Designation: ~~F2260 – 03 (Reapproved 2012)$^{\varepsilon 1}$~~ F2260 – 18

Standard Test Method for Determining Degree of Deacetylation in Chitosan Salts by Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy[1]

This standard is issued under the fixed designation F2260; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

~~ε^1 NOTE—Editorial changes were made to subsections 2.2, 2.3, and 4.5 in November 2012.~~

1. Scope

1.1 This test method covers the determination of the degree of deacetylation in chitosan and chitosan salts intended for use in biomedical and pharmaceutical applications as well as in Tissue Engineered Medical Products (TEMPs) by high-resolution proton NMR (^{1}H NMR). A guide for the characterization of chitosan salts has been published as Guide F2103.

1.2 The test method is applicable for determining the degree of deacetylation (% ~~DA)~~DDA) of chitosan chloride and chitosan glutamate salts and is valid for % ~~DA~~DDA values from 50 up to and including 99. It is simple, rapid, and suitable for routine use. Knowledge of the degree of deacetylation is important for an understanding of the functionality of chitosan salts in TEMP formulations and applications. This test method will assist end users in choosing the correct chitosan for their particular application. Chitosan salts may have utility in drug delivery applications, as ~~a~~ scaffold or matrix material, and in cell and tissue encapsulation applications.

1.3 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate ~~safety~~ safety, health, and ~~health~~environmental practices and determine the applicability of regulatory limitations prior to use.*

1.5 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

F386 Test Method for Thickness of Resilient Flooring Materials Having Flat Surfaces

F2103 Guide for Characterization and Testing of Chitosan Salts as Starting Materials Intended for Use in Biomedical and Tissue-Engineered Medical Product Applications

2.2 *United States Pharmacopeia Document:*

USP 35-NF30 <761> Nuclear Magnetic Resonance[3]

2.3 *European Pharmacopoeia Document:*

European Pharmacopoeia Monograph 2008:1774 Chitosan Chloride[4]

3. Terminology

3.1 *Definitions:*

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[1] This test method is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.42 on Biomaterials and Biomolecules for TEMPs.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from U.S. Pharmacopeia (USP), 12601 Twinbrook Pkwy., Rockville, MD 20852-1790, http://www.usp.org.

[4] Available from European Directorate for the Quality of Medicines (EDQM), Publications and Services, European Pharmacopoeia, BP 907, F-67029 Strasbourg, France.

3.1.1 *chitosan, n*—a linear polysaccharide consisting of β(1→4) linked 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-D-glucopyranose (GlcN). Chitosan is a polysaccharide derived by *N*-deacetylation of chitin.

3.1.2 *degradation, n*—change in the chemical structure, physical properties, or appearance of a material. Degradation of polysaccharides occurs via cleavage of the glycosidic bonds. It is important to note that degradation is not synonymous with decomposition. Degradation is often used as a synonym for depolymerization when referring to polymers.

3.1.3 *degree of deacetylation, n*—the fraction or percentage of glucosamine units (GlcN: deacetylated monomers) in a chitosan polymer molecule.

3.1.4 *depolymerization, n*—reduction in the length of a polymer chain to form shorter polymeric units.

3.1.5 *water soluble chitosan salt, n*—an ionic compound between chitosan molecule (cation) and a negatively charged anion (e.g. glutamate, acetate, lactate, chloride) that is soluble in water.

4. Significance and Use

4.1 The degree of deacetylation of chitosan salts is an important characterization parameter since the charge density of the chitosan molecule is responsible for potential biological and functional effects.

4.2 The degree of deacetylation (% ~~DA)~~DDA) of water-soluble chitosan salts can be determined by ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR). Several workers have reported on the NMR determination of chemical composition and sequential arrangement of monomer units in chitin and chitosan. The test method described is primarily based on the work of Vårum et al. (1991),[5] which represents the first publication on routine determination of chemical composition in chitosans by solution state ^{1}H NMR spectroscopy. This test method is applicable for determining the % ~~DA~~DDA of chitosan chloride and chitosan glutamate salts. It is a simple, rapid, and suitable method for routine use. Quantitative ^{1}H NMR spectroscopy reports directly on the relative concentration of chemically distinct protons in the sample, consequently, no assumptions, calibration curves or calculations other than determination of relative signal intensity ratios are necessary.

4.3 In order to obtain well-resolved NMR spectra, depolymerization of chitosans to a number average degree of polymerization (DP_n) of ~15 to 30 is required. This reduces the viscosity and increases the mobility of the molecules. Although there are several options for depolymerization of chitosans, the most convenient procedure is that of nitrous acid degradation in deuterated water. The reaction is selective, stoichiometric with respect to GlcN, rapid, and easily controlled (Allan & Peyron, 1995).[6] The reaction selectively cleaves after a GlcN-residue, transforming it into 2,5-anhydro-D-mannose (chitose), consequently, depletion of GlcN after depolymerization is expected. On the other hand, the chitose unit displays characteristic ^{1}H NMR signals the intensity of which may be estimated and utilized in the calculation of % ~~DA,~~DDA, eliminating the need for correction factors. Using the intensity of the chitose signals, the number average degree of polymerization can easily be calculated as a control of the depolymerization.

4.4 Samples are equilibrated and analyzed at a temperature of 90 ± 1°C. Elevated sample temperature contributes to reducing sample viscosity and repositions the proton signal of residual water to an area outside that of interest. While samples are not stored at 90°C but only analyzed at this elevated temperature, the NMR tubes should be sealed with a stopper to avoid any evaporation. At a sample pH* of 3.8-4.3 (see 6.1.5 below), artifactual deacetylation of the sample does not occur during the short equilibration and analysis time.

4.5 A general description of NMR can be found in <761> of the USP 35-NF30.

5. Materials

5.1 *Chemicals:*
5.1.1 Chitosan chloride or chitosan glutamate sample.
5.1.2 D_2O (99.9 %).
5.1.3 DCl (deuterium chloride), 0.1 M and 1 M in D_2O.
5.1.4 NaOD (sodium deuteroxide), 0.1 M and 1 M in D_2O.
5.1.5 $NaNO_2$.
5.1.6 0.15 M TMSP (sodium 3-trimethylsilylpropionate-2,2',3,3'-d_4) in D_2O.

5.2 *Instruments:*
5.2.1 Analytical balance (0.1 mg).
5.2.2 Laboratory shaking device.
5.2.3 pH meter or pH paper.
5.2.4 5 mm NMR tubes.

[5] Vårum, K. M., Anthonsen, M. W., Grasdalen, H., and Smidsrød, O., "Determination of the Degree of N-acetylation and the Distribution of N-acetyl Groups in Partially N-deacetylated Chitins (Chitosans) by High-Field N.M.R. Spectroscopy," *Carbohydr. Res.*, Vol 211, 1991, pp. 17–23.

[6] Allan, G. G. and Peyron, M., "Molecular Weight Manipulation of Chitosan 1: Kinetics of Depolymerization by Nitrous Acid," *Carbohydr. Res.*, Vol 277, 1995, pp. 257-272.

5.2.5 NMR spectrometer (300 MHz field strength or higher is recommended although analysis at 100 MHz is possible), with variable temperature option, capable of maintaining 90 ± 1°C sample temperature during analysis, Analog-digital conversion (ADC) with a minimum of 16 ~~bit~~bits is recommended.

6. Procedure

6.1 *Sample Preparation:*

6.1.1 Dissolve 33 mg chitosan chloride or 47 mg chitosan glutamate in 3.3 mL D_2O by gentle shaking until completely dissolved.

6.1.2 Add 250 µL of 1 M DCl and shake. Check that the sample pH* is <2.

6.1.3 Add 100 µL freshly made $NaNO_2$ solution (10 mg/mL in D_2O).

6.1.4 Store the sample at room temperature in the dark for 4 h.

6.1.5 Use 0.1 M or 1 M NaOD to adjust the sample to pH* 3.8 to 4.2.

6.1.6 Transfer 0.7 mL of the sample solution to an NMR tube.

6.1.7 Add 5 µL of 0.15 M TMSP for chemical shift referencing.

NOTE 1—For a sample in 100 % D_2O, the pH reading on a pH meter is 0.4 units lower than the true pD, due to an isotope effect on the glass electrode. The meter reading in such solvents is normally reported uncorrected and designated pH*.

6.2 *Technical Parameters*—The most important parameters used for quantitative ^1H NMR analysis of the degree of deacetylation in chitosan salts are as follows:

6.2.1 *Acquisition:*

6.2.1.1 ^1H NMR acquisition should be performed at 90°C with the sample spinning at 20 Hz using a standard one-dimensional pulse program.

Nucleus	^1H
Proton spectral width	10 ppm (approx. –0.5→9.5 ppm)
Number of scans	64
Relaxation delay	5 s
Pulse angle	90°
Acquisition time	4.096 s
Number of data points	determined by spectral width (in Hz) and acquisition time; 32768 at 400 MHz.

Typical temperature equilibration time is <15 min and spectrum acquisition time is approximately 10 min or less.

6.2.1.2 The use of digital filters and appropriate digital signal processing is recommended for good baseline performance.

6.2.2 *Processing:*

6.2.2.1 Use exponential window with 0.5 Hz line broadening and zero-fill to 64k data points before Fourier transformation.

6.2.2.2 Relative areas of proton signals are estimated by numeric integration of the relevant ^1H NMR signals; K1, H1D, H1A, H2D and HAc (for chitosan chloride only) (Figs. 1 and 2). Correct phasing and flat baseline ~~is~~are essential for good results.

6.3 *Calculations*—For chitosan chloride, signal intensities of H1D and H2D may be averaged. Similarly, intensities of H1A and HAc/3 (3 protons in HAc) may be averaged, to give a better estimate of the relative occurrence of GlcN- and GlcNAc-units. This gives a more precise estimate of % ~~DA.~~DDA. Averaging of the two acetylated signals cannot be performed with chitosan glutamate, due to severe overlap of HAc with glutamate signals (Figs. 1 and 2).

6.3.1 The relative number of GlcN-units in the polymer before depolymerization can be expressed as:

$$D = K1 + (H1D + H2D)/2 \tag{1}$$

where K1, H1D and H2D are estimates of the corresponding signal intensities from the ^1H NMR spectrum (Figs. 1 and 2).

6.3.2 The relative number of GlcNAc-units in the polymer before depolymerization can be expressed as:

$$A = (H1A + (HAc/3))/2 \quad \text{(chitosan chloride)} \tag{2}$$

$$A = H1A \quad \text{(chitosan glutamate)}$$

where H1A and HAc are estimates of the corresponding signal intensities from the ^1H NMR spectrum (Figs. 1 and 2).

6.3.3 Degree of deacetylation (%) is calculated according to the following equation:

~~% DA = Degree of deacetylation (%) = 100 %*D/(D+A)~~ ~~(3)~~

$$\%\ DDA = \text{Degree of deacetylation } (\%) = 100\ \%*D/(D+A) \tag{3}$$

6.3.4 The number average degree of polymerization (DP_n) may be estimated as a control of the degradation as:

$$DP_n = (K1 + A + D)/K1 \tag{4}$$