



Certificate of Analysis

Standard Reference Material[®] 3266

Hypericin Calibration Solution

This Standard Reference Material (SRM) consists of a solution of hypericin in methanol with approximately 1.4 % (mass fraction) pyridine. SRM 3266 is intended primarily for use in calibration of instruments and techniques used for the determination of hypericin. A unit consists of five 2 mL ampoules, each containing approximately 1.2 mL of the hypericin solution.

Development of SRM 3266 was a collaboration between the National Institute of Standards and Technology (NIST) and the National Institutes of Health, Office of Dietary Supplements (NIH ODS).

Certified Values: The certified values for hypericin are based on the masses used in the gravimetric preparation of the solution and from the analytical results determined using liquid chromatography with absorbance detection (LC-abs) and liquid chromatography with mass spectrometric detection (LC-MS). 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]. Values are provided in mass fraction units (mg/g) and mass concentration units (mg/mL) [2]. The mass concentration was obtained by multiplying the certified value in mass fraction units by the density of the solution at 21 °C (0.79301 mg/mL). The uncertainty provided with each value is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence. The expanded uncertainty is calculated as $U = ku_c$, where u_c incorporates the observed difference between the results from the methods and their respective uncertainties, as well as uncertainty related to purity correction, consistently with the ISO Guide and with its Supplement 1, and $k = 2$ is a coverage factor corresponding to approximately 95 % confidence [3-5]. The expanded uncertainty for the mass concentration incorporates additional uncertainty components related to density measurement and variability of density due to changes in temperature over the interval 18 °C to 24 °C.

Analyte	Mass Fraction (mg/g)	Mass Concentration (mg/mL)	Coverage Factor (<i>k</i>)
Hypericin	0.021 20 ± 0.000 69	0.016 81 ± 0.000 56	2

Expiration of Certification: The certification of **SRM 3266** is valid, within the measurement uncertainty specified, until **01 January 2017**, provided the SRM is handled and stored in accordance with instructions given in this certificate (see “Instructions for Handling, 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.

Support for the development of SRM 3266 was provided in part by NIH ODS. Technical consultation was provided by J.M. Betz (NIH ODS).

The overall direction and coordination of the technical measurements leading to the certification of this SRM were performed by L.C. Sander, K.E. Sharpless, and S.A. Wise of the NIST Analytical Chemistry Division.

Preparation of the SRM solution and analytical measurements were performed by R. Lieberman of the NIST Analytical Chemistry Division.

Stephen A. Wise, Chief
Analytical Chemistry Division

Consultation on the statistical design of the experimental work and evaluation of the data were provided by J.H. Yen of the NIST Statistical Engineering Division.

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

INSTRUCTIONS FOR HANDLING, STORAGE, AND USE

Handling: This SRM consists of a solution of methanol, pyridine, and hypericin contained in tip-sealed borosilicate glass ampoules with pre-scored stems. Therefore, all appropriate safety precautions, including use of gloves during handling, should be taken. Unopened ampoules should be stored under normal laboratory conditions in an upright position inside the original container supplied by NIST.

Storage: Sealed ampoules, as received, should be stored in the dark at temperatures at or below $-20\text{ }^{\circ}\text{C}$.

Use: Prior to removal of a test portion for analysis, an ampoule of the SRM should be equilibrated to room temperature and the contents mixed thoroughly. Test portions for use should be withdrawn immediately after opening the ampoules, and should be processed or diluted without delay for the certified value to be valid within the stated uncertainty. The stability of hypericin in previously opened and stored ampoules has not been investigated.

PREPARATION AND ANALYSIS⁽¹⁾

Solution Preparation: The solution was prepared gravimetrically at NIST from methanol and a primary standard for hypericin obtained from Blaze Science Industries LLC (Lawndale, CA). The solution was mixed overnight (18 h) and aliquoted into 2 mL amber glass ampoules that had been purged with argon prior to addition of the solution. The ampoules were then flame-sealed. The masses of the primary standards and the total mass of the solution were used to calculate the gravimetric mass fraction. The mass fraction was adjusted for the purity estimates of the primary standard, which was determined using liquid chromatography with ultraviolet and visible detection at 210 nm, 270 nm, and 590 nm; Karl Fischer titration; and thermogravimetric analysis (TGA).

Analytical Approach: Aliquots from 10 ampoules, selected using a stratified random sampling scheme, were analyzed in duplicate using LC-abs at 590 nm. Four independent calibration solutions were gravimetrically prepared. For LC-abs, an external standard calibration approach was used. A representative chromatogram and the separation conditions are presented in Figure 1. A second set of aliquots from 10 ampoules, also selected using a stratified random sampling scheme, were analyzed in duplicate using LC-MS. Rhodamine 6G was used as an internal standard.

Homogeneity Assessment: The homogeneity of hypericin mass fractions was assessed at NIST by using the analytical approach described above. An analysis of variance did not show inhomogeneity for the test portions analyzed.

Value Assignment: For calculation of assigned values, the mean of the available sets of data were averaged. The certified value is the mean of the mass fraction determined by gravimetric preparation and the means from the two chromatographic methods.

⁽¹⁾Certain commercial equipment, instruments or materials are identified in this certificate to adequately specify 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.

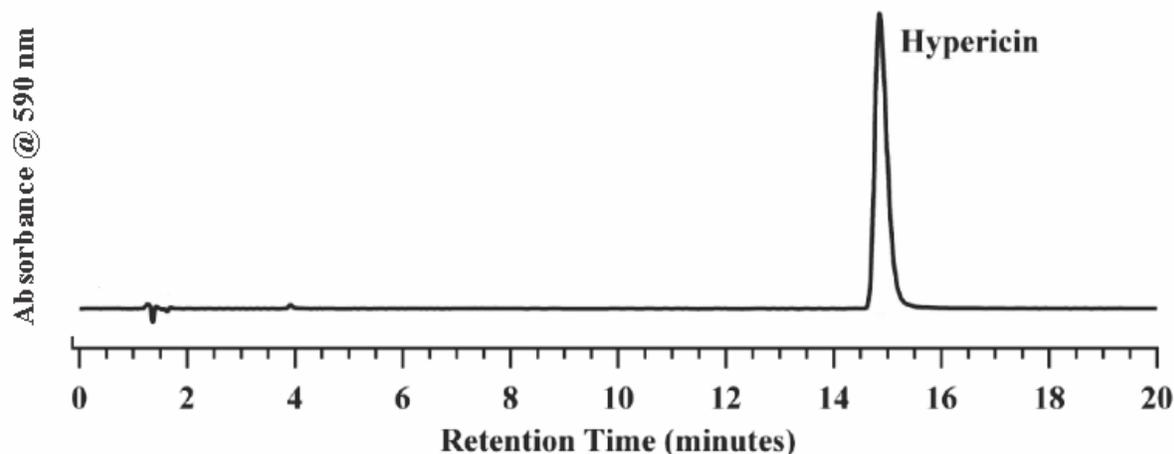


Figure 1. The chromatogram was obtained using a Halo C18 column (MacMod Analytical, Inc, Chadds Ford, PA) with dimensions of 150 mm \times 4.6 mm ID and containing 2.7 μ m diameter fused-core particles. The isocratic mobile phase consisted of 40 % water containing 0.25 % triethylamine (TEA) adjusted to pH 4.5, and 60 % acetonitrile containing 0.25 % TEA, at a flow rate of 1 mL/min. (All percentages represent volume fractions.)

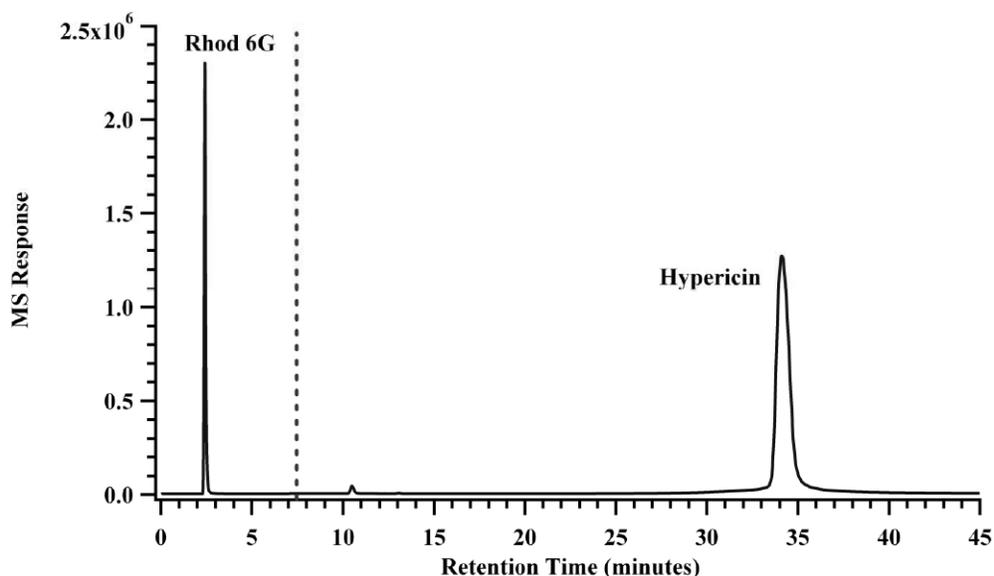


Figure 2. Selected ion chromatogram using an Ascentis Express reversed-phase amide column (150 mm \times 4.6 mm i.d., 2.7 μ m diameter fused-core particles). The isocratic mobile phase consisted of 20 % 100 mmol/L ammonium formate buffer (adjusted to pH 4.0 with formic acid) and 80 % acetonitrile. (All percentages represent volume fractions.) Rhodamine 6G and hypericin were monitored at m/z 444.0 and m/z 503.5, respectively, using electrospray ionization. The dotted line indicates a change from positive ion mode to negative ion mode.

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 Jan 2012).
- [2] 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 Jan 2012).

- [3] 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/utis/common/documents/jcgm/JCGM_100_2008_E.pdf (accessed Jan 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://www.nist.gov/pml/pubs/index.cfm> (accessed Jan 2012).
- [4] JCGM 101:2008; *Evaluation of Measurement Data – Supplement 1 to the Guide to the Expression of Uncertainty in Measurement – Propagation of Distributions Using a Monte Carlo Method*; Joint Committee for Guides in Metrology (JCGM) (2008); available at http://www.bipm.org/utis/common/documents/jcgm/JCGM_101_2008_E.pdf (accessed Jan 2012).
- [5] Efron, B.; Tibshirani, R.J.; *An Introduction to the Bootstrap*; Chapman & Hall, London, UK (1993).

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) 926-4751, e-mail srminfo@nist.gov; or via the Internet at <http://www.nist.gov/srm>.