National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 3276

Carrot Extract in Oil

This Standard Reference Material (SRM) is intended primarily for use in validating analytical methods for the determination of tocopherols and fatty acids in carrot extract in oil and similar matrices. This SRM may also be used for quality assurance when assigning values to in-house control materials. A unit of SRM 3276 consists of five ampoules of the oil, each containing approximately 1 mL of material.

The development of SRM 3276 was a collaboration between the National Institute of Standards and Technology (NIST) and the National Institutes of Health (NIH), Office of Dietary Supplements (ODS).

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 of selected tocopherols and fatty acids are provided in Tables 1 and 2. Values were derived from the combination of results provided by NIST and, in the case of tocopherols, by collaborating laboratories. The certified values in this material are the equally weighted means of the individual sets of NIST results and the medians of the individual sets of measurements made by collaborating laboratories. 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].

Expiration of Certification: The certification of **SRM 3276** is valid, within the measurement uncertainty specified, until **30 September 2017**, provided the SRM is handled and stored in accordance with 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 Certification: 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) will facilitate notification.

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 3276 was provided in part by NIH-ODS. Technical consultation was provided by J.M. Betz of NIH-ODS.

Acquisition and preparation of the material was coordinated by K.E. Sharpless. Analytical measurements at NIST were performed by K. Putzbach, C.A. Rimmer, M.M. Schantz, J.B. Thomas, and T. Yarita of the NIST Analytical Chemistry Division.

Statistical analysis was provided by J.H. Yen of the NIST Statistical Engineering Division and D.L. Duewer of the NIST Analytical Chemistry Division.

Support aspects involved in the issuance of this SRM were coordinated through the NIST Measurement Services Division.

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Certificate Issue Date: 27 August 2012

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WARNING TO USERS

For laboratory use only. Not for human consumption.

INSTRUCTIONS FOR STORAGE AND USE

Storage: The material should be stored under refrigeration (0 °C to 4 °C), in an unopened ampoule, until required for use.

Use: Prior to removal of a test portion for analysis, the contents of an ampoule of material should be allowed to warm to room temperature and mixed thoroughly. Test portions used for NIST analyses described below were 70 mg to 90 mg for tocopherols and 20 mg to 100 mg for fatty acids.

PREPARATION AND ANALYSIS[1]

Material Acquisition and Preparation: Carrot extract in oil (6.32 kg) was combined with 4.24 g butylated hydroxytoluene (BHT) to yield a final BHT mass fraction of approximately 670 mg/kg. To facilitate dissolution of the BHT, the mixture was sonicated, manually shaken, and stirred overnight using a magnetic stir bar. SRM 3276 was ampouled under argon with each ampoule containing approximately 1 mL of oil.

Analytical Approach for Determination of Tocopherols: Value assignment of the mass fractions of tocopherols in SRM 3276 was based on the combination of measurements from three different analytical methods at NIST and from laboratories participating in the NIST Micronutrients Measurement Quality Assurance Program (QA Program). NIST provided measurements by using a combination of three liquid chromatorgraphy (LC) methods with different detection methods (i.e., absorbance [abs] and fluorescence) as described below. The laboratories in the QA Program used their usual methods.

NIST Analyses for Tocopherols: Tocopherols were measured by using combinations of three LC methods with absorbance or fluorescence 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 tocopherols in the SRM. A single internal standard solution was used for the calibrants and samples.

Sample Preparation: Two 20 mg to 40 mg test portions from each of six ampoules were diluted in about 250 mg (mass known) of ethanol for LC/abs Method 1. Single 70 mg to 80 mg test portions were diluted with approximately equal volumes of an ethanolic tocol solution (internal standard), and then with an additional 750 mg ethanol for LC/abs Method 2. Samples were analyzed by the LC methods below.

LC/abs Method 1: An isocratic LC method with a methanol/triethylamine/acetonitrile mobile phase and a polymeric C_{18} column was used for determination of tocopherols. Absorbance detection wavelength was at 292 nm.

LC/abs Method 2: An isocratic LC method with a methanol/water mobile phase and a C_{30} column held at 5 °C was used for LC/abs determination of tocopherols. Absorbance detection wavelength was 295 nm.

LC/fluorescence Method: An isocratic LC method with a methanol/water mobile phase and a C_{30} column was used for LC/fluorescence determination of tocopherols. The excitation wavelength was 298 nm; the emission wavelength was measured 325 nm.

Analytical Approach for Determination of Fatty Acids: Value assignment of the mass fractions of the fatty acids in SRM 3276 was based on the combination of measurements from two different analytical methods at NIST. NIST provided measurements by using a combination of two gas chromatography (GC) methods with different detection methods (i.e., flame ionization detection [FID] and mass spectrometry [MS]) as described below. 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.

[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.

Sample Preparation Method 1: Two 20 mg test portions from each of eight ampoules were combined with chloroform and an internal standard solution. 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 1 mL. Samples were analyzed by GC-FID.

Sample Preparation Method 2: Two 100 mg test portions from each of four ampoules were combined with benzene and an internal standard solution. Approximately 0.1 mL of MethPrep II (0.1 mol/L methanolic (*m*-trifluoromethylphenyl)trimethylammonium hydroxide, Alltech, Deerfield, IL) was added, samples were mixed for 1 min, and allowed to sit for at least 1 h prior to analysis by GC/MS.

GC-FID: GC-FID was performed using a 0.25 mm × 100 m fused silica capillary column. A typical separation is provided in the Appendix; note that the run time was longer than necessary for elution of the fatty acids to ensure that residual oil was eluted from the column.

GC/MS: GC/MS was performed using a 0.25 mm × 100 m fused silica capillary column. The MS was operated both in the scan mode (200 amu to 400 amu) and in the single-ion monitoring (SIM) mode.

Homogeneity Assessment: The homogeneity of tocopherols was assessed at NIST by using LC/abs Method 1 described above. An analysis of variance did not show inhomogeneity for a 20 mg to 40 mg sample. The fatty acids were also found to be homogeneously distributed in 20 mg and 100 mg test portions using the GC-FID method described above.

Value Assignment: For the tocopherols, the three individual NIST data set means were averaged with the median result of data provided by the QA Program laboratories to obtain the assigned value. For the fatty acids, the two NIST data set means were averaged to obtain the assigned value.

Values for β-carotene isomers in this SRM were originally certified. In 2008, the values were found to have changed. New values for β-carotene isomers were assigned and values were changed from certified to reference in 2008. As a result of continued instability, the reference values for β-carotene isomers were removed from the Certificate of Analysis in 2012.

Certified Mass Fraction Values for Tocopherols: Each certified value, expressed as a mass fraction, is an equally weighted mean of the individual sets of results provided by NIST using LC/abs Method 1, LC/abs Method 2, and LC/fluorescence, and the median of results provided by QA Program laboratories. The uncertainty in the certified value, calculated according to the method described in the ISO Guide [2] and a published method for combined results [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 and within-laboratory 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 1. Certified Mass Fraction Values for Tocopherols in SRM 3276

	Mass Fraction (μg/g)	k
δ-Tocopherol	373 ± 34	2.45
γ-Tocopherol	443 ± 64	2.57

Certified Mass Fraction Values for Fatty Acids: Each certified value, expressed as a mass fraction, is an equally weighted mean of the results from NIST using GC-FID and GC/MS. The uncertainty in the certified value, calculated according to the method described in the ISO Guide [2] and a published method for combined results [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 and within-laboratory 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 Mass Fraction Values for Selected Fatty Acids (as Triglycerides) in SRM 3276

	Mass Fraction (%)	k
Hexadecanoic Acid (C16:0) (Palmitic Acid)	1.36 ± 0.05	2.45
(Z)-9-Hexadecenoic Acid (C16:1 n-7) (Palmitoleic Acid)	0.0147 ± 0.0014	2.78
Heptadecanoic Acid (C17:0) (Margaric Acid)	0.0213 ± 0.0017	2.31
Octadecanoic Acid (C18:0) (Stearic Acid)	1.14 ± 0.02	2.26
(Z)-9-Octadecenoic Acid (C18:1 n-9) (Oleic Acid)	3.68 ± 0.06	2.23
(Z)-11-Octadecenoic Acid (C18:1 n-7) (Vaccenic Acid)	0.519 ± 0.012	2.31
(Z,Z)-9,12-Octadecadienoic Acid (C18:2 n-6) Linoleic Acid	6.64 ± 0.11	2.26
(Z,Z,Z)-9,12,15-Octadecatrienoic Acid (C18:3 n-3) (Linolenic Acid)	0.816 ± 0.014	2.26
Eicosanoic Acid (C20:0) (Arachidic Acid)	0.0578 ± 0.0025	2.45
(Z)-11-Eicosenoic Acid (C20:1 n-9) (Gondoic Acid)	0.353 ± 0.006	2.45
Docosanoic Acid (C22:0) (Behenic Acid)	0.126 ± 0.016	2.78
Tetracosanoic Acid (C24:0) (Lignoceric Acid)	0.0242 ± 0.0018	2.57

REFERENCES

[1] May, W.; Parris, R.; Beck, C.; Fassett, J.; Greenberg, R.; Guenther, F.; Kramer, G..; Wise, S.; Gills, T.; Colbert, J.; Gettings, R.; MacDonald, B.; *Definitions of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements;* NIST Special Publication 260–136; U.S. Government Printing Office: Washington, DC (2000); available at http://www.nist.gov/srm/publications.cfm (accessed Aug 2012).

[2] JCGM 100:2008; *Evaluation of Measurement Data — Guide to the Expression of Uncertainty in Measurement* (ISO GUM 1995 with Minor Corrections); Joint Committee for Guides in Metrology (JCGM) (2008); available at http://www.bipm.org/utils/common/documents/jcgm/JCGM_100_2008_E.pdf (accessed Aug 2012); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297; U.S. Government Printing Office: Washington, DC (1994); available at http://physics.nist.gov/Pubs/ (accessed Aug 2012).

[3] Levenson, M.S.; Banks, D.L.; Eberhardt, K.R.; Gill, L.M.; Guthrie, W.F.; Liu, H.-K.; Vangel, M.G.; Yen, J.H.; Zhang, N.F.; *An Approach to Combining Results From Multiple Methods Motivated by the ISO GUM*; J. Res. Natl. Inst. Stand. Technol.; Vol. 105, p. 571 (2000).

[4] Thompson, A.; Taylor, B.N.; *Guide for the Use of the International System of Units (SI)*; NIST Special Publication 811; U.S. Government Printing Office: Washington, DC (2008); available at http://www.nist.gov/pml/pubs/index.cfm/ (accessed Aug 2012).

Certificate Revision History: 27 August 2012 (Extension of certification period; reference values for carotenoids removed; editorial changes), 15 January 2009 (Extension of certification period; carotenoid values updated and changed to reference values; editorial changes); 30 July 2007 (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 at: telephone (301) 975-2200; fax (301) 948-3730; e-mail srminfo@nist.gov; or via the Internet http://www.nist.gov/srm.

Appendix

Typical chromatogram from the fatty acid analysis of SRM 3276 using GC-FID on an SP 2560 (Supelco, Bellefonte, PA) is shown below in Figure A1.

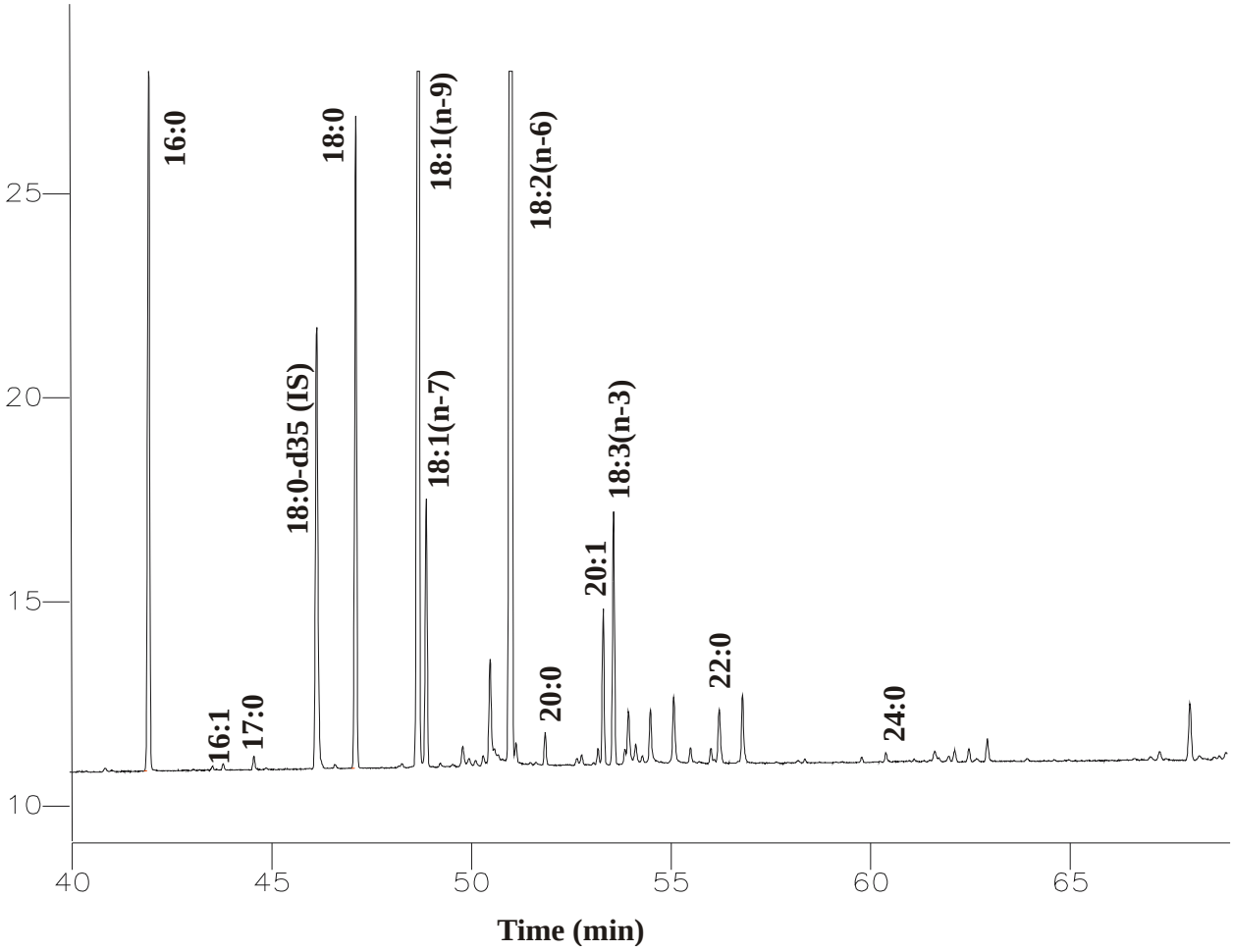

Figure A1. The column was held isothermally at 100 ºC for 4 min and then temperature programmed at 2.5 ºC/min to 240 ºC for 50 min. The injection port and FID were maintained at 240 ºC. All injections were done in the split mode (1 µL) with helium as a carrier gas at a constant flow rate of 1 mL/min.