	±	0.0054
(Z)-9-Octadecenoic Acid (C18:1 n-9) (Oleic Acid)	3.24	±	0.15
(Z)-11-Octadecenoic Acid (C18:1 n-7) (Vaccenic Acid)	0.0547	±	0.0030
(Z,Z)-9,12-Octadecadienoic Acid (C18:2 n-6) (Linoleic Acid)	0.824	±	0.055
(Z,Z,Z)-9,12,15-Octadecatrienoic Acid (C18:3 n-3) (Linolenic Acid)	0.194	±	0.025
Eicosanoic Acid (C20:0) (Arachidic Acid)	0.0097	±	0.0002
Docosanoic Acid (C22:0) (Behenic Acid)	0.0066	±	0.0002
Tetracosanoic Acid (C24:0) (Lignoceric Acid)	0.0107	±	0.0003

^(a) Each certified concentration value, expressed as a mass fraction, is an equally weighted mean of results provided by GC-FID and GC/MS. The uncertainty in the certified value, calculated according to the method described in the NIST and ISO/JCGM 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-method, within-method, 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.

Table 3. Reference Concentration Values for Selected Fatty Acids (as Triglycerides) in SRM 3250^(a)

Fatty Acids	Mass Fraction (%, dry-mass basis)		
Tridecanoic Acid (C13:0)	0.0076	±	0.0014
Pentadecanoic Acid (C15:0)	0.0047	±	0.0006
Heptadecanoic Acid (C17:0)	0.0061	±	0.0007
(Z)-11-Eicosenoic Acid (C20:1 n-9) ^(b) (Gondoic Acid)	0.0173	±	0.0006

^(a) Each reference concentration value, expressed as a mass fraction, is an equally weighted mean of results provided by GC-FID and GC/MS. The uncertainty in the reference value, calculated according to the method described in the NIST and ISO/JCGM 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-method, within-method, 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) Value obtained using only GC/MS.

Table 4. Reference Concentration Values for Free Fatty Acids in SRM 3250^(a)

	Mass Fraction (mg/g, dry-mass basis)
Octanoic Acid (C8:0) (Caprylic Acid)	0.781 ± 0.036
Dodecanoic Acid (C12:0) (Lauric Acid)	7.21 ± 0.36
Tridecanoic Acid (C13:0)	0.017 ± 0.001
Tetradecanoic Acid (C14:0) (Myristic Acid)	5.96 ± 0.21
Pentadecanoic Acid (C15:0)	0.012 ± 0.001
Hexadecanoic Acid (C16:0) (Palmitic Acid)	8.72 ± 0.45
(Z)-9-Hexadecenoic Acid (C16:1 n-7) (Palmitoleic Acid)	0.216 ± 0.014
Heptadecanoic Acid (C17:0)	0.093 ± 0.006
Octadecanoic Acid (C18:0) (Stearic Acid)	2.02 ± 0.09
(Z)-9-Octadecenoic Acid (C18:1 n-9) (Oleic Acid)	33.7 ± 1.9
(Z)-11-Octadecenoic Acid (C18:1 n-7) (Vaccenic Acid)	0.789 ± 0.053
(Z,Z)-9,12-Octadecadienoic Acid (C18:2 n-6) (Linoleic Acid)	5.70 ± 0.48
(Z,Z,Z)-9,12,15-Octadecatrienoic Acid (C18:3 n-3) (Linolenic Acid)	1.35 ± 0.05
Eicosanoic Acid (C20:0) (Arachidic Acid)	0.146 ± 0.008
Docosanoic Acid (C22:0) (Behenic Acid)	0.056 ± 0.005
Tetracosanoic Acid (C24:0) (Lignoceric Acid)	0.096 ± 0.003

^(a) Each reference concentration value, expressed as a mass fraction, is the mean of results provided by GC-FID. The uncertainty in the reference value, calculated according to the method described in the ISO/JCGM Guide [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 within-method 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.

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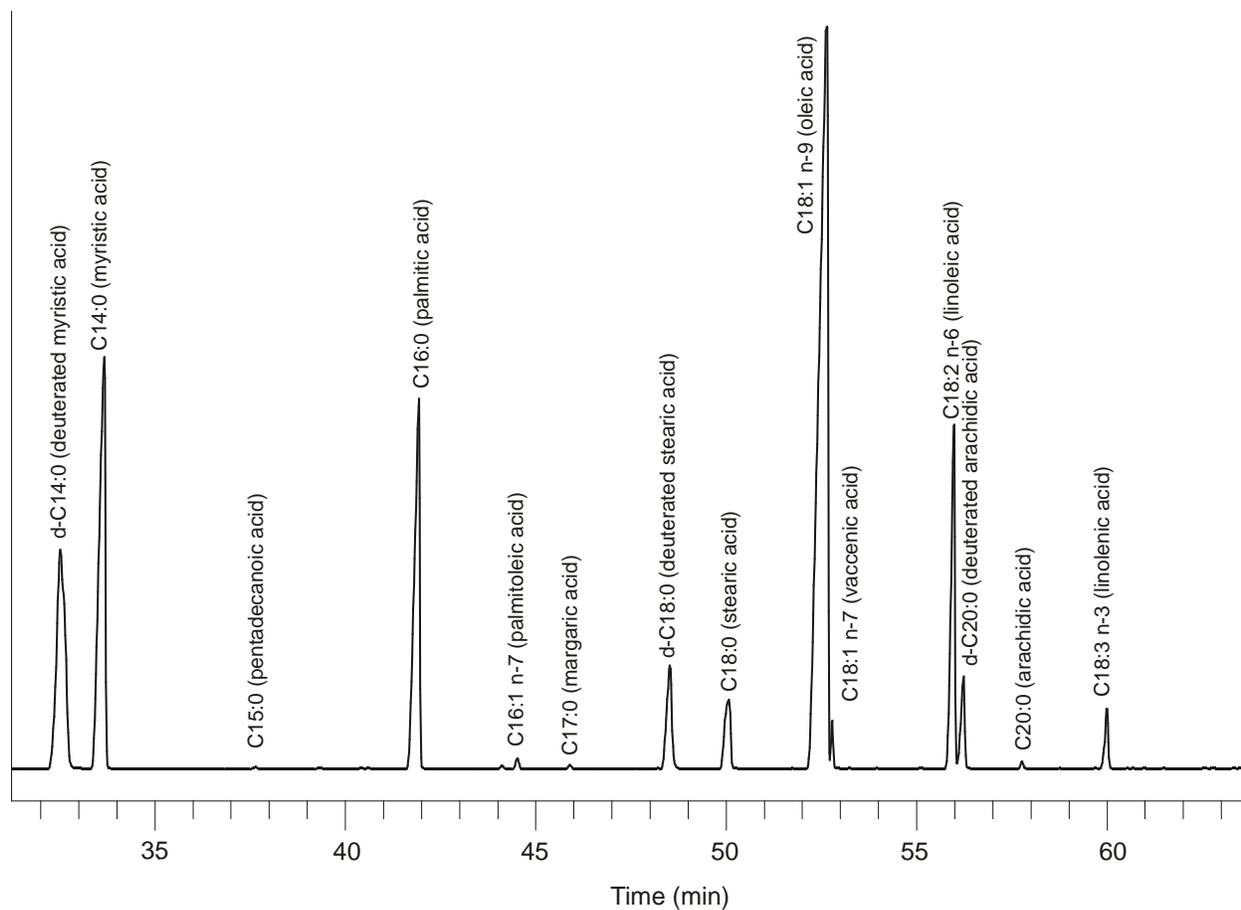
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Certificate Revision History: 08 April 2014 (Extension of certification period, editorial changes); 05 January 2009 (Original certificate).

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; fax (301) 948-3730; e-mail srminfo@nist.gov; or via the Internet at <http://www.nist.gov/srm>

APPENDIX A

Typical chromatogram for the measurement of fatty acids in SRM 3250 by using GC-FID on a 0.25 m × 100 m fused silica capillary column containing a nonbonded biscyanopropyl polysiloxane phase (SP 2560, Supelco, Bellefonte, PA). The column was held isothermally at 100 °C for 4 min and then temperature programmed at 2.5 °C per min to 240 °C where it was held for 50 min. The injection port and FID were maintained at 240 °C. All injections were done in the split mode (1 μL) with helium as a carrier gas at a constant flow rate of 1 mL/min.



APPENDIX B

Top: Typical chromatogram obtained for the measurement of phytosterols by using GC-FID with a 30 m dimethylpolysiloxane fused silica capillary column (HP-1MS, Agilent Technologies, Wilmington, DE). The column was held isothermally at 250 °C for 10 min and then temperature programmed at 4 °C per min to 280 °C for 22.5 min. The injection port and FID were maintained at 280 °C. All injections were done in the split mode (1 □) 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 3250 obtained by using LC/MS with a 15 cm ACE C₁₈ column (Advanced Chromatography Technologies, Aberdeen, Scotland). The isocratic mobile phase consisted of 10 % water, 90 % ethanol (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.

