National Institute of Standards & Technology

Report of Investigation

Reference Material® 8327

Peptide Reference Material for Molecular Mass and Purity Measurements

Reference Material (RM) 8327 is intended for use in evaluating the performance and reliability of methods for the determination of molecular mass from 1 000 g/mole to 3 000 g/mole and purity of peptides from 80 % to 100 % relative purity [1,2]. Three synthetic peptides, termed A, B, and C, were designed to provide long-term stability, a range of purities, and a range of molecular masses [1,2]. Each peptide material is a consensus sequence designed in collaboration with the Peptide Standards Project Committee (PSPC) of the Association of Biomolecular Resource Facilities (ABRF) [3]. A unit of RM 8327 consists of three lyophilized synthetic peptides (A, B, and C) with 1.0 mg of each peptide in a vacuum rubber-sealed amber glass vial. The vials are contained in a sample box and enclosed in a resealable plastic bag.

Reference Sequence Information: The reference sequence information on each of the three peptides of RM 8327 is listed in Table 1.

Reference Values for the Total UV Absorbance Area Percent Purity: The total UV absorbance area percent purity of the peptide material is defined as the percent of UV absorbance exhibited by the peptide material relative to the total UV absorbance at 216 nm and 226 nm exhibited by other components in the sample separated by High Performance Liquid Chromatography (HPLC) and Capillary Electrophoresis (CE). The detected signals on the chromatograph output of the HPLC and the CE are plotted as a function of the UV absorbance versus the run time of the chromatograph. Integration of the absorbance signal over time results in the area percent purity. These value assignments are based on a method-specific protocol recommended by PSPC of ABRF. Reference values for the total UV absorbance area percent purities of the peptides are listed in Table 2. A NIST reference value [4] is a noncertified value that is the best estimate of the true value; however, the value does not meet NIST criteria for certification and is provided with associated uncertainties that may reflect only measurement precision and may not include all sources of uncertainty.

Reference Values for the Percent Peptide Mass Purity: The percent peptide mass purity is defined as the percent mass of the peptide per mass of the lyophilized material in the sample as determined from amino acid analysis and UV absorbance measurements. Measurements of the UV absorbance at 280 nm of each peptide in water were compared to the mg/L extinction coefficients calculated from the amino acid sequence of the peptide [6] to determine the peptide material mass purity. Reference values for the peptide mass purity are presented in Table 3.

Verified Calculated Values for the Peptide Molecular Mass: The calculated monoisotopic molecular masses based on ^{12}C were determined from the masses of each of the atoms of the amino acid sequence of the peptide. The experimental values of the monoisotopic molecular masses of the peptides were determined from Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) and Electrospray Ionization-Mass Spectrometry (ESI-MS) measurements. Both values are presented in Table 4.

Expiration of Reference Values: The reference values of this RM are valid until **30 September 2015**, within the measured uncertainties specified, provided the RM is handled and stored in accordance with the instructions given in this report (see "Notice and Warning to User"). However, the reference values are invalid if the RM is contaminated or modified.

Maintenance of RM Reference Values: NIST will monitor this RM over the period of its validity. If substantive technical changes occur that affect the reference values before the expiration, NIST will notify the purchaser. Registration (see attached sheet) will facilitate notification.

The overall direction and coordination of technical measurements leading to the reference and experimental values were performed by H. Remmer of ABRF and F.P. Schwarz of the NIST Biochemical Science Division.

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Analyses were performed by D.H. Atha, F.P. Schwarz, and I. Turko of the NIST Biochemical Science Division and by N. Ambulos and P. Campbell of the University of Maryland (College Park, MD), K. Darlak of Peptides International, Inc. (Louisville, KY), J. Johnson of the University of Texas (Austin, TX), M. Lively of Wake Forest University (Winston-Salem, NC), and H. Remmer of the University of Michigan (Ann Arbor, MI).

The statistical analysis of the data used for the reference and experimental values was performed by D.D. Leber of the NIST Statistical Engineering Division.

The support aspects involved in the issuance of this RM were coordinated through the NIST Measurement Services Division.

NOTICE AND WARNING TO USER

Storage: Recommended storage conditions are containment of the RM unit in a resealable bag with desiccant at –20 °C to minimize possible side reactions such as aspartimide formation or α/β–isomerization. Although similar peptides generally show excellent stability when stored as a lyophilized powder, the participating laboratories will continue to monitor the stability of the material and its physical and chemical properties with time and advise NIST on any possible side reactions that may occur.

INSTRUCTIONS FOR USE

The lyophilized peptide is contained in an amber glass vial vacuum sealed with a septum. The amount of lyophilized peptide material in each vial is 1.0 mg. Sample removal is achieved by injection of water through the septum into the vial with a syringe, agitation of the vial for solubilization of the peptide, and subsequent removal of the solution with a syringe. The septum reseals the vial. The vial is then placed in the sample box. The sample box is enclosed in a resealable plastic bag with desiccant, and then stored in a freezer at –20 °C.

RM 8327 may be used for evaluating the performance and reliability of methods for the determination of molecular mass in Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) and in Electrospray Ionization-Mass Spectrometry (ESI-MS), for the determination of area percent peptide purity in High Pressure Liquid Chromatography (HPLC) and Capillary Electrophoresis (CE), and for the determination of amino acid mass purity in amino acid analysis (AAA) and in UV spectrophotometric analysis (UV) [2].

Table 1. Sequence Information on the Peptides of RM 8327

Peptide	Sequence	Number of Amino Acid Residues
A	DAEPDILELATGYR	14
B	KAQYARSVLLEKDAEPDILELATGYR	26
C	RQAKVLLYSGR	11

Table 2. Reference Values for Total UV Absorbance Area Percent Purities of Peptide Material in RM 8327

Peptide	Total UV Absorbance Area [a] (%)		U [b]	k [c]
A	94.0	±	4.6	2.776
B	86.5	±	12.6	2.776
C	97.1	±	3.6	2.776

[a] The reference values stated above for the peptide material purities are the means of the average measurements obtained by each of the five laboratories/methods as described.

[b] The uncertainty in the reference values is expressed as an expanded uncertainty, $U = ku_c$, calculated according to the methods in the ISO and NIST Guides [5]. The quantity u_c represents, at the level of one standard deviation, the combined uncertainty due to the potential effects within and between the laboratories/methods in the assessment of the peptide material purities, which includes the calibration and method effects.

[c] The quantity k is the coverage factor used to specify the approximate confidence level of the expanded uncertainty interval about the mean. The value of the coverage factor, $k = 2.776$, is determined from the Student's t-distribution with 4 effective degrees of freedom and a confidence level of 95 %.

Table 3. Reference Values for the Percent Peptide Mass Purity in RM 8327

Peptide	Peptide Mass Purity [a] (%)		U [b]	k [c]
A	69	±	11	4.303
B	73	±	14	4.303
C	67	±	16	4.303

[a] The reference values stated above for the peptide mass purity are the means of the average measurements obtained by each of the three laboratories/methods as described.

[b] The uncertainty in the reference values is expressed as an expanded uncertainty, $U = ku_c$, calculated according to the methods in the ISO and NIST Guides [5]. The quantity u_c represents, at the level of one standard deviation, the combined uncertainty due to the potential effects within and between the laboratories/methods in the assessment of the peptide mass purities, which includes the calibration and method effects.

[c] The quantity k is the coverage factor used to specify the approximate confidence level of the expanded uncertainty interval about the mean. The value of the coverage factor, $k = 4.303$, is determined from the Student's t-distribution with 2 effective degrees of freedom and a confidence level of 95 %.

Table 4. Comparison of the Calculated and Experimental Mass Values of the Peptides in RM 8327

Peptide	Calculated Isotopic Molecular Mass (g/mol)	Experimental Molecular Mass [a] (g/mol)		U [b]	k [c]
A	1561.8	1561.60	±	0.40	3.182
B	2948.6	2949.2	±	1.7	3.182
C	1289.8	1289.78	±	0.37	3.182

[a] The experimental values stated above for the peptide molecular masses are the means of the averages of the eight measurements obtained by four laboratories/methods as described.

[b] The uncertainty in the experimental values is expressed as an expanded uncertainty, $U = ku_c$, calculated according to the methods in the ISO and NIST Guides [5]. The quantity u_c represents, at the level of one standard deviation, the combined uncertainty due to the potential effects within and between the laboratories/methods in the assessment of the peptide molecular masses, which includes the calibration and method effects.

[c] The quantity k is the coverage factor used to specify the approximate confidence level of the expanded uncertainty interval about the mean. The value of the coverage factor, $k = 3.182$, is determined from the Student's t-distribution with 3 effective degrees of freedom and a confidence level of 95 %. The experimental values are in good agreement with the calculated molecular masses of the peptides.

SYNTHESIS AND ANALYSIS[1]

Synthesis: The peptides were synthesized at the University of Maryland Biopolymers Core facility on a commercial peptide synthesizer at a 10 mM scale for Peptides A and B and a 7.5 mM scale for Peptide C, respectively, using Fmoc-Arg(Pbf)-PEG-PS resin in dimethylformamide (DMF) for all three peptides. The amino acids were reagent quality and employed the side chain protective groups. Couplings were performed two times for one hour and were monitored by quantitative Ninhydrin test. Each peptide was then cleaved, filtered, ether-precipitated, and washed three times with cold ether. Peptides A and C were purified by ion exchange chromatography followed by C18 reversed phase (RP)-HPLC. Peptide B was purified by two steps of C18 RP-HPLC, performed at the University of Michigan Protein Structure Facility and Anaspec, Inc. The purified peptides were dissolved in double-distilled purified water to a concentration of 1.00 mg/mL, dispensed into amber glass vials, and lyophilized in the vial. The vials were then vacuum sealed with a rubber septum cap.

Analytical Methods for the Reference Total UV Absorbance Area Percent Purity: The area percent purity of the peptide material is defined as the percent UV absorbance exhibited by the peptide material relative to the total UV absorbance exhibited by other components in the sample separated by HPLC and CE. The detected signals on the chromatograph output of the HPLC and the CE are plotted as a function of the UV absorbance versus the run time of the chromatograph. Integration of the absorbance signal over time results in the area percent purity. The CE measurements were performed on samples of approximately 0.5 mg/mL in high-purity deionized water (≥18 MΩ) by D.H. Atha at NIST and P. Campbell at the University of Maryland using a commercial analytical High Performance Capillary Electrophoresis (HPCE) instrument employing a UV detector set at 214 nm. The analytical HPLC measurements were performed by P. Campbell at the University of Maryland, K. Darlak of Peptides International, Inc., and H. Remmer of the University of Michigan. An analytical HPLC was used with a C18 reversed phase column and a UV detector set at 226 nm. The sample concentrations were 0.5 mg/mL in high-purity deionized water and a 30 μL aliquot was injected into the HPLC column operating in the gradient mode.

Analytical Methods for the Reference Percent Peptide Mass Purity: The percent peptide mass purities were obtained from amino acid analysis (AAA) and UV measurements. The AAA determinations were performed by J. Johnson at the University of Texas, and M. Lively at Wake Forrest University using alanine amino acid based analysis. Four 20 μL replicates each of peptides A, B and C at a concentration of 1.0 mg/mL in high-purity deionized water were subjected to standard vapor-phase 6 N HCl-phenol hydrolysis at 150 °C for 1.5 hours. Internal standards (Norvaline for primary amino acids and Sarcosine for secondary amino acids) were added to the samples prior to the hydrolysis to control for errors due to sample loss, injection variation, and micro variations in dilution. The samples were then analyzed on a commercial amino acid analyzer employing automated precolumn derivatization of the hydrolyzed primary amino acids with *o*-phthalaldehyde (OPA) and of the secondary amino acids with 9-fluoromethyl-chloroformate (FMOC). The derivatized amino acids were eluted from a narrow bore 5 μm reverse phase column using a solution gradient. The OPA-tagged amino acids were detected at 338 nm/390 nm and the FMOC-tagged amino acids were detected at 266 nm/324 nm using a diode-array UV detector. The derivatized amino acids were then monitored by a fluorimeter operating at an excitation/emission wavelength of 340 nm/450 nm for the OPA-tagged samples and 266 nm/305 nm for the FMOC-tagged samples. The UV measurements were performed from 250 nm to 350 nm on 0.5 mg/mL highly purified deionized water solutions of the peptide by F.P. Schwarz at NIST using a UV dual-beam spectrophotometer. The optical densities were recorded at 280 nm and 350 nm and the value at 350 nm was subtracted from the value at 280 nm to yield the net UV absorbance at 280 nm. These values were compared to the calculated optical densities of 0.8196 for 1 mg/mL of peptide A, 0.868 for 1 mg/mL of peptide B and 0.992 for 1 mg/mL of peptide C at 280 nm from the website, www.scripps.edu/~cdputnam/protcalc.html, to yield the peptide mass purity percent.

Analytical Methods for the Experimental Molecular Mass: The molecular mass of each peptide was determined by two methods, Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) and Electrospray Ionization (ESI) mass spectrometry. The MALDI-TOF measurements were performed by I. Turko of NIST, M. Lively at Wake Forest University, and H. Remmer at the University of Michigan. Aliquots of approximately 1 mg/mL of the peptide solutions were diluted to final concentrations of 3.2 μM for peptide A, 3.4 μM for peptide B, and 3.9 μM for peptide C. An 0.5 μL aliquot of the diluted solution was then spotted on the

[1] Certain commercial equipment, instrumentation, or materials are identified in this report 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.

MALDI plate and then a 0.5 µL aliquot of the matrix solution was immediately added to the spot. A MALDI spectrum representing the average of 100 laser shots was then acquired. The resulting mass-to-charge ratios, m/z, were for the species $(M + H)^+$ so that the final mass was determined by subtracting 1.0087 from the mass-to-charge ratio of this species. The ESI-MS measurements for the determination of mass were performed by H. Remmer at the University of Michigan on samples of 6.4 µM of peptides A and C and 3 µM of peptide B. A tube containing 100 µL aliquot of the diluted solution was placed in an autosampler, which was then operated at an injection volume of 20 µL.

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[6] Protein Calculator. www.scripps.edu/~cdputnam/protcalc.html (accessed Feb. 2007).

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