Designation: F 2259 – 03

Standard Test Method for Determining the Chemical Composition and Sequence in Alginate by Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy[1]

This standard is issued under the fixed designation F 2259; 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.

1. Scope

1.1 This test method covers the determination of the composition and monomer sequence of alginate 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 alginate has been published as Guide F 2064.

1.2 Alginate, a linear polymer composed of β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) linked by β-(1—>4) glycosidic bonds, is characterized by calculating parameters such as mannuronate/guluronate (M/G) ratio, guluronic acid content (G-content), and average length of blocks of consecutive G monomers (that is, $N_{G>1}$). Knowledge of these parameters is important for an understanding of the functionality of alginate in TEMP formulations and applications. This test method will assist end users in choosing the correct alginate for their particular application. Alginate may have utility as a scaffold or matrix material for TEMPs, in cell and tissue encapsulation applications, and in drug delivery formulations.

1.3 *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 and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*

E 386 Practice for Data Presentation Relating to High-Resolution Nuclear Magnetic Resonance (NMR) Spectroscopy[2]

F 2064 Guide for Characterization and Testing of Alginates as Starting Materials Intended for Use in Biomedical and Tissue-Engineered Medical Products Application[3]

2.2 *United States Pharmacopeia Document:*

USP 24-NF19 <761> Nuclear Magnetic Resonance[4]

3. Terminology

3.1 *Definitions:*

3.1.1 *alginate*, *n*—a polysaccharide substance extracted from brown algae, mainly occurring in the cell walls and intercellular spaces of brown seaweed and kelp. Its main function is to contribute to the strength and flexibility of the seaweed plant. Sodium alginate, and in particular calcium cross-linked alginate gels are used in Tissue Engineered Medical Products (TEMPs) as biomedical matrices, controlled drug delivery systems, and for immobilizing living cells.

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

4. Significance and Use

4.1 The composition and sequential structure of alginate determines the functionality of alginate in an application. For instance, the gelling properties of an alginate are highly dependent upon the monomer composition and sequential structure of the polymer. Gel strength will depend upon the guluronic acid content (F_G) and also the average number of consecutive guluronate moieties in G-block structures ($N_{G>1}$).

4.2 Chemical composition and sequential structure of alginate can be determined by ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy (NMR). A general description of NMR can be found in <761> of the USP24-NF19. The NMR methodology and assignments are based on data published by Grasdalen et al. (1979, 1981, 1983).[5,6,7] The NMR technique has made it

[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.

Current edition approved Apr. 10, 2003. Published May 2003.

[2] *Annual Book of ASTM Standards*, Vol 03.06.

[3] *Annual Book of ASTM Standards*, Vol 13.01.

[4] Available from U.S. Pharmacopeia (USP), 12601 Twinbrook Pkwy., Rockville, MD 20852.

[5] Grasdalen, H., Larsen, B., and Smidsrød, O., "A P.M.R. Study of the Composition and Sequence of Uronate Residues in Alginates.," *Carbohydr. Res.*, 68, 1979, pp. 23-31.

[6] Grasdalen, H., Larsen, B., and Smidsrød, O., "^{13}C-NMR Studies of Monomeric Composition and Sequence in Alginate," *Carbohydr. Res.*, 89, 1981, pp. 179-191.

possible to determine the monad frequencies F_M(fraction of mannuronate units) and F_G(fraction of guluronate units), the four nearest neighboring (diad) frequencies F_{GG}, F_{MG}, F_{GM}, F_{MM}, and the eight next nearest neighboring (triad) frequencies F_{GGG}, F_{GGM}, F_{MGG}, F_{MGM}, F_{MMM}, F_{MMG}, F_{GMM}, F_{GMG}. Knowledge of these frequencies enables number averages of block lengths to be calculated. N_G is the number average length of G-blocks, and $N_{G>1}$ is the number average length of G-blocks from which singlets (-MGM-) have been excluded. Similarly, N_M is the number average length of M-blocks, and $N_{M>1}$ is the number average length of M-blocks from which singlets (-GMG-) have been excluded. ^{13}C NMR must be used to determine the M-centered triads and $N_{M>1}$. This test method describes only the ^1H NMR analysis of alginate. Alginate can be well characterized by determining F_G and $N_{G>1}$.

4.3 In order to obtain well-resolved NMR spectra, it is necessary to reduce the viscosity and increase the mobility of the molecules by depolymerization of alginate to a degree of polymerization of about 20 to 50. Acid hydrolysis is used to depolymerize the alginate samples. Freeze-drying, followed by dissolution in 99 % D_2O, and another freeze-drying before dissolution in 99.9 % D_2O yields samples with low ^1H_2O content. TTHA is used as a chelator to prevent traces of divalent cations to interact with alginate. While TTHA is a more effective chelator, other agents such as EDTA and citrate may be used. Such interactions may lead to line broadening and selective loss of signal intensity.

4.4 Samples are analyzed at a temperature of 80 ± 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.

5. Materials

5.1 *Chemicals*:

5.1.1 Alginate sample.

5.1.2 Deionized water (Milli-Q Plus or equivalent; conductivity <10 µS/cm).

5.1.3 HCl (1M, 0.1 M).

5.1.4 NaOH (1M, 0.1 M).

5.1.5 D_2O (99-99.9 %, 99.9 %).

5.1.6 TTHA (triethylenetetraminehexaacetic acid) (0.3 M in D_2O, adjust pH* to 5-5.5 using DCl or NaOD).

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*.

5.2 *Instruments*:

5.2.1 Analytical balance (0.1 mg).

5.2.2 Laboratory shaking device.

5.2.3 pH meter.

5.2.4 Water bath (100°C).

5.2.5 Freeze dryer.

5.2.6 NMR spectrometer (300 MHz field strength or higher is recommended), capable of maintaining 80 ± 1°C sample temperature during analysis.

[7] Grasdalen, H., "High-field ^1H-NMR Spectroscopy of Alginate: Sequential Structure and Linkage Conformations," *Carbohydr. Res.*, 118, 1983, pp. 255-260.

6. Procedure

6.1 *Sample Preparation*:

6.1.1 Prepare 100 mL of a 0.1 % (w/v) alginate solution.

6.1.2 Adjust the pH with HCl (1 M, 0.1 M) to pH 5.6, and put the alginate sample in a water bath at 100°C for 1 h.

6.1.3 Adjust the pH with HCl (1 M, 0.1 M) to pH 3.8, and put the alginate sample back to the water bath at 100°C for 30 min.

6.1.4 Adjust the pH with NaOH (1 M, 0.1 M) to pH 7-8, and freeze-dry the sample overnight.

6.1.5 Dissolve the alginate sample in 5 ml 99-99.9 % D_2O, and freeze dry it again.

6.1.6 Dissolve 10 to 12 mg of the sample in 1 mL 99.9 % D_2O.

6.1.7 Add 0.7 mL of the alginate solution to a NMR tube, and then add 20 µL 0.3 M TTHA to the same tube.

6.2 *Technical Parameters*—The most important parameters used for quantitative ^1H NMR analysis of alginate are as follows:

6.2.1 *Acquisition*:

6.2.1.1 ^1H NMR acquisition should be performed at 80°C with sample spinning at 20 Hz using a standard one-dimensional pulse program.

Nucleus	^1H
Proton spectral width	–0.5→9.5 ppm
Number of scans	64
Relaxation delay	2 s
Proton pulse angle	90°
Acquisition time	4.096 s
Number of data points sampled	determined by spectral width (in Hz) and acquisition time; 32768 at 400 MHz.

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. Correct phasing and flat baseline are essential for good results.

6.3 *Calculations*:

6.3.1 ^1H NMR data are calculated from a set of equations/relations. These relations are based on 2 principles: (*1*) maximal averaging of the data, (*2*) ensure consistency (for example, $F_M = F_{\underline{M}M} + F_{\underline{M}G}$). The relations utilize the integrated intensities of the signals A, B1, B2, B3, B4 and C shown in Fig. 1. The assignments of the ^1H NMR signals in Fig. 1 are as following:

red-a	alpha reducing ends
Signal A	G (proton 1)
red-b	beta reducing ends
Signal B1	G$\underline{G}$M (proton 5)
Signal B2	M$\underline{G}$M (proton 5)
Signal B3	$\underline{M}$G (proton 1)
Signal B4	$\underline{M}$M (proton 1)
Signal C	$\underline{G}$G (proton 5)

6.3.1.1 The chemical composition and the sequence in alginate are determined from the signal intensities, which reflect the quantities of the respective frequencies.